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A CHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF THREE SOUTH AFRICAN PLANTS

by

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On top of all the drama, I cannot forget to say thank you my darling wife Agnes. Your support is always awesome and never did you let me down. Mom, Dad it is finally done and this one I did for you. To my brother, sisters and children Sean and Michelle it is never the end of the road, life is bright but you have to work on it and with God's direction you will see the road ahead.

Lastly, thank you to all who have contributed in the content of this thesis by analyzing my samples and testing for biological activity. This goes out to the analytical function at CSIR, WITS University and North West for mass spectra, and our very own Natasha for anticancer screening.

To the reader, be blessed when reading this thesis. One chapter in my Life is done and new chapter begins, which is also acknowledged by the new member in my family Rotondwa. Keep watching the space……

ADONAI I will never seize to worship you. May your peace that surpass all understanding continue to be in me and the reader through Christ Jesus (Phil. 4:7)

AMEN

ABSTRACT

Three plant species (*Phylica paniculata* Willd., *Pergularia daemia* Forssk. and *Monsonia angustifolia* E.Mey. ex A. Rich.) were investigated for their anticancer properties. Extracts from the plants were tested *in vitro* for cytotoxicity effects against three highly sensitive cancer cell lines (namely melanoma UACC62, renal TK10 and breast MCF7). In all three plants, the organic (methanol/dichloromethane, 1:1 v/v) extracts exhibited a significant inhibition of the growth of cancer cells which warranted further purification to isolate the active principle(s). Structural elucidation of the active compounds was based on proton and carbon-13 nuclear magnetic resonance experiments.

Bioassay-guided fractionation of the methanol/dichloromethane extract of *P. paniculata* resulted in the isolation of ursolic acid as the active constituent along with another triterpenoid, α-amyrin that showed a weak cytotoxic effect.

Repeated flash chromatography of the organic extract of *P. daemia* afforded the isolation of the active coroglaucigenin, a 19-hydroxycardenolide, and four compounds that were characterized as β-sitosterol, β-sitosteryl glucoside, α-amyrin and 3-*O*-acetyl-α-amyrin. All these compounds are reported to have been previously isolated from related plant species. Although it has been reported that other cardenolides have cytotoxic effects, this is the first report on the cytotoxicity against cancer cell lines of coroglaucogenin.

Purification of *M. angustifolia* extracts resulted in the isolation of five lignans (*viz.* 5-methoxyjusticidin A, justicidin A, chinensinaphthol, retrochinensinaphthol methyl ether and suchilactone) that previously have been predominantly found in the *Justicidin* genus. These compounds were tested for their cytotoxic activity against the cancer cell lines and two justicidin compounds, 5-methoxyjusticidin A and justicidin A, showed good activity. 5-Methoxyjusticidin A was selected by the National Cancer Institute, USA, for testing in their 60 cell line screen, but research on this compound was not continued because of lack of sufficient potency.

The extracts and compounds isolated from *M. angustifolia* were also evaluated in relevant *in vitro* and *in vivo* biological assays for erectile dysfunction and libido. Male rats that were dosed with the extract mixture (organic and aqueous) demonstrated a significant increase in mating frequency with female rats as compared to male rats dosed with the

vehicle control. An increase in the number of pregnant rats as compared to the vehicle control was also observed. The organic extract was tested *in vitro* and inhibited the phosphodiesterase 5 (PDE 5) enzyme and also effectively relaxed the pre-contracted smooth muscle. The five lignans isolated from the *M. angustifolia* extract showed varying degrees of the inhibition of the PDE 5 enzyme. Four compounds, 5-methoxyjusticidin A, chinesinaphthol, suchilactone and retrochinesinaphthol methyl ether relaxed rabbit corpus cavernosum smooth muscle significantly. However, in both the above-mentioned *in vitro* assays the extracts and isolated compounds displayed a much lower activity that sildenafil (Viagra). Structural modifications of these lignans may lead to enhanced activity and bioavailability.

ABBREVIATIONS USED IN TEXT

AR	Analytical reagent
c	Concentration in g/100 mL
CH_2Cl_2 or DCM	Dichloromethane
COSY	Correlated SpectroscopY
DAD	Diode array detector
DEPT	Distortionless Enhancement Polarization Transfer
EI-MS	Electron Impact Mass Spectroscopy
ESI-MS	Electrospray Ionization Mass Spectroscopy
EtOAc	Ethyl acetate
EtOH	Ethanol
FAB	Fast Atom Bombardment
GC	Gas Chromatography
HIV-1	Human Immunodeficiency Virus 1
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HR-MS	High Resolution Mass Spectrometry
HSQC	Heteronuclear Single Quantum Correlation
Hz	Units in Hertz
J	Spin-spin coupling constant in Hz
MeOH	Methanol
min	Minutes
m.p.	Melting point
NMR	Nuclear Magnetic Resonance
NOE	Nuclear Overhauser effect
ppm	Parts per million
sp.	Species
TLC	Thin-Layer Chromatography
UV	Ultra Violet Spectroscopy
VLC	Vacuum Liquid Chromatography
^oC	Degrees Celsius
1D	One Dimensional

2D	Two Dimensional
[α]	Specific Rotation
δ	NMR chemical shift in ppm

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Chapter 1

INTRODUCTION

As a result of inaccessibility to health care facilities, people from disadvantaged communities often turn to traditional healers (owners of indigenous knowledge) who prescribe indigenous natural medicines.[1] This system of medicines needs to be evaluated scientifically, given due recognition and developed to possibly improve its efficacy and safety.[2]

The chemical constituents of most of the local medicinal plants in use are still unknown. Therefore, it is of importance to characterize the chemical composition of the medicinal plants and document the indigenous knowledge acquired from the healers or herbalists. This study will deal with the phytochemical investigation of selected plants that are endemic to South Africa.

In the past, scientific investigations of folk medicine have led to the development of marketable products as pharmaceuticals (single component drugs) or nutraceuticals (botanical extracts) and in the laboratory of natural product chemists, the search for anti-HIV, antiviral, antitumor, etc. agents continues. South Africa is one of the richest countries with natural resources (plants, animals, marine organisms and insects) and this biodiversity has the potential to be a primary source of new leads for drug discovery. Many of the indigenous plant species used traditionally in South Africa are endemic and consumers are trading them for their use to a large market, both nationally and internationally.

At the CSIR, plants are being investigated and evaluated for medicinal properties. In this programme, plants that are received by the CSIR's Bioprospecting research group from traditional healers and botanists are evaluated for biological activity, specifically anticancer activity. The information provided to CSIR is governed under collaborative agreements that also stipulate secrecy of the knowledge provided. The South African National Biodiversity Institute (SANBI) in Pretoria assists in the identification of the collected plants. Bioactive compounds are isolated by bioassay-guided fractionation of the plant

extracts. Unraveling of the complex chemical compositions of the bioactive plants will be the focus of this study.

In this investigation, three plants from different families were selected for phytochemical investigation on the basis of their antitumour activity observed in the CSIR's in-house anticancer screen on three cell lines, namely melanoma, breast and renal cancer cell lines. The plants under investigation are *Phylica paniculata* (Rhamnaceae), *Pergularia daemia* (Asclepiadaceae) and *Monsonia angustifolia* (Geraniaceae).

For each of the three plants, the aim of this study was to:

- Prepare a herbarium specimen for identification by SANBI
- Confirm the biological activity of the plant extracts
- Develop HPLC fingerprinting and TLC methods for guiding fractionation
- Isolation and biological evaluation of the pure compounds
- Elucidation of the structures of the compounds by using NMR spectroscopy and MS spectrometry

All plant collections were primarily screened for anticancer activity in the available in-house anticancer screen. Wherever possible, extracts and compounds were also assayed against other activities. Based on information received from traditional healers, *Monsonia angustifolia* extracts and isolated compounds were also assayed for treatment of erectile dysfunction.

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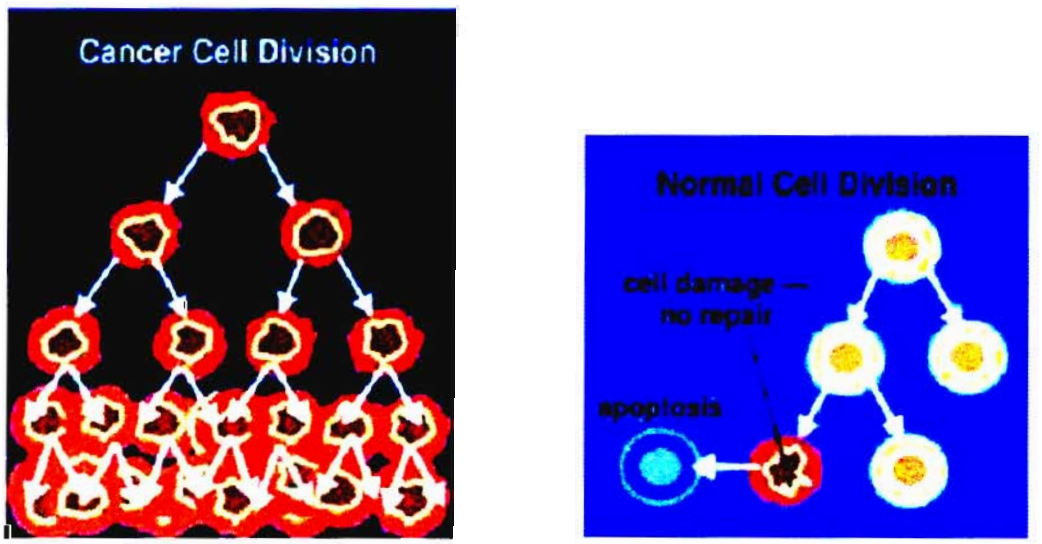
Chapter 2

LITERATURE OVERVIEW OF PLANTS AS A SOURCE OF ANTICANCER DRUGS AND COMPOUNDS WITH OTHER PHARMACOLOGICAL PROPERTIES.

2.1. INTRODUCTION TO CANCER

Cancer is a disease that begins in cells that are behaving abnormally. The human body is formed out of basic units called cells. A group of cells form tissue. Normally, cells grow and divide to produce more cells when the body needs them. Sometimes, however, cell growth becomes abnormal and ends up forming too many cells without control or order, creating a mass of excess tissue called a tumour. These tumours can be classified as benign (non-cancerous) and malignant or neoplasm (cancerous). When normal cells are damaged beyond repair, they are eliminated by a process known as apoptosis. Cancer cells are formed when the damaged cells avoid apoptosis and continue to multiply in an unregulated manner (see Fig. 2.1).



(Courtesy of http://en.wikipedia.org/wiki/Cancer, 15/06/2006)

Figure 2.1: The process of normal cell growth and formation of a tumour.

Most cancers are named after the organ they are associated with like lung cancer for cancer that originated in the lungs, breast cancer for cancer originating from the breast and skin originating cancer also known as melanoma. When the cancer cells migrate to another organ, the new cells take the name of the old. For example, if lung cancer migrates to the liver and causes cancer, then the new cancer is called metastasis lung cancer.

2.2. CANCER AND ITS IMPACT

The American Cancer Society estimates that 1,399,790 people (720,280 men and 679,510 women) will be diagnosed with and 564,830 people will die of cancer of all sites in 2006.[1] Approximately 12.5 million new cases are reported to have been diagnosed each year and considerable research is underway to obtain a drug that can function as a vaccine against cancer or that will cure and kill cancer cells. In the USA and other developed countries, cancer is presently responsible for about 25% of all deaths.[2]

The word carcinoma is the medical term for a malignant tumour derived from epithelial cells. It is translated from *carcinos* (Latin), also meaning crab. Galen used "*oncos*" to describe *all* tumours, the root for the modern word oncology.[3]

Most cancers can be treated and some cured, depending on the specific type, location and stage when it was diagnosed. Cancer is usually treated with a combination of surgery, chemotherapy and radiotherapy. As research develops, available treatments are becoming more specific for the type of cancer pathology. Drugs that target specific cancers already exist for many cancers. If untreated, cancers may eventually cause illness and death, though this is not always the case.

According to information obtained from the National Cancer Institute (NCI) website on 7 November 2006, the most common cancer types are:

> Bladder cancer, Breast cancer, Colon and Rectal cancer, Endometrial cancer, Kidney (Renal cell), Leukaemia cancer, Lung cancer, Melanoma cancer, Non-Hodgkin's Lymphoma cancer, Pancreatic cancer, Prostate cancer, Skin (non-melanoma) cancer, Thyroid cancer and Ovarian cancer.

2.3. PLANTS AS SOURCES OF ANTICANCER DRUGS

Most of the research to discover and develop new anticancer drugs to be used for therapy is focused on the investigation of natural resources based on the indigenous plants used for folk medicine. Other resources include biological marine resources like sea sponges. The NCI records show that most of the important drugs came from plant origin and this is supported by the published anticancer agents namely, the vinca alkaloids (vinblastine and vincristine) isolated from *Catharanthus roseus*, the semi-synthetic derivatives teniposide and etoposide, from podophyllotoxin, taxol (or paclitaxel) and camptothecins.[4] The great majority of these compounds isolated from plants never made it to clinical trials or failed at the very early stages of the trials because of cytotoxicity towards the host cells or the loss of potency *in vivo*. In order to eliminate these unwanted effects of the compounds, synthetic chemists do structural modifications by changing or introducing new functionalities in the molecules. This process of structure activity relationships (SAR) is aimed at improving the activity of the compound but at the same time reducing the toxicity level.

At the present moment significant progress has been made in the research aimed at establishing therapeutic cancer vaccines that can reliably and consistently induce tumour destruction or improve patient survival.[5] Evidence was also obtained that patients get improved antitumour immune responses when using cancer vaccines, inducing cancer regression or increased survival.

The reported beneficial isolation of potential antigens from plants led to the identification of certain classes of compounds that can be useful as anticancer agents.[6] These also include molecules that have been discovered after modifications of the natural product leads[7] and the focus will be on some important classes of compounds namely, alkaloids, diterpenoids, triterpenoids and lignans. It is however worth noting that Mukherje *et al.*[8] reported that since 1955, NCI has screened more than 114 000 plant-derived extracts and it was reported that several anticancer agents obtained from natural plants are in clinical use.[9] Cragg *et al.*[10] reported that five plant-derived compounds (vincristine, vinblastine, etoposide, teniposide and taxol) have advanced to human clinical trials but of these only taxol was discovered in the NCI programme.

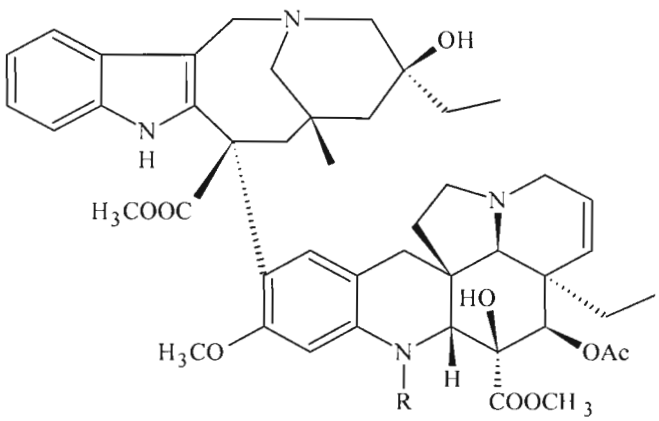
2.3.1. Alkaloids

Alkaloids are nitrogen-containing compounds that are mostly in cyclic form. This class of compounds can again be grouped into different subgroups but for the purpose of this study they will be discussed in general.

Vinca Alkaloids

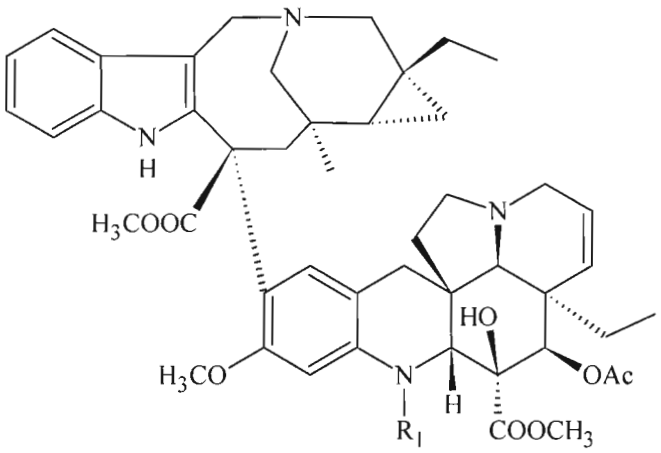
Research work conducted on the *Vinca rosea* extracts (also known as *Catharanthus roseus*) led to the discovery of their anti-neoplastic activity. The isolation of highly active vinblastine, vincristine, leorosine and leorosidine (**2.1** to **2.4**) was achieved and the compounds were developed as commercial drugs.[10,11]

The vinca alkaloids are useful as chemotherapeutic drugs for the treatment of the following type of cancers: Hodgkin's disease, non-Hodgkin's lymphomas, leukaemia, renal, cervical, breast, testicular and small cell lung cancer.



2.1: Vinblastine R = CH_3

2.2: Vincristine R = CHO

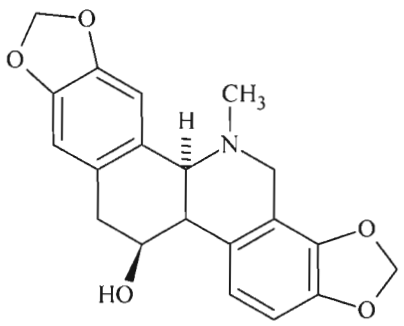


2.3: Leurosine $R_1 = CH_3$

2.4: Leurosidine $R_1 = CHO$

Ukrain

A recent systematic review was conducted on the pharmacological activity and clinical effectiveness of a chelated benzophenanthridine alkaloid known as ukrain, a semi-synthetic compound derived from chelidonine (**2.5**) isolated from the extract of the common weed *Chelidonium majus* L. Ukrain is a compound that is formed from thiophosphoric acid conjugated to three molecules of chelidonine (**2.5**).[12]

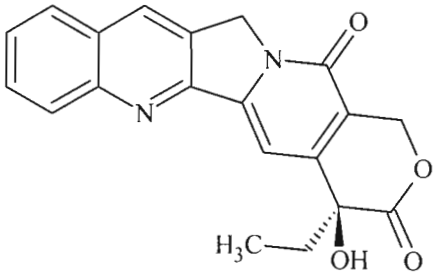


2.5: Chelidonine

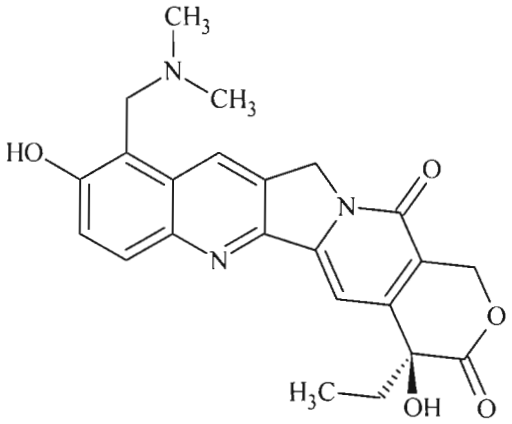
Ukrain has been reported as the only known product which at present does not harm normal cells but reduces the cancer cells and boosts the immune system.[13] Clinical trials have shown that this compound has curative effects on a range of cancers.

2.3.2. Campothecin and analogues

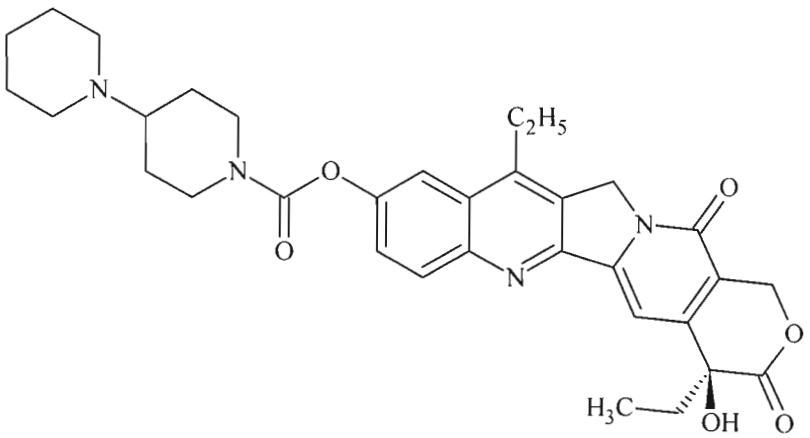
Campothecin (CPT, **2.6**) was discovered in the early 1960s as an anticancer agent which inhibits the DNA topoisomerase I *in vitro* as well as mouse leukaemia cells, colon and pancreatic cancer.[14] It was first extracted from the stem wood of the Chinese tree *Camptotheca acuminate* and became an important anticancer agent.[15,16]



2.6: CPT



2.7: Topotecan

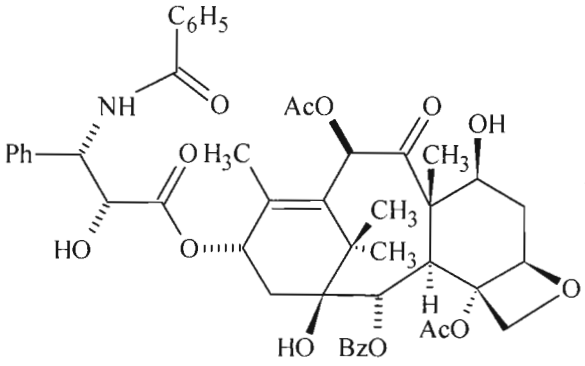


2.8: Irinotecan

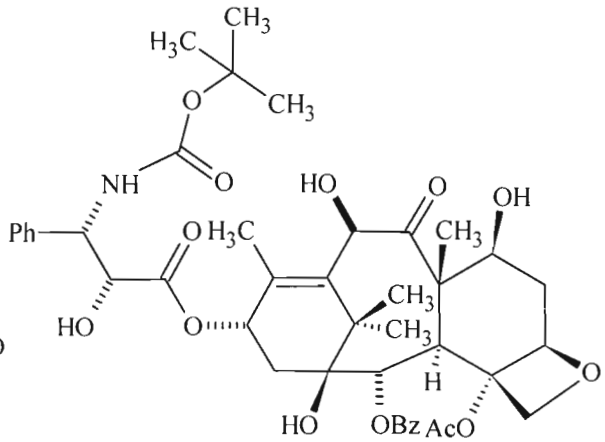
The two synthetic analogues of CPT (**2.6**), topotecan, (**2.7**) and irinotecan (**2.8**) are used for the treatment of breast, liver, prostate, ovarian, pancreatic and colon cancers under the label of Glaxo-SmithKline and Pfizer as Hycamtin and Camptosar respectively.[17,18]

2.3.3. Taxol and analogues

Paclitaxel (**2.9**), also known by the generic name taxol, is a complex polyoxygenated diterpenoid isolated from *Taxus brevifolia*.[19] This compound and derivatives thereof were later isolated from other species of *Taxus*.



2.9: Taxol

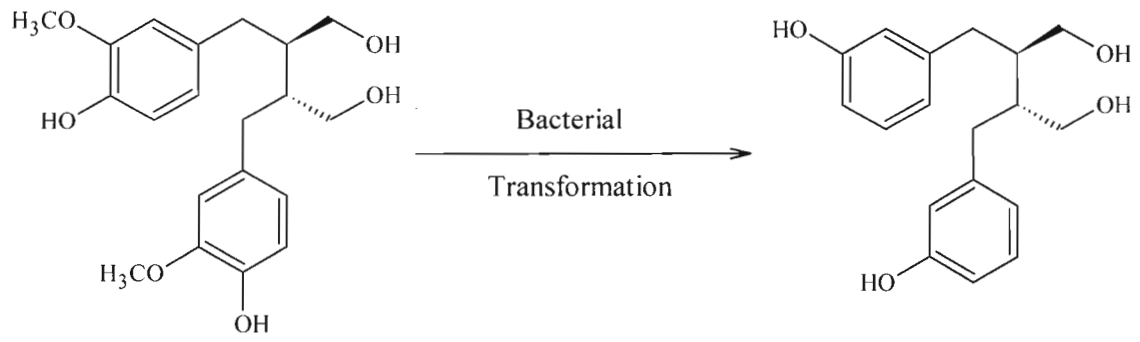


2.10: Taxotere

Taxol was reported to have shown promising results in phase I and phase II clinical trials in lung, ovarian and breast cancers and squamous cell carcinoma of the head and neck. The FDA gave approval in 1992 to the NCI for developing and using taxol to treat metastasis ovarian cancer but bioavailability of the drug still presents a challenge.[20] The bioavailability problem is caused by the drugs partial solubility in water. Synthetic modifications prepared, solved this problem and a semi-synthetic derivative taxotere (docetaxel) **2.10** was reported to show improved pharmacology compared to taxol.[21,22]

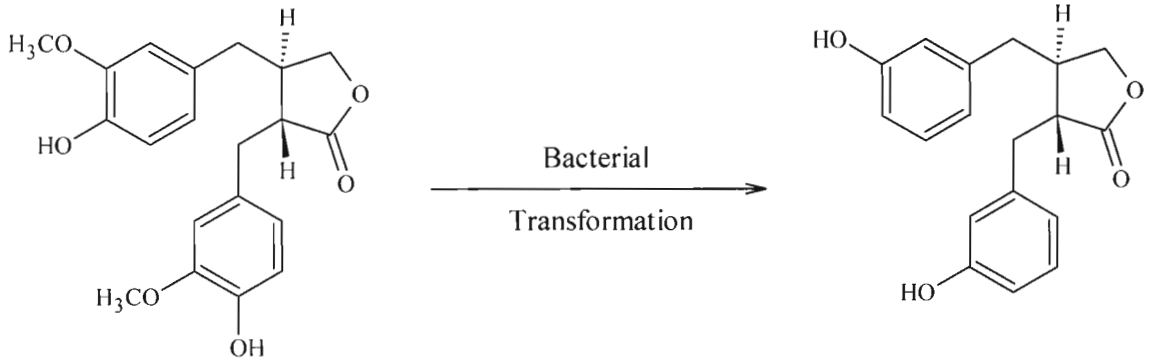
2.3.4. Lignans and analogues

Lignans are a large group of natural products that are formed by the coupling of two C6-C3 arylpropyl units.[23] There are two major lignans that are found in most plants especially those that are edible. These lignans, secoisolariresinol (**2.11**) and matairesinol (**2.12**), get transformed by bacterial action into mammalian lignans enterodiol (**2.13**) and enterolactone (**2.14**), respectively (Scheme 2.1). These mammalian lignans are believed to help stop the onset of breast and prostate cancers.[24] Matairesinol (**2.12**) is also a precursor in the formation of the podophyllotoxin (**2.15**).[25]



2.11: Secoisolariciresinol

2.13: Enterodiol



2.12: Matairesinol

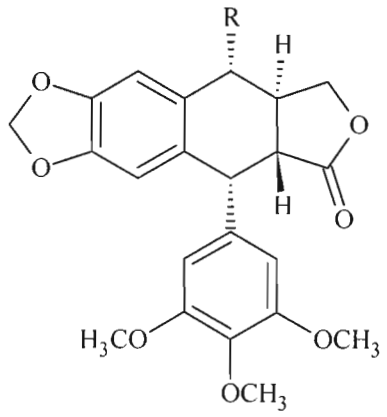
2.14: Enterolactone

Scheme 2.1: The major lignans and their bacterial-converted mammalian intermediates.

Podophyllotoxin (**2.15**) and desoxypodophyllotoxin (**2.16**) are well-known natural aryltetralin lignans and the former was isolated from *Podophyllum* species, but initially from *P. emodi.* The natural derivative desoxypodophyllotoxin was isolated from *Anthriscus sylvestris* and *Pulsatilla koreana.*[26]

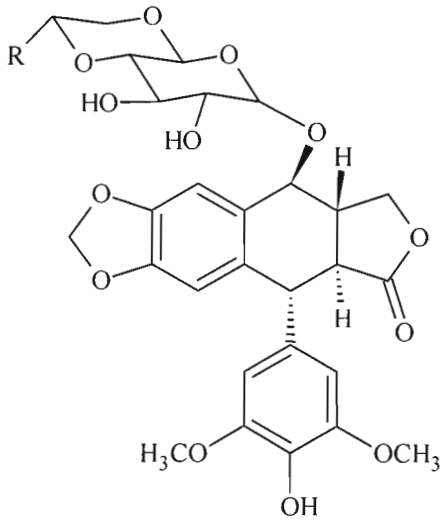
Both of these compounds show cytotoxic effects against cancer cell lines and their semi-synthetic derivatives etoposide (**2.17**) and teniposide (**2.18**) are in use as chemotherapeutic agents against various cancers.[27] Etoposide is the most widely-used anticancer agent and the NCI database records its use mainly for the treatment of leukaemia.[28]

There are other semi-synthetic analogues of podophyllotoxin that were prepared and exhibited anticancer activity against numerous cancer cells. Most of these compounds failed because of their non-specific toxicity to both normal and cancer cells, as well as bioavailability problems. These compounds also show antiviral[29,30] and anti-HIV activities.[31]

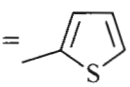


2.17: Podophyllotoxin R = OH

2.16: Desoxypodophyllotoxin R = H



2.17: Etoposide $R = CH_3$

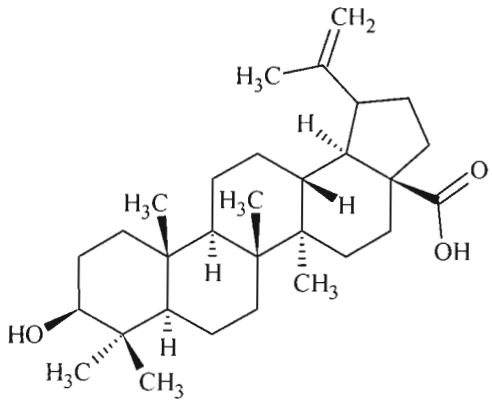
2.18: Teniposide R = 

2.3.5. Triterpenoids

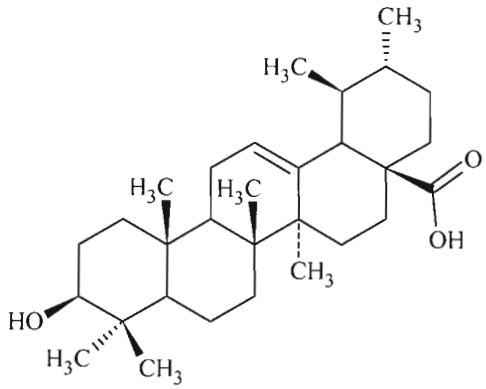
Another plant-derived lupane type triterpenoid, betulinic acid (**2.19**) has been isolated from many taxonomically diverse plant genera and it demonstrated a variety of activities like antibacterial, anti-inflammatory, antimalarial, inhibition of HIV replication and cytotoxicity against a range of cancer cell lines.[32]

Two well-known triterpenoids, ursolic acid (**2.20**) and oleanolic acid (**2.21**), are said to be associated with weak anti-inflammatory and antitumour activities. Attempts to prepare synthetic analogues in order to improve the potency led to the synthesis of 2-cyano-3,12-dioxo-olean-1,9-dien-28-oic acid (CDDO, **2.22**), which exhibited potent *in vitro* and *in vivo* anticancer activity against epithelial ovarian carcinoma cell lines.[33]

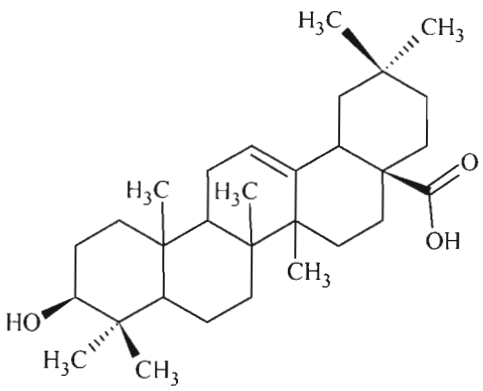
Compounds developed from natural products in the 1990s like vinblastine, vincristine, combretastatin and maytansine exert their action through the depolymerization of tubulin.[6] Taxanes stabilize against depolymerization whereas camptothecin derivatives like topotecan and irinotecan promote their cytotoxic action through the inhibition of topoisomerase I.[6]



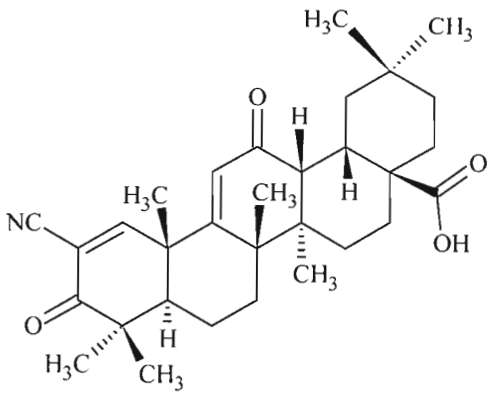
2.19: Betullinic acid



2.20: Ursolic acid



2.21: Oleanolic acid



2.22: CDDO

2.4. INTRODUCTION TO ERECTILE DYSFUNCTION/LIBIDO

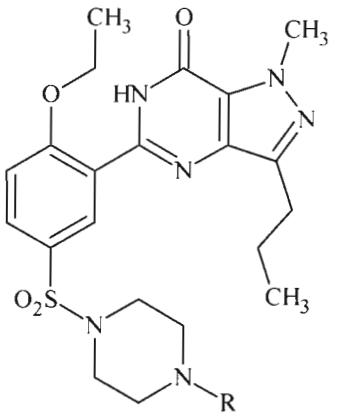
Sexual dysfunction (SD) is a clinical problem that affects both males and females. The causes thereof may be both organic and psychological. Organic aspects of SD are typically caused by underlying vascular diseases such as those associated with hypertension or diabetes mellitus, prescription medication and psychiatric disease such as depression. Physiological factors include fear, performance anxiety and interpersonal conflict. SD impairs sexual performance, diminishes self-esteem and disrupts personal relationships thereby inducing personal distress. In the clinic, SD disorders have been divided into female sexual dysfunction (FSD) disorders and male sexual dysfunction (MSD) disorders.[34]

FSD is best defined as the difficulty or inability of a woman to find satisfaction in sexual expression which can be related as libido. Male sexual dysfunction (MSD) is generally associated with erectile dysfunction, also known as male erectile dysfunction (ED).[35] Erectile dysfunction has been defined as the repeated inability to achieve or sustain an erection for sexual performances. ED affects the lives of over 150 million men worldwide.[36] Most underlying factors that contributes to the onset of ED includes conditions like stress, diseases (e.g. diabetes) and blocking of penile arteries from the side effects of some prescription drugs.

Penile erection is a carefully orchestrated series of events with the central nervous system in the role of conductor. Even when the penis is at rest, the nervous system is at work. When a man is at rest, part of the sympathetic nervous system limits the flow of blood to the penis, keeping it limp. Regardless of where the stimulant signals come from, the excitory nerves in the penis respond by releasing the pro-erectile neurotransmitters such as nitric oxide (NO) and acetylcholine. These chemical messengers signal the muscles of the penile arteries to relax causing more blood to flow into the organ. Spongy chambers inside the penis fill up with blood causing the erection and consequently compress the veins that drain the blood.

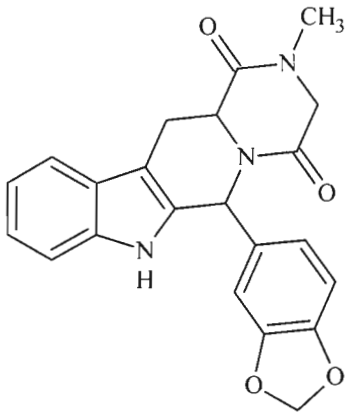
2.5. THE COMMERCIAL PRODUCT VIAGRA

The commercial product Viagra (**2.23**) also known as sildenafil, works by slowing the breakdown of one of the chemicals that keeps the muscles relaxed, thereby holding the blood in the penis for a longer period (see flow diagram in Fig. 2.2).



2.23: Sildenafil $R = CH_3$

2.24: Vardenafil $R = CH_2CH_3$



2.25: Tadalafil

Viagra was developed in 1998 by Pfizer as the first synthetic oral drug treatment for male ED. Sildenafil is a potent inhibitor of the phosphodiesterase 5 (PDE 5) enzyme.[37,38] This compound acts by triggering the sexual stimulant that leads to the enhanced release of nitrous oxide which is synthesized via the non-adrenergic non-cholinergic (NANC) nerve and endothelial cells. NO activates the enzyme guanilate cyclase that increases the intracellular concentration of cyclic guanosine 3',5'-monophosphate (cGMP) by conversion from guanosine triphosphate (GTP). The increase in cGMP concentration causes a decrease in intracellular Ca^{2+} levels in the corpus cavernous smooth muscle followed by relaxation of the smooth muscle. During this process in the corpus carvenosa, some of the cGMP is hydrolysed to 5'-guanosine monophosphate (GMP) by the PDE 5 enzyme. Thus, sildenafil and its analogues act by limiting the hydrolysis of cGMP to GMP through selective inhibition of the PDE 5 enzyme[39,40] and thereby increasing the intracellular concentrations of cGMP and facilitating corpus cavernous smooth muscle relaxation. It is now understood that sildenafil citrate is an effective and safe inhibitor of phosphodiesterase 5, the predominant isoenzyme in the human corpus cavernosum.

Decreased levels of phosphodiesterase allow increased production of nitrous oxide which, in turn, stimulates blood flow to the corpus cavernosum and concomitantly causing an erection.

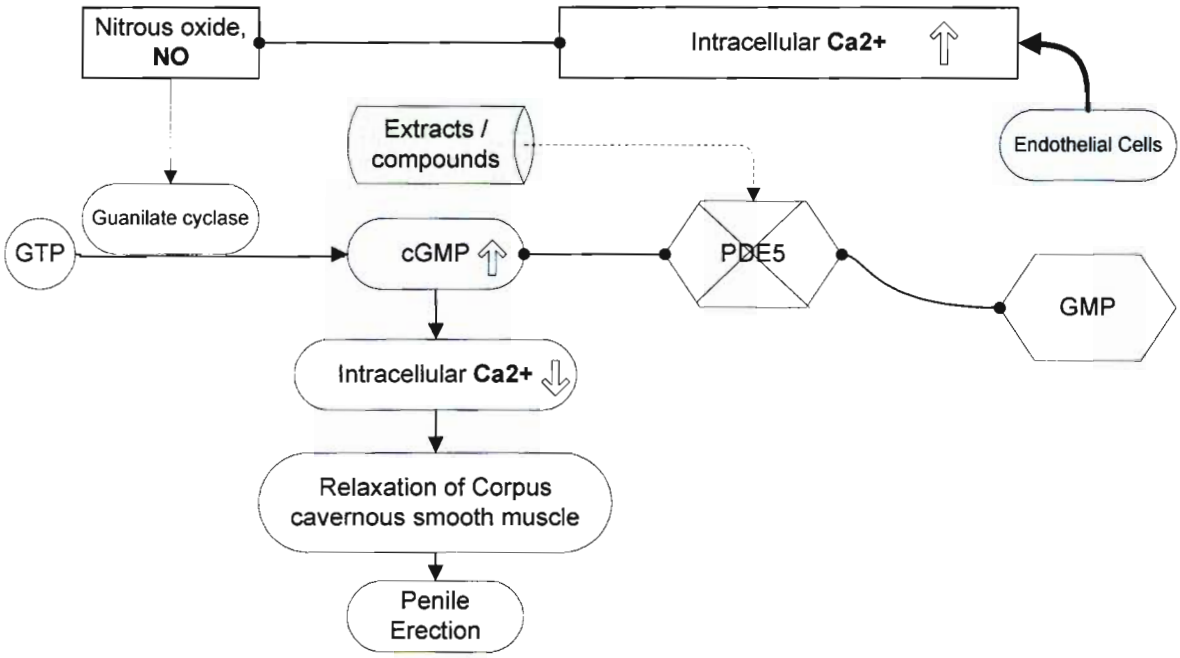


Figure 2.2: Pathway leading to penile erection.

Sildenafil has been reported to be 10 fold less effective at the same concentrations as compared to the corresponding synthetic analogues, Vardenafil (Levitra, **2.24**) and Tadalafil (Cialis, **2.25**).[41]

2.6. NATURAL TREATMENTS OF ERECTILE DYSFUNCTION

The introduction of Viagra and other synthetic analogues presented a solution for the ED problem but these compounds also present some side effects. There is thus a necessity to develop a product with fewer side effects. Researchers are still focusing on the research in natural products and specifically in the plant kingdom. There have been several reports documenting the use of extracts made from herbal products for the treatment of ED. A review of plant-derived compounds used for ED was published by Drewes *et al.*[42]

Plants and their active components, reported to be used for the treatment of erectile dysfunction are:[42]

Ginkgo biloba

Zingiber officinale[42]

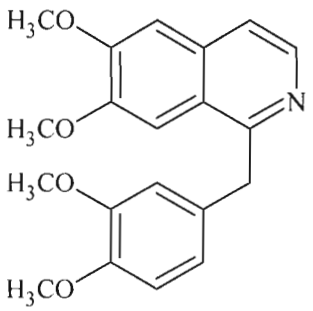
Eurycoma longifolia

Withania somnifera

Saw palmetto (Serenoa repens)

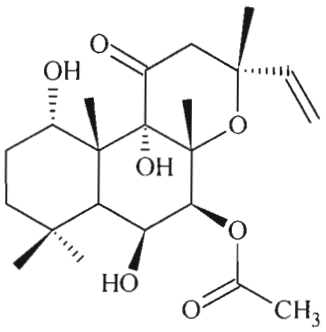
Ptychopetalum olacoides also known by the common name Muira puama

Papaver somniferum contains papaverine[42]



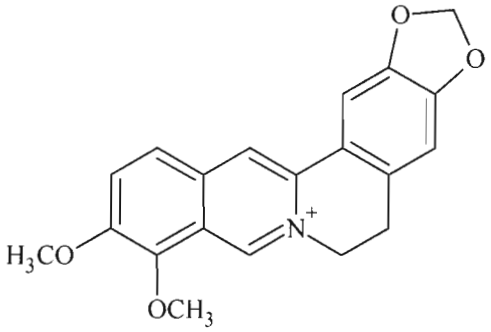
Papaverine

Plectranthus barbatus which contain forskolin



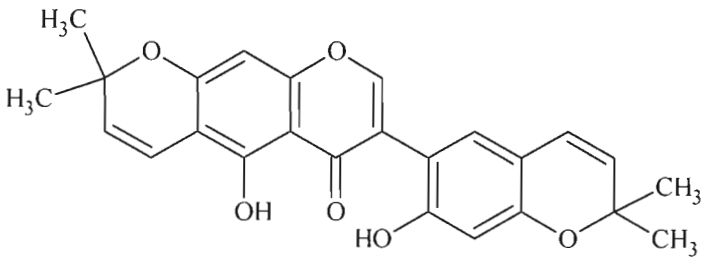
Forskolin

Coptis chinensis which contains berberine



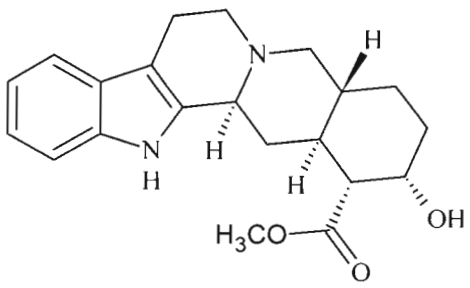
Berberine

Eriosema kraussianum which contains the pyranoisoflavone kraussianone



Kraussianone

Pausinystalia yohimbe Pierre (formerly *Corynanthe yohimbe*) *containing* yohimbine, an alkaloid.[42]



Yohimbine

Most of these products act by the same mechanism as the synthetic sildenafil although this is not the only mechanistic orchestration of events that can lead to the onset of the concomitant treatment of ED. For the purpose of this study only the route that follows the inhibition of the PDE 5 was studied.

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Chapter 3

MATERIAL AND METHODS

General materials and methods that were used in some or all subprojects are described here in order to avoid repetitions. However, specific materials and methods used in this study are described in the corresponding chapters.

3.1. PLANT COLLECTION

The plants were collected as part of a collaborative initiative of the CSIR with the National Cancer Institute of the United States (NCI) to evaluate South African plants for anticancer properties against a panel of cancer cell lines hosted at the CSIR and the NCI. The source of the plant material was either from the Traditional Healers Committee (THC) who has a vast knowledge of indigenous plant use or botanists who collected plants at random. The collections were made all over South Africa based on the availability of the plant material for collection. Plant specimens were sent to the South African National Biodiversity Institute (SABNI) in Pretoria for identification.

3.2. PROCESSING AND EXTRACTION OF PLANT MATERIAL

The plant material was temporarily stored in a cold room maintained at 4 ^{o}C until further processing. The plants (roots and/or stems and/or leaves) were weighed, dried in the oven maintained at 45 ^{o}C long enough to achieve proper drying. The drying process was monitored every 24 hours and resulted in at most 3 days of drying period. Grinding of oven-dried plant material was done at the CSIR using a hammer mill equipped with a mesh pore size of 2 mm. The dried, ground plant material was then subjected to exhaustive extraction as outlined in the flow diagram (Fig 3.1).

3.2.1. Extraction

Aqueous extraction

For aqueous extractions, one of two routes was followed (see Fig. 3.1).

(A) Route 1

The dried material was first extracted with deionised water, enough to cover the plant material. The mixture was stirred occasionally for 4 h and then filtered. The aqueous filtrate was freeze-dried and the remaining plant material was placed in an oven for drying to prepare the material for the organic solvent extraction.

(B) Route 2

The dried, ground plant material was extracted in the extraction vessel by adding enough deionised water to cover the plants. The mixture was boiled for an hour as per the traditional method of preparation of herbal medicines. The cooled extract was filtered and the filtrate was freeze-dried. The plant residues were discarded.

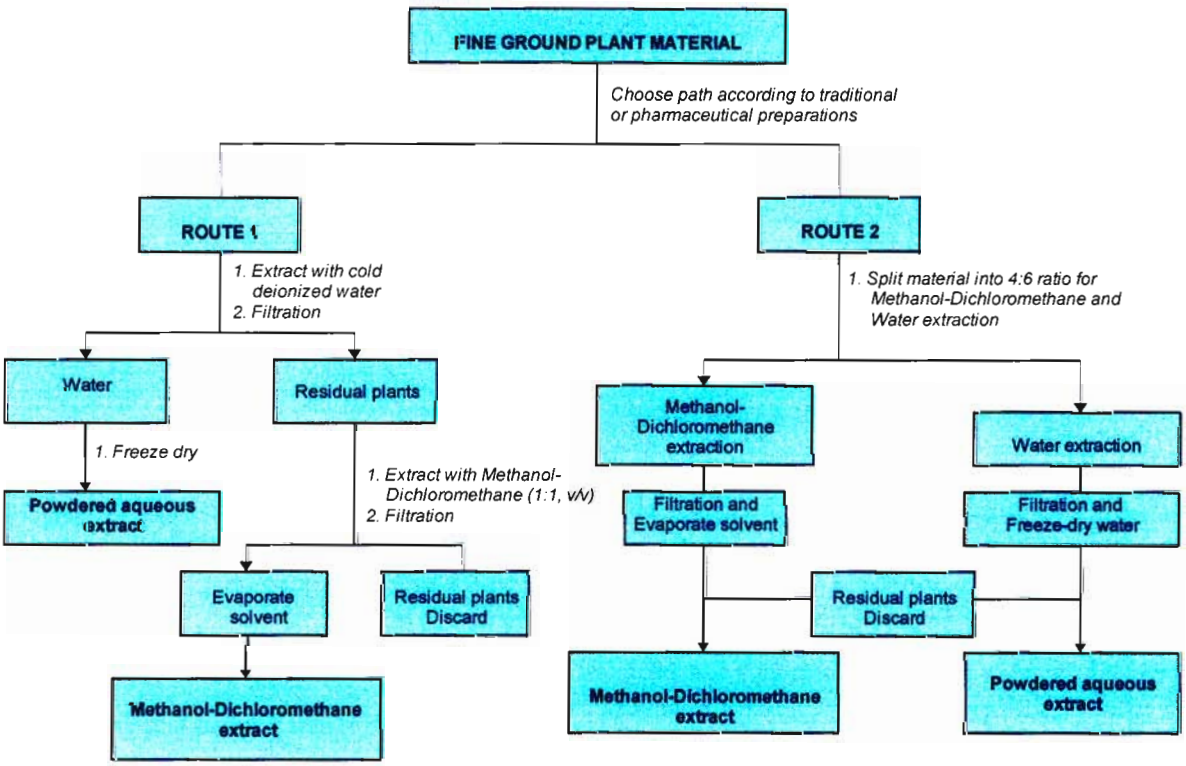

Figure 3.1: Flow diagram of the general extraction process

Organic extraction

The protocol followed for the organic solvent extraction was the same for both routes but the pathway leading to their preparation was different.

The oven-dried, ground plant material was transferred to the extraction vessel. Sufficient organic solvent [methanol-dichloromethane (1:1 v/v)] was added to cover the plant material. The extraction mixture was left to stand for four to twenty four hours with occasional stirring. The solvent was filtered off and evaporated under reduced pressure at a temperature of not more that 60 ^{o}C. The extract was transferred to a tared sample bottle and the residual plant material was discarded.

3.2.2. Liquid-liquid partitioning

The general separation procedure accomplished by liquid-liquid partitioning involved the partitioning of the organic extract (refer to section 3.2.1.) between methanol-water (90%, v/v) and hexane three times in a separating funnel. The hexane layers were combined and evaporated under reduced pressure and the aqueous residue was diluted with water, concentrated by evaporation of the methanol under reduced pressure and extracted three times with dichloromethane. The combined dichloromethane layers were evaporated under reduced pressure. The water layer was concentrated by evaporating under vacuum and thereafter it was subjected to freeze-drying. The schematic diagram in Fig. 3.2 illustrates the extraction procedure.

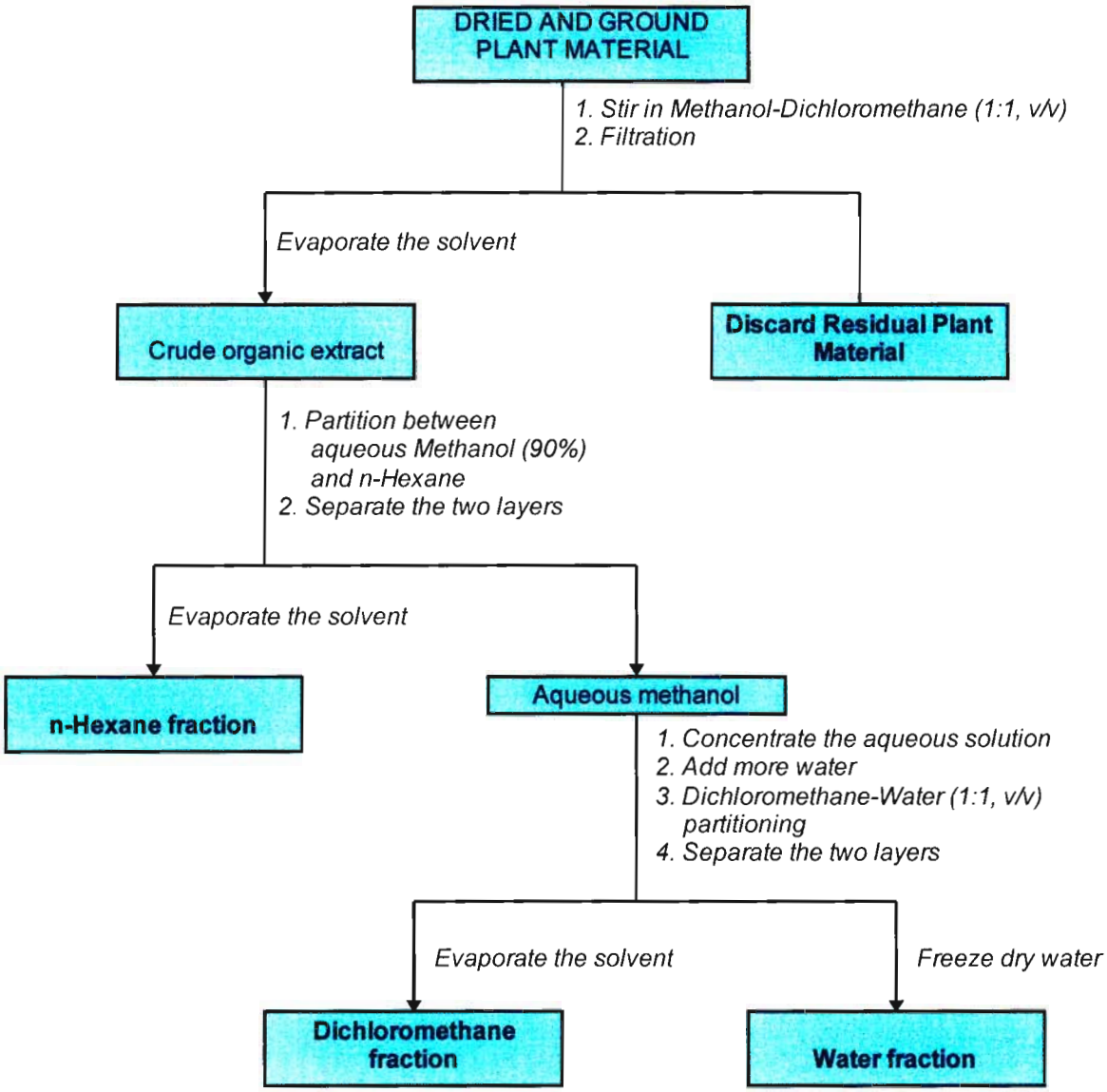

Figure 3.2: General liquid-liquid partitioning procedure

3.3. CHROMATOGRAPHY

3.3.1. Thin-Layer Chromatography

Thin-layer chromatography (TLC) was carried out using either aluminium base sheets covered with silica gel 60 F254 (Merck 5554) or pre-coated glass plates (Merck, SIL G-25 UV_{254}, 0.25 mm, 20 x 20 cm). Standard chromatograms of plant extracts and isolates were prepared by applying a concentrated sample to a silica gel TLC plate and developing it with suitable solvent combinations. Chromatograms were detected using ultraviolet (UV) light (short wave 254 nm and long wave 366 nm). The general TLC spraying reagent used

was vanillin-H_2SO_4 reagent (1 g vanillin dissolved in 100 ml of 85% H_2SO_4) and the sprayed TLC plate was heated at 100 °C for a few minutes.

3.3.2. High Performance Liquid Chromatography (HPLC) and detectors (UV-MS)

HPLC-UV-MS experiments were performed on a Waters 2690 Alliance HPLC Separations Module, linked to a Waters 996 Photo Diode Array detector (PDA) and interfaced to a triple pole Quattro LC Micro mass spectrometer. The mass spectrometer was operated in the positive or negative mode using electrospray ionisation ($ESI^{+/-}$). Chromatography was performed on an ODS Hypersil C18 (5 µm) HPLC column (250 x 4.6 mm) equipped with a Hypersil C18, 5 µm guard column. Typical eluting solvents were acetonitrile-water in a gradient system at a flow rate of 1 ml/min. The column temperature was set at 40 °C and the solvents were pre-filtered through a 0.45 µm filtering membrane.

If not stated otherwise, analyte sample concentrations of between 2500 and 5000 ppm (i.e. 1.0 – 5.0 mg/ml) were prepared by dissolving the sample in the appropriate elution solvent. Injected amounts usually equalled 20 µl of extract solution per acquisition. The samples were manually filtered through a 0.45 µm filtering disk before feeding into the auto sampler for injection through a column. The flow was split after the column to the variable PDA UV detector and the mass spectrometer at a ratio of 15:1 respectively.

3.3.3. GC/MS

GC/MS was performed on an Agilent (Hewlett Packard) HP 6890/5973 GC/MSD instrument equipped with a fused silica capillary column, film (5% phenyl methyl silicone) thickness 0.25 µm and data processed using an HP computer with a Wiley 138.1 library for comparison.

3.3.4. VLC and CC

Sorbents for vacuum liquid chromatography (VLC) and column chromatography (CC) were either silica gel 60 normal grade (0.063-0.200 mm Merck 70 - 230 mesh) or flash grade (0.040 – 0.063, Merck 230 – 400). Chromatography was performed with CP grade solvents.

3.4. STRUCTURE ELUCIDATION

3.4.1. NMR Spectroscopy

NMR spectra were recorded on either a Varian Unity 400 MHz spectrometer or a Varian Unity Inova 500 MHz spectrometer operating at 400 and 500 MHz for ^{1}H and 100 and 125 MHz for ^{13}C using standard pulse programs. The internal reference standard used was tetramethylsilane (TMS). Spectra were obtained for solutions in deuterium solvents.

Multiplicity for ^{13}C was deduced from DEPT experiments; s = C, d = CH, t = CH_2, q = CH_3. Structural assignments were based on spectra resulting from one or more of the following NMR experiments: ^{1}H, ^{13}C, DEPT, ^{1}H-^{1}H COSY, ^{1}H-^{13}C direct correlation (HMQC), ^{1}H-^{13}C long-range correlation (HMBC) and ^{1}H difference NOE.

3.4.2. Mass Spectrometry

Mass spectral data was obtained on a GC-EIMS (70 eV) at the CSIR laboratories in the Biosciences platform. FABMS was obtained from a Micromass Model AutoSpec ETOF EI^{+} (70 eV) spectrometer at North West University (Potchefstroom). HREIMS were obtained from the University of the Witwatersrand using a Model VG 705EQ operating in positive EI mode.

3.4.3. Optical Rotation

Optical rotations were measured using a Perkin Elmer 241 Polarimeter equipped with a 1 mL cell, cell length 10.000 cm. Rotations were recorded using the Na lamp at 589 nm.

3.5. DESCRIPTION OF BIOLOGICAL ASSAYS

3.5.1. *In vitro* anticancer screen

Assay Laboratory: CSIR Biosciences, Bioprospecting Platform, Pretoria (RSA)

Methodology

The anticancer screening programme at the CSIR housing three cell lines was initiated as a collaborative project with the National Cancer Institute (NCI) in the USA aimed at the discovery of new anticancer drugs. The protocol used for the anticancer screening at CSIR was successfully transferred from the NCI and the cancer cell lines were provided by the NCI under the collaborative agreement.

The three cell line pre-screening method was used in the testing of the extracts and the compounds that were isolated from the extracts. Extracts were initially screened at the CSIR at a single dose (100 µg/ml concentration) to determine their anticancer activity against the three cell lines viz. **MCF7** (breast), **TK10** (renal), and **UACC62** (melanoma). Extracts that exhibited a growth inhibition of above 75% for two or three cell lines were subjected to further screening at five serial dilution concentrations. The results obtained from this test were presented as dose-response curves (Fig. 3.3) for each cell line.

The dose-response curve is a plot of the percent growth inhibition (GI) of the cancerous cells against the concentration of the extract or the compound presented in parts per million (ppm or µg/ml). The response parameters were interpolated values from these graphs representing the concentrations at which the GI was +50, 0, or -50 respectively interpreted as fifty percent growth inhibition (GI_{50}), total growth inhibition (TGI) and the fifty percent lethal concentration (LC_{50}).

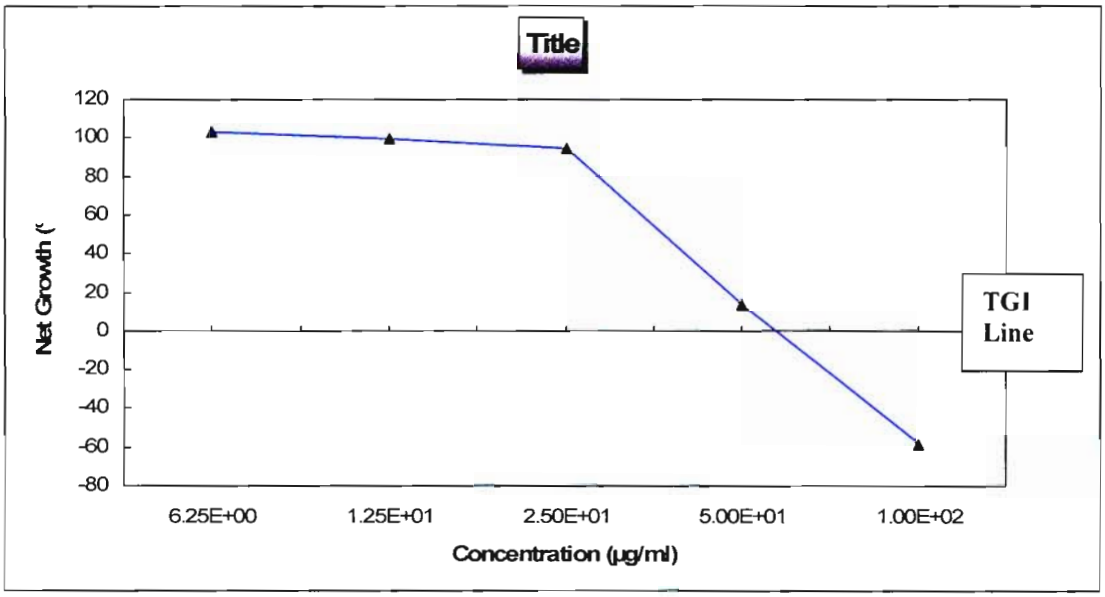

Figure 3.3: Typical graph representing the plotted anticancer screen results

The following criteria were used to interpret the activity:

Inactive = TGI (Total Growth Inhibition) > 50 ppm

Weak = TGI between 15 ppm and 50 ppm for two or three cell lines

Moderate = TGI between 6.25 ppm and 15 ppm for two or three cell lines

Potent = TGI < 6.25 ppm for two or three cell lines

3.5.2. *In vitro* 60 cell lines anticancer screen[1,2,3]

Assay Laboratory: National Cancer Institute (NCI) in the USA

The screening was a two-stage process, beginning with the evaluation of all extracts or compounds against the 60 cell lines at a single dose of 100 ppm or 100 µM. The output from the single dose screen was reported as a mean graph. Compounds and extracts which exhibit significant growth inhibition were evaluated against the 60 cell panel at five concentration levels.

Selected plant extracts and compounds that showed some cytotoxicity against the three cancer cell lines at CSIR preliminary screen were assayed by the NCI against their 60 cancerous cell lines. The panel of cancer cell lines includes sub-cells from leukaemia (6), non-small lung cell (NSLC, 8), colon (6), CNS (4), melanoma (8), ovarian (6), renal (7),

prostate (2) and breast (6) (the values in brackets are the number of sub-cells in each cell line). A statistical programme was used to obtain the log GI_{50} value (logarithm of the concentration at 50% growth inhibition) to categorize the resulting anticancer activity as interpreted by the NCI.

Methodology

The protocol used by the NCI to screen the compounds and extracts is the same as the one adopted by the CSIR and it has been published in scientific papers[4,5,6] and the protocol can also be obtained from the NCI website.

Using the absorbance measurements time zero (Tz), control growth, (C), the percentage growth was calculated at each of the drug concentration levels. Three dose-response parameters were calculated for each experimental agent. Growth inhibition of 50 % (GI_{50}) was calculated as a concentration where 50% of the net protein increase is reduced (as measured by SRB staining) in control cells during the drug incubation. The drug concentration resulting in total growth inhibition (TGI) was also calculated. The LC_{50} (concentration of drug resulting in a 50% reduction in the measured protein at the end of the drug treatment as compared to that at the beginning) indicating a net loss of cells following treatment was calculated equal to -50. Values were calculated for each of these three parameters if the level of activity was reached; however, if the effect was not reached or was exceeded, the value for that parameter was expressed as greater or less than the maximum or minimum concentration tested. Significant potency was considered for at least two of the three cell lines.

Weak = log GI_{50} between 1.10 ppm and 1.50 ppm
Moderate = log GI_{50} between 0.00 ppm and 1.10 ppm
Potent = log $GI_{50} < 0.00$ ppm

The extracts that showed weak to potent inhibition of cancerous cell growth were fractionated to isolate the compounds responsible for the activity. The compounds were also assayed at five different concentrations. The results obtained from this test were presented as dose-response curves for each cell line.

3.5.3. *In vitro* cytotoxicity assay against a mammalian cell line, Chinese Hamster Ovarian cells (CHO)

Assay Laboratory: Department of Medicine, Division of Pharmacology, University of Cape Town (RSA)

Methodology

The samples were tested for *in vitro* cytotoxicity against a mammalian cell line, Chinese hamster ovarian cells (CHO), using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. All samples were tested in triplicate in a single experiment. The MTT-assay was used as a colorimetric assay for cellular growth and survival and compares well with other available assays.[7,8] The tetrazolium salt MTT was used to measure all growth and chemosensitivity.

Aqueous samples were dissolved in water and organic samples were dissolved in methanol-water (1:9). The samples that did not dissolve completely or formed a suspension were dissolved in DMSO. The samples that did not dissolve even when using DMSO were tested as a suspension. The initial concentration of stock solutions was 2 mg/ml for all samples. Samples were stored at -20 °C until use. The highest concentration of methanol to which the cells were exposed to had no measurable effect on the cell viability (data not shown). Emetine was used as the positive control in all experiments. The initial concentration of emetine was 100 µg/ml, which was serially diluted in complete medium with 10-fold dilutions to give 6 concentrations, the lowest being 0.001 µg/ml. The same dilution technique was applied to all other samples with an initial concentration of 100 µg/ml to give 5 concentrations, with the lowest concentration being 0.010 µg/ml.

The 50% inhibitory concentration (IC_{50}) values for these samples were obtained from dose-response curves, using a non-linear dose-response curve fitting analyses via Graph Pad Prism v.2.01 software.

3.5.4. Antibacterial screen

Assay Laboratory: University of Pretoria, Pharmacology department, South Africa

Methodology

The crude extracts supplied were re-suspended to a known concentration of 20 mg/ml. The extract was suspended in a small amount of DMSO and made up to the desired concentration with water.

The test organisms in the investigations of antibacterial activity included two Gram-positive bacteria, *Enterococcus faecalis* and *Staphylococcus aureus*, and two Gram-negative species, *Pseudomonas aeruginosa* and *Escherichia coli*. A micro plate serial dilution method[9] was used to evaluate the antibacterial activity of extracts. This method allows the calculation of minimal inhibitory concentration (MIC) values for active plant extracts against each bacterial species. A two-fold serial dilution of plant extract was prepared in 96-well micro titre plates and bacterial culture was added to each well. The presence of bacterial growth was detected by the addition of a tetrazolium salt, which was reduced to a red-coloured formazan by biologically active organisms; in this case the dividing bacteria. When the solution in the well remains clear after addition of the extract, bacterial growth was inhibited by that concentration of plant extract. Neomycin was used as a positive antibiotic control and appropriate negative solvent controls were included.

In order to establish the possible compounds responsible for the inhibition of bacterial growth, the components of the sample were separated by TLC using ethyl acetate:methanol:water [45:5:4.4] [EMW] as solvent system.

Bioautography was performed as a supplementary qualitative analysis for the presence of antibacterial compounds. After the diffusion of the eluent at room temperature, the TLC plate was sprayed with a suspension of *S. aureus*. After overnight incubation, the plate was sprayed with a tetrazolium salt to identify the presence of antibacterial compounds in the samples. The active compounds appeared as white spots (no bacterial growth) on a pink background of dividing bacteria.

3.5.5. Rabbit corpus cavernosum smooth muscle relaxation / contraction assay

Assay Laboratory 1: University of Pretoria, South Africa

Methodology

The bioassay was performed as described by Levin et al.[10] with some minor changes. Strips (12 mm long and 1-2 mm thick) of rabbit corpus cavernosum smooth muscle were dissected and mounted in an organ-bath chamber containing Krebs-PSS solution with the following composition: NaCl = 7.01 g/l, KCl = 0.34 g/l, KH_2PO_4 = 0.1 g/l, $NaHCO_3$ = 1.99 g/l, $CaCl_2$ = 0.2 g/l, $MgSO_4$ = 0.3 g/l and glucose = 1.8 g/l. One end of the muscle was tied to the inside bottom of the perfusion bath and the other end to a thin wire connected to a Harvard isotonic force transducer for isotonic tension measurements. Changes in isotonic tension were recorded on a computerized calibrating program. The corpus cavernosum muscle was perfused with 2ml Krebs-PSS buffered saline and oxygenated with 95% O_2 and 5% CO_2 for 5 min to ascertain a stable baseline recording. This was followed by perfusion with 2 ml of $CaCl_2$ in Krebs-PSS (17.8 mg/ml) for muscle contraction. Baseline tension was set at the point of maximal contraction following the addition of $CaCl_2$ into the experimental bath. The extracts to be analyzed were added after a stable contraction baseline. The same procedure (and concentration) was repeated for the positive control, Sildenafil. The contraction/relaxation was reported relative to sildenafil (Viagra) tested at 78 ng/ml. In these experiments the stimulation frequency used for rabbit strips was 9 Hz.

Assay Laboratory 2: MDS Pharma, Taiwan

Methodology

Corpus cavernosum obtained from New Zealand-derived albino male rabbits weighing 2.5 - 3.0 kg and sacrificed by CO_2 overexposure was used. A strip from the basal area of the corpus cavernosum was removed and placed under 2 g tension in a 10 ml bath containing Krebs solution pH 7.4 at 32 ^{o}C and sub-maximal isometrically recorded tonic contraction was induced by phenylephrine (3 μM). Test substance (30 μM)-induced relaxation by 50 percent or more (≥50%) within 5 minutes, relative to the control 0.3 μM sodium nitroprusside response, indicates significant relaxation.

Reference Data:

Compound	EC_{50} (μM)
Acetylcholine	0.093
Dipyridamole	>30
Nifedipine	0.075
PGE_1 (Prostaglandin E_1)	4.2
Prazosin	0.156
Rolipram	0.22
Sildenafil	0.042
*Sodium nitroprusside	0.074

*Indicates standard reference agent used.

The method used is cited also in publications by Angulo et al.[11] and Thompson et al.[12].

3.5.6. Phosphodiesterase 5 enzyme (PDE5) inhibition assay

Assay Laboratory: MDS Pharma, Taiwan

Methodology

PDE5 partially purified from human platelets was used. Test compound and/or vehicle were incubated with 3.5 μg enzyme[#] and 1 μM cGMP containing 0.01 μM [^{3}H]cGMP in Tris buffer pH 7.5 for 20 minutes at 30 °C. The reaction was terminated by boiling for 2 minutes and the resulting GMP is converted to guanosine by addition of 10 mg/ml snake venom nucleotidase and further incubation at 30 °C for 10 minutes. Unhydrolyzed cGMP is bound to AG1-X2 resin and remaining [^{3}H]guanosine in the aqueous phase is quantitated by scintillation counting. Compounds were screened at 100 μM.

[#]since enzyme activity may change from lot to lot; the concentration used was adjusted if necessary.

Reference Data:

Compound	IC_{50} (μM)
Dipyridamole	5.9
*IBMX(3-isobutyl-1-ethylxanthine)	63
Milrinone	>100
Sildenafil	0.0041

*Indicates standard reference agent used.

The method is also published in Hidaka et al.[13] and Nicholsen at al.[14].

3.6. REFERENCES

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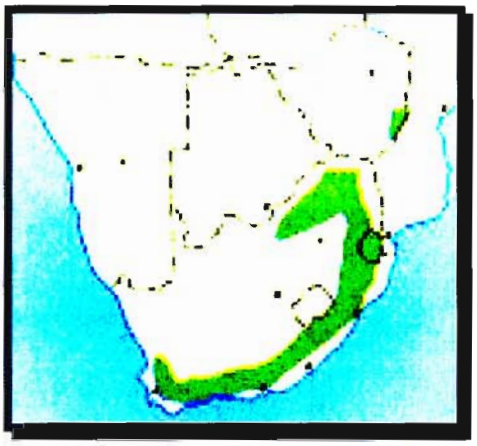
Phylica paniculata Willd. (Fig. 4.1) is a perennial shrub or small tree with grey-green foliage that occurs on moist slopes and bush and forest margins.[2] It can be easily confused with *P. buxifolia* because of the type of leaves and flowers that are cream and aromatic (compare in Fig. 4.1). *P. buxifolia* is common from the Cape Peninsula area (Table Mountain) to Caledon and grows on mountain slopes near the sea and in rocky places. The distribution of *P. paniculata* is from the Hex River valley and Wittenberg to Zimbabwe[2] (Fig. 4.2).



"Pictures courtesy of the Field guide to Trees of Southern Africa"[3]

Figure 4.2: Distribution of *Phylica paniculata* in Southern Africa

P. paniculata was collected by a botanist who was contracted by CSIR to collect endemic plants at random. The crude extract inhibited the growth of cancerous cells *in vitro*. This chapter reports the isolation, identification and activity of the active ingredient and another structurally related compound.

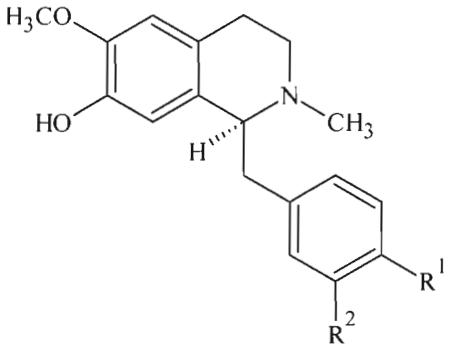
4.1.2. Ethnopharmacology

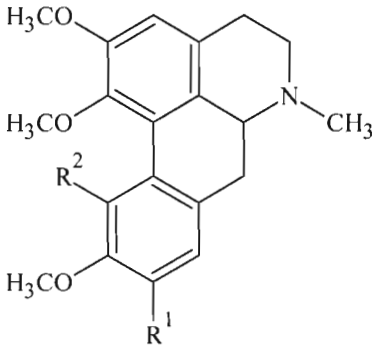
Desktop literature studies revealed no published ethnopharmacological information on this species and its related species.

4.2. PHYTOCHEMICAL STUDIES

A literature study did not yield any published information on the phytochemistry of *P. paniculata*. Four alkaloids, reticuline **(4.1)**, *N*-methylcoclaurine **(4.2)**, lauroscholtzine **(4.3)** and isocorydine **(4.4),** were isolated from the related species *P. rogersii*.[4,5] These alkaloids

have antiplatelet aggregation activity, weak neuromuscular inhibition, cardiac depressant, antimicrobial properties and no anti-tussive activity.[6]





4.1: Reticuline $R^1 = OCH_3$, $R^2 = OH$

4.2: Coclaurine $R^1 = OH$, $R^2 = H$

4.3: Lauroscholtzine $R^1 = OH$, $R^2 = H$

4.4: Isocorydine $R^1 = H$, $R^2 = OH$

From other genera of the Rhamnaceae family alkaloids, polyphenols, anthracenones, quinones, tetracosanolides, procyanidin glycosides and triterpenoids have been isolated.

4.3. ISOLATION OF THE ACTIVE COMPOUND

Plant material of *P. paniculata* was collected from a farm near Marikana, Northwest Province. The plant material was dried, ground, extracted and partitioned as indicated in Fig. 4.3. The extract and fractions were assayed for growth inhibition of three cancer cell lines (melanoma **UACC-62**, renal **TK-10**, and breast **MCF-7**) in the CSIR laboratories. The activity was associated with the dichloromethane fraction **4-D** (Fig. 4.3) and column chromatography of this extract yielded an active fraction labeled **4-F2.** Spectroscopic analyses of this fraction led to the identification of a pentacyclic triterpenoid ursolic acid (**4.5**). This compound was isolated as a white amorphous powder with molecular formula $C_{30}H_{48}O_3$.

The amount of ursolic acid isolated was insufficient to conduct all spectroscopic and experiments for the proper identification of this compound. Thus, from the same batch of collected plants, the medium polar components were isolated following a method summarised in Fig. 4.4. This protocol was followed because the dichloromethane fraction

obtained by liquid–liquid partitioning in the first isolation method was selectively active. TLC analysis confirmed that the dichloromethane extract **4-G** (Fig. 4.4) contained the same compounds as the dichloromethane fraction **4-D** (Fig 4.3). The two fractions also exhibited similar anticancer activity. Repeated column chromatography of the combined fractions **4-D** and **4-G** on silica gel yielded ursolic acid (**4.5**) and a second pure compound, α-amyrin (**4.6**).

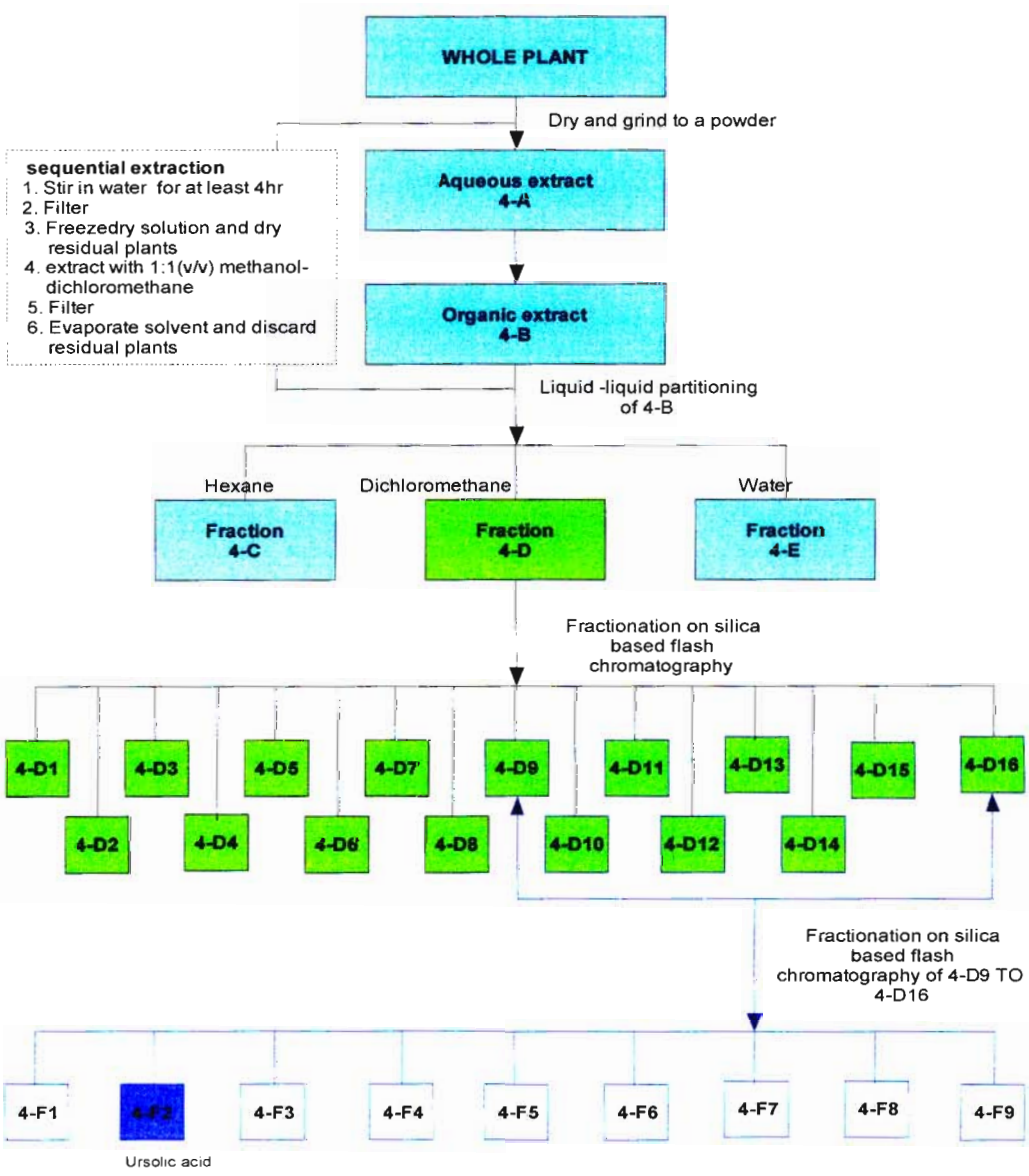


Figure 4.3: General isolation procedure followed to isolate the bioactive compound.

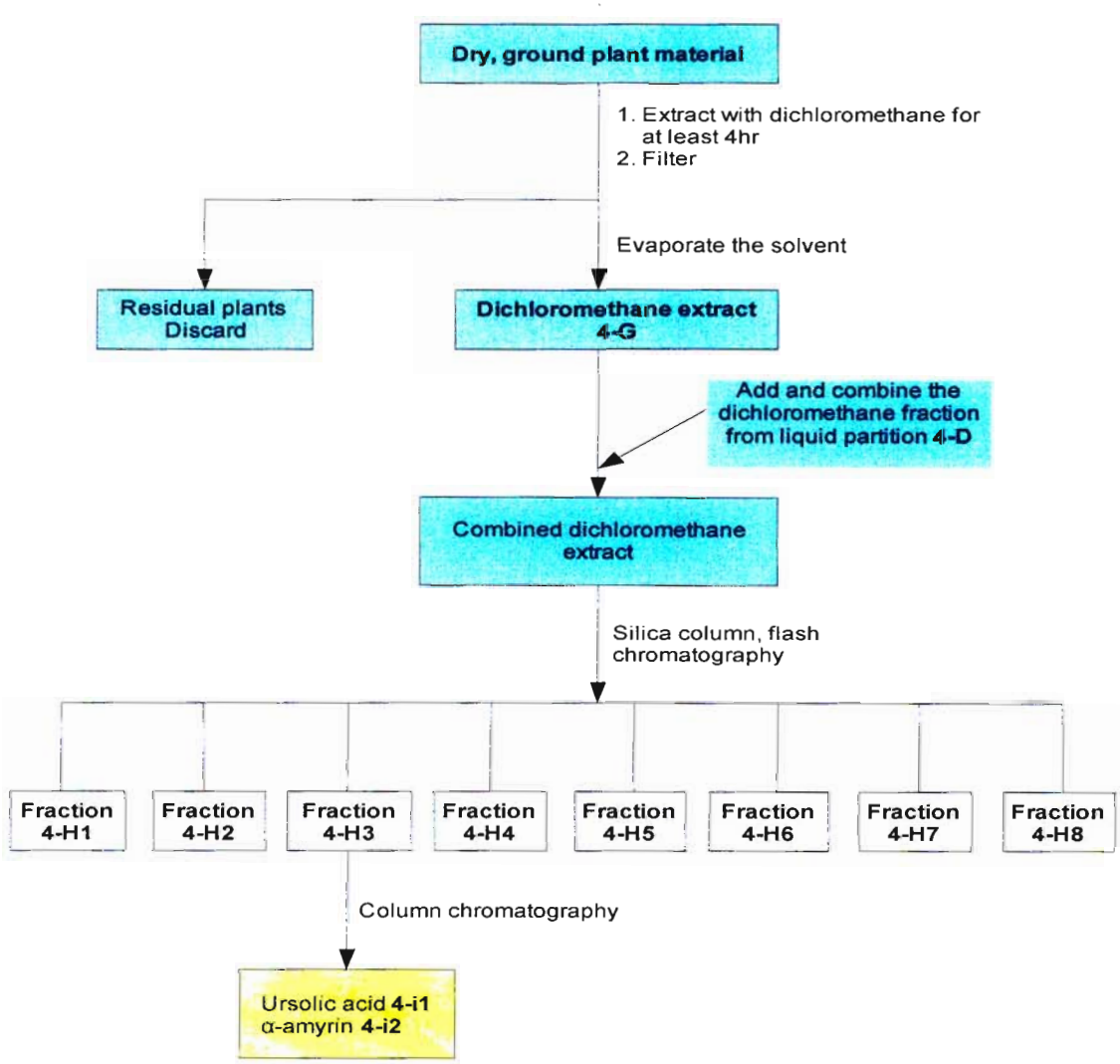
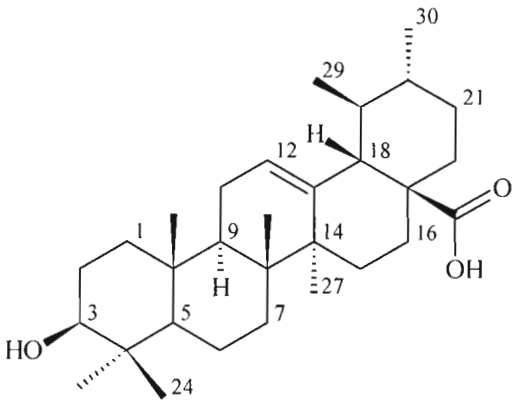


Figure 4.4: Schematic illustration of the isolation of α-amyrin and ursolic acid

4.3.1. Characterisation of ursolic acid



4.5: Ursolic acid

Ursolic acid (**4.5**) isolated from the organic extract of the leaves of *Phylica paniculata* was not visible on a TLC plate under either long (360 nm) or short (254 nm) wavelength UV light. However, after spraying the plate with vanillin spraying reagent and heating it at 100 ^{o}C for a few minutes, the compound appeared as a pink spot on a clear background.

NMR data of the compound revealed thirty resonance signals in the ^{13}C spectrum, suggesting that the compound could be a triterpenoid. Structural elucidation of triterpenoids is challenging, especially to characterize the complex saturated alkyl region. In the ^{13}C spectrum, there is evidence for a carbonyl function (δ_C 180.3), a trisubstituted alkene (δ_C 138.0 and 125.4), an oxygen-containing methine (δ_C 78.8) and seven methyl groups. In the ^{1}H NMR spectrum, five tertiary methyl (δ_H 0.76, 0.74, 0.88, 0.91 and 1.01) and two secondary methyl groups (δ_H 0.79 and 0.86) were identified. Correlations between protons of two geminal methyl groups and the signal at δ_C 78.8 confirmed the presence of a hydroxyl group at C-3.

Three well-known and dominant types of triterpenoids were initially considered namely, ursane, oleanane and lupane derivatives. A review article summarising ^{13}C NMR data of 99 of triterpenoids[7] assisted us with the assignment of the structure. Comparison with the published data enabled the assignment of a C-28 carboxylic acid at δ_C 180.3 and located the double bond (δ_C 125.4 and 138.0) between C-12 and C-13. The presence of a 29-methyl group attached to C-19 is believed to cause deshielding of the double bond carbon C-12 and shielding of C-13 as compared to the resonances of the oleanolic acid double bond.[8,9]

From the proton spectrum, five methyl singlets (δ_H 0.76, 0.74, 0.88, 0.91, and 1.01) could be assigned together with two doublets (δ_H 0.79, J_{HH} = 6.0, δ_H 0.86, J_{HH} = 7.0). The olefin proton in position C-12 was assigned to a resonance signal at δ_H 5.08.

The structure of compound **4.5** was assigned as ursolic acid. The structure was confirmed by comparison of the obtained NMR data with the published literature data together with the reported MS data as well as optical rotations.[10,11]

In the EI^+ mass spectrum, a molecular ion at m/z 456 agrees with a molecular formula $C_{30}H_{48}O_3$. The library match from the GC-MS instrument identifies ursolic acid with a 97% possibility match.

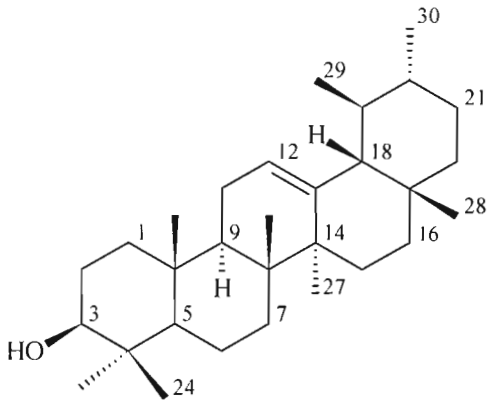
Table 4.1 : ^{13}C (100 MHz) and ^1H (400 MHz) data for ursolic acid (**4.5**) in $CDCl_3$ + CD_3OD.

	Compound 4.5		Literature[11,†]	
C	δ_C	δ_H (*J* in Hz)*	δ_C	δ_H (*J* in Hz)
1	38.6	1.16-1.51 (m,2H)	39.2	1.00-1.58
2	28.6	1.85 (m,2H)	28.2	1.81
3	78.7	3.12 (m,1H)	78.2	3.44 (dd)
4	38.5		39.6	
5	55.1	0.87 (m,1H)	55.9	0.88 (d)
6	18.2		18.8	1.58-1.39
7	32.9		33.7	1.59-1.39
8	39.3		40.1	
9	47.4		48.1	1.65
10	36.8		37.5	
11	23.1		23.7	1.96
12	125.4	5.18 (s, broad,1H)	125.7	5.49 (s)
13	138.0		139.3	
14	41.9		42.6	
15	29.5		28.8	1.22-2.33
16	24.1		25.0	2.14-2.01
17	47.7		48.1	
18	52.7	2.12 (d, 1H, *J* =11.2)	53.6	2.63 (d)
19	38.9		39.5	1.49
20	38.7		39.4	1.05
21	30.5		31.1	1.40-1.49
22	36.7		37.4	1.97
23	27.9	0.91 (s, 3H)*	28.8	1.24 (s)
24	15.2	0.74 (s, 3H)*	16.5	1.02 (s)
25	15.4	0.70 (s, 3H)*	15.7	0.92 (s)
26	16.7	0.87 (s, 3H)*	17.5	1.06 (s)
27	23.3	1.01 (s, 3H)*	24.0	1.24 (s)
28	180.3		179.7	
29	16.8	0.85 (d, 3H, *J* = 5.9)	17.5	1.02 (d)
30	21.0	0.79 (d, 3H, *J* = 6.3)	21.4	0.97 (d)

† The experiments were done at 313 K with compounds dissolved in pyridine-d_5
* The assignments of the methyl signals can be interchanged.

There have been a number of reassignments of the carbon signals of this compound since its early characterization[12,13] but assignments were recently confirmed by 2D NMR experiments (see Table 4.1). It was also cited that the resonance assignments of this compound has been revised as the ^{13}C shifts for C-24/C-25 are interchanged.[10]

4.3.2. Characterisation of α-amyrin (4.6)



4.6: α–Amyrin

This triterpenoid showed a single spot on TLC in various solvent systems but with an R_f value in close proximity to that of ursolic acid. In the ^{13}C NMR spectrum of compound **4.6,** 30 resonances were observed. A closer inspection of the data showed the ^{13}C data to be similar to that of ursolic acid (**4.5**). The main difference is the absence of the carboxylic acid resonance peak at δ_C 180 (C-28). Inspection of the spectral data pointed to the substitution of the C-19 carboxylic group with a methyl group (δ_C 28.2). Two signals at δ_C 126.0 and 138.1 were assigned to olefinic carbons.

As with ursolic acid (**4.5**), compound **4.6** gave 2 doublet methyl signals δ_H 0.75 and δ_H 0.87 assigned to C-29 and C-30, respectively. The chemical shifts observed for C-12 and C-13 are in agreement with an urs-12-ene skeletal structure. Singlet signals representing three protons each were identified upfield from TMS signal at δ_H 0.95, 0.74, 0.75, 1.04, 1.21, 0.89, 0.82 (J = 6.3 Hz) and 0.91 (J = 6.3 Hz) for respective positions C-23 to C-30 that were assigned to the methyl protons in a quaternary environment.

The structure of compound **4.6** was assigned as α-amyrin. The EI^+ mass spectrum of the compound shows a molecular ion peak at *m/z* 426, which supported the molecular formula of $C_{30}H_{50}O$. Comparison with the ^{13}C NMR of uvaol[7] (28-hydroxy-α-amyrin) led to the suggested structure of compound **4.6**.

Table 4.2: ^{13}C (100 MHz) and ^{1}H (400 MHz) data for α-amyrin (**4.6**) in $CDCl_3$.

	Compound 4.6 (α-amyrin)		**Literature**[14,15,16]	
C	δ_C	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
1	38.9		38.7	
2	27.2		27.2	
3	79.2	3.18 (dd, *J* = 10.7 & 5.6)	79.0	3.25 (m,)
4	38.8		38.7	
5	55.4		55.2	
6	18.4		18.3	
7	33.1		32.9	
8	39.6		40.0	
9	47.7		47.7	
10	37.1		36.9	
11	23.7		23.3	
12	126.0	5.08 (t, *J* = 3.5)	124.4	5.24 (t, *J* = 3.5)
13	138.1		139.5	
14	42.1		42.0	
15	28.9		28.7	
16	24.3		26.6	
17	36.6		33.7	
18	52.9		58.9	
19	39.0		39.6	
20	39.2		39.6	
21	30.8		31.2	
22	36.8		41.5	
23	28.3	0.95 (s)	28.1	0.99 (s)
24	15.6	0.74 (s)	15.6	0.79 (s)
25	15.7	0.75 (s)	15.6	0.95 (s)
26	17.1	1.04 (s)	16.8	1.01 (s)
27	23.4	1.21 (s)	23.2	1.07 (s)
28	28.2	0.89 (s)	28.1	0.84 (s)
29	17.2	0.82 (d, *J* =6.3)	17.4	0.79 (d, *J* = 5.5)
30	21.3	0.91 (d, *J* = 6.3)	21.3	0.91 (d, *J* = 5.5)

The ^{1}H NMR spectra data showed a doublet of a doublet at δ_H 3.18 which was assigned to the proton attached to position 3 adjacent to the hydroxyl group. The splitting pattern (doublet of a doublet) resulted from the interaction between the diastereotopic protons in position 2, one situated in the alpha and the other in the beta position. The coupling constants observed for H-3 (*J* = 10.7 and 5.6 Hz) confirms the axial orientation of this proton and the β-orientation of the 3-hydroxyl group.

A triplet resonance signal (δ_H 5.08, *t*, J= 3.5 Hz) was assigned to the C-12 protons which is characteristic of the coupling observed between a proton on C-12 and the methylene protons on the sp^3-hybridised C-11.

4.3.3. Analysis of plant extracts and primary fractions by liquid chromatography.

The crude extracts were analysed by HPLC with the pump gradient starting with 90:10 water-acetonitrile and changing gradually to 100% of acetonitrile after 75 minutes. The water fraction (Fig 4.5) managed to extract most of the highly polar compounds with a retention time of less than 15 minutes where the solvent gradient was still predominately water. The dichloromethane extracted most of the compounds in the mid-polar region. There was one distinct major peak at 62.79 minutes that still remains to be identified. This peak representing an unidentified compound was selectively extracted by dichloromethane. Apart from this compound there were several other peaks which are representative of the minor compounds. Lastly, after 60 minutes when the solvent was almost 100% organic, the least polar compounds were detected.

At a glance it was concluded that the attempt to separate the organic extract into three fractions containing compounds of different polarity was a success. The UV fingerprint of the methanol-dichloromethane extract is a total representative of the fractions obtained from the partitioning as a means of primary fractionation.

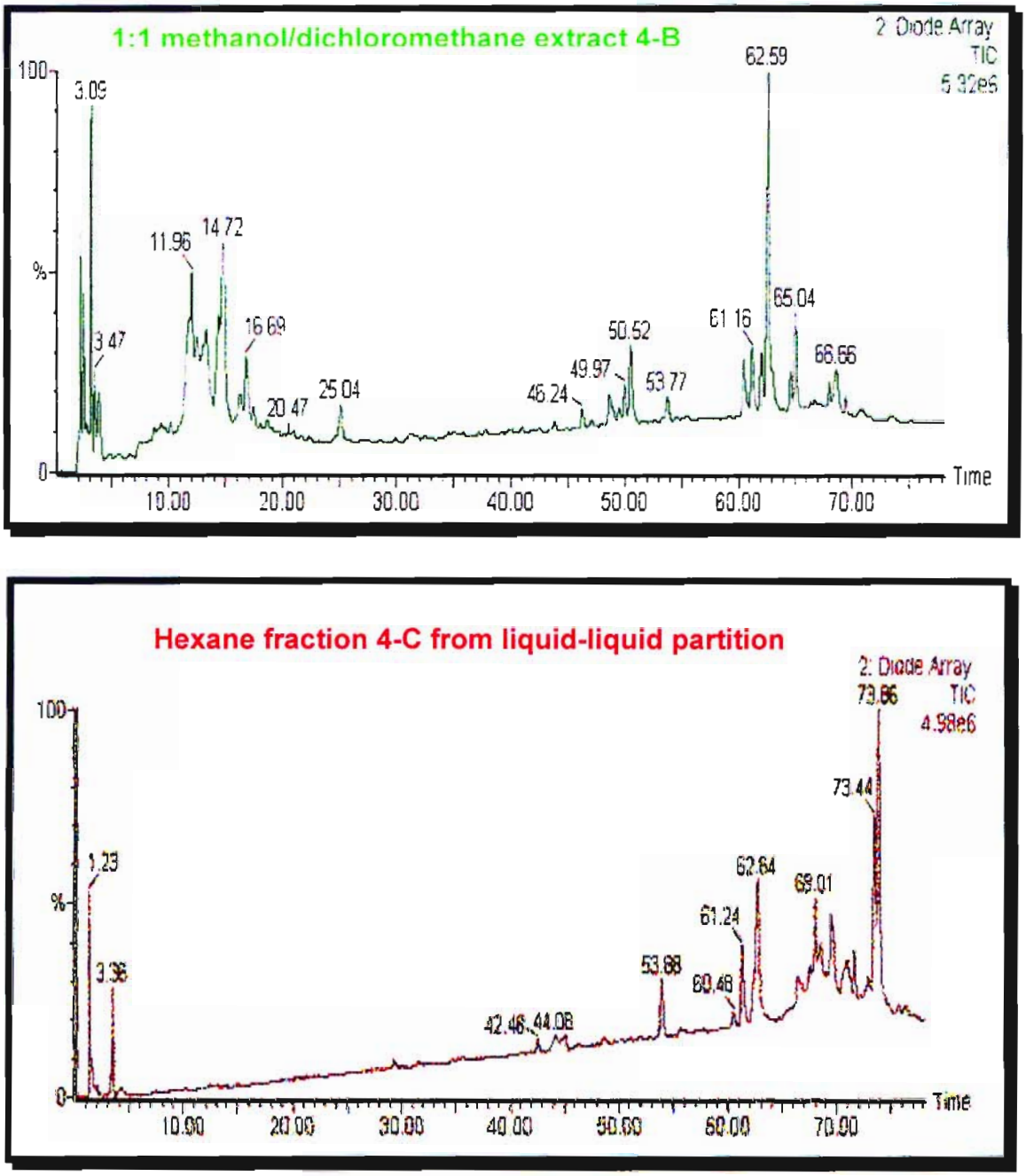
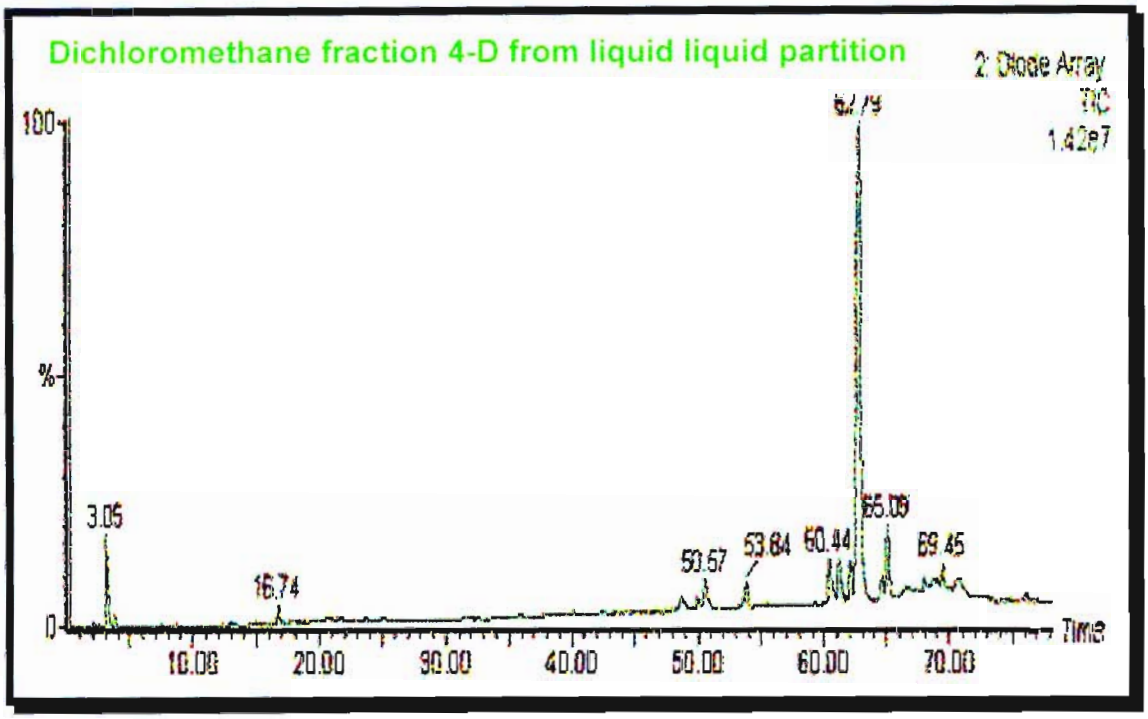


Figure 4.5: LC-DAD chromatograms of the organic methanol-dichloromethane extract prepared of the whole plant and of the fractions obtained for the liquid-liquid partitioning using hexane, dichloromethane and water.



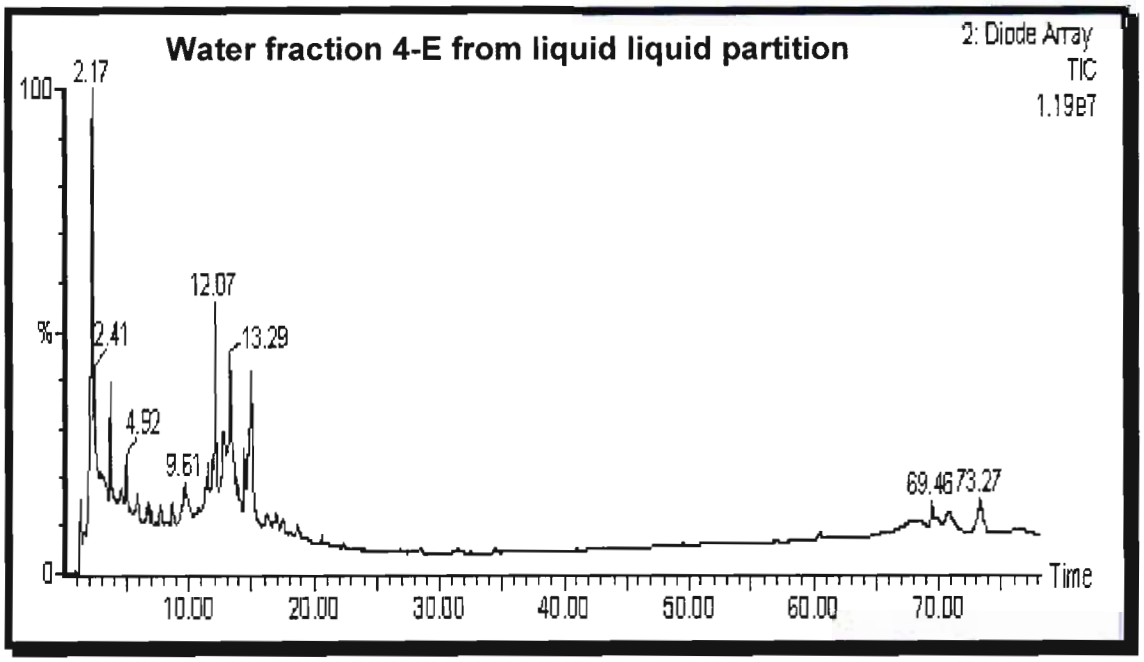


Figure 4.5. Continued: LC-DAD chromatograms of the organic methanol-dichloromethane extract prepared of the whole plant and of the fractions obtained for the liquid-liquid partitioning using hexane, dichloromethane and water.

4.4. BIOLOGICAL ACTIVITY

The plant extract and the compounds obtained were tested for anticancer activity against three highly sensitive cell lines *viz.* melanoma UACC-62, renal TK-10, and breast MCF-7 in the CSIR laboratories.

Extracts of the whole plant were prepared as shown in Fig 4.3. Sequential extraction yielded an aqueous extract **4-A** and a methanol-dichloromethane extract **4-B**. These extracts were tested for anticancer activity at a single dose concentration (100 μg/ml) and only the organic extract showed a noteworthy inhibition of the growth of cancerous cells. The results obtained from the CSIR laboratories were confirmed by the National Cancer Institute (NCI) who obtained a weak activity (log GI_{50} = 1.33 μg/ml) for the extract with no selectivity to the different cells. This finding does not rule out a non-selective cytotoxic effect of the extract on the cells of all types.

Table 4.3: Anticancer activity of the extracts

Sample	Extract type	In-house Cancer Results*[f]	NCI Results*[f]
4-A	aqueous	Not active	Not done
4-B	organic	Weak	Weak, no selectivity

* The criteria to used for classifying results is defined in chapter 3, section 3.5.1 and 3.5.2
Weak → NCI: logGI_{50} between 1.10ppm and 1.50ppm and **CSIR:** TGI between 15ppm and 50ppm
[f] Test concentration 100ppm

The same extract **4-B** was re-tested (at CSIR) at five serial dilutions at concentrations between 6.25 and 100 μg/ml and the result thereof is given in the form of a graph in Fig. 4.5.

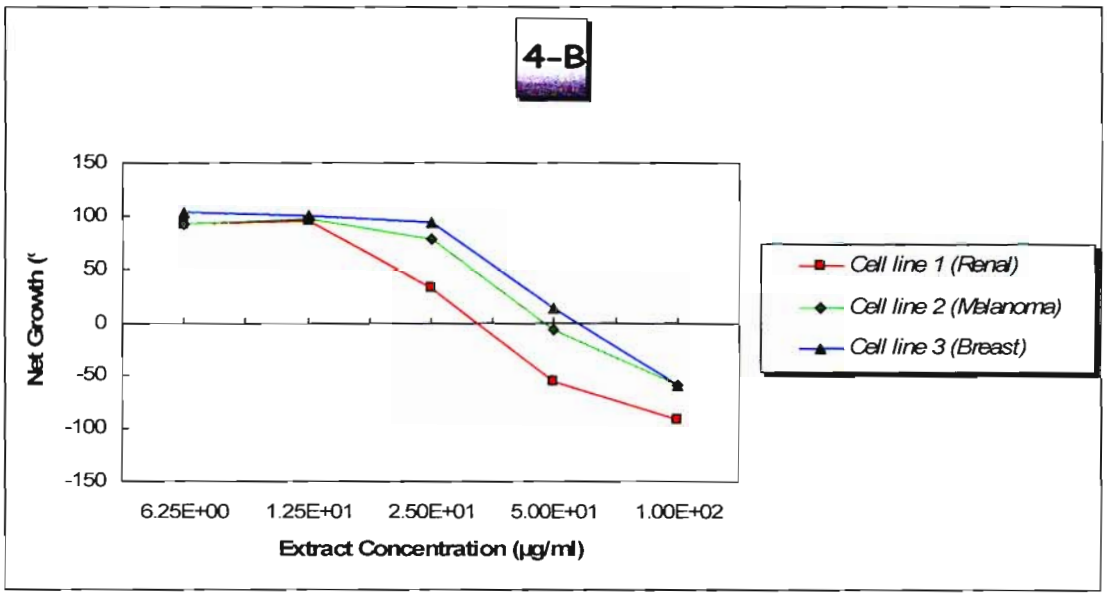


Figure 4.5: Anticancer activity of the methanol-dichloromethane extract

TGI was calculated for **4-B** as 50 µg/ml, but the renal cell line had selective TGI at a concentration less than 50 µg/ml. According to the set criteria, total inhibition of the growth of cancerous cell achieved at extract concentrations less than 50.0 µg/ml is considered weak. Based on this result, it was decided to identify and isolate the active compound(s) by chromatographic fractionation of the organic extract **4-B**.

The hexane (**4-C**), dichloromethane (**4-D**) and water (**4-E**) based fractions obtained from liquid-liquid partitioning were evaluated at five dose response for their cytotoxic activity against the three cell lines. The result of this experiment is shown in Fig. 4.6 as a dose-response graph.

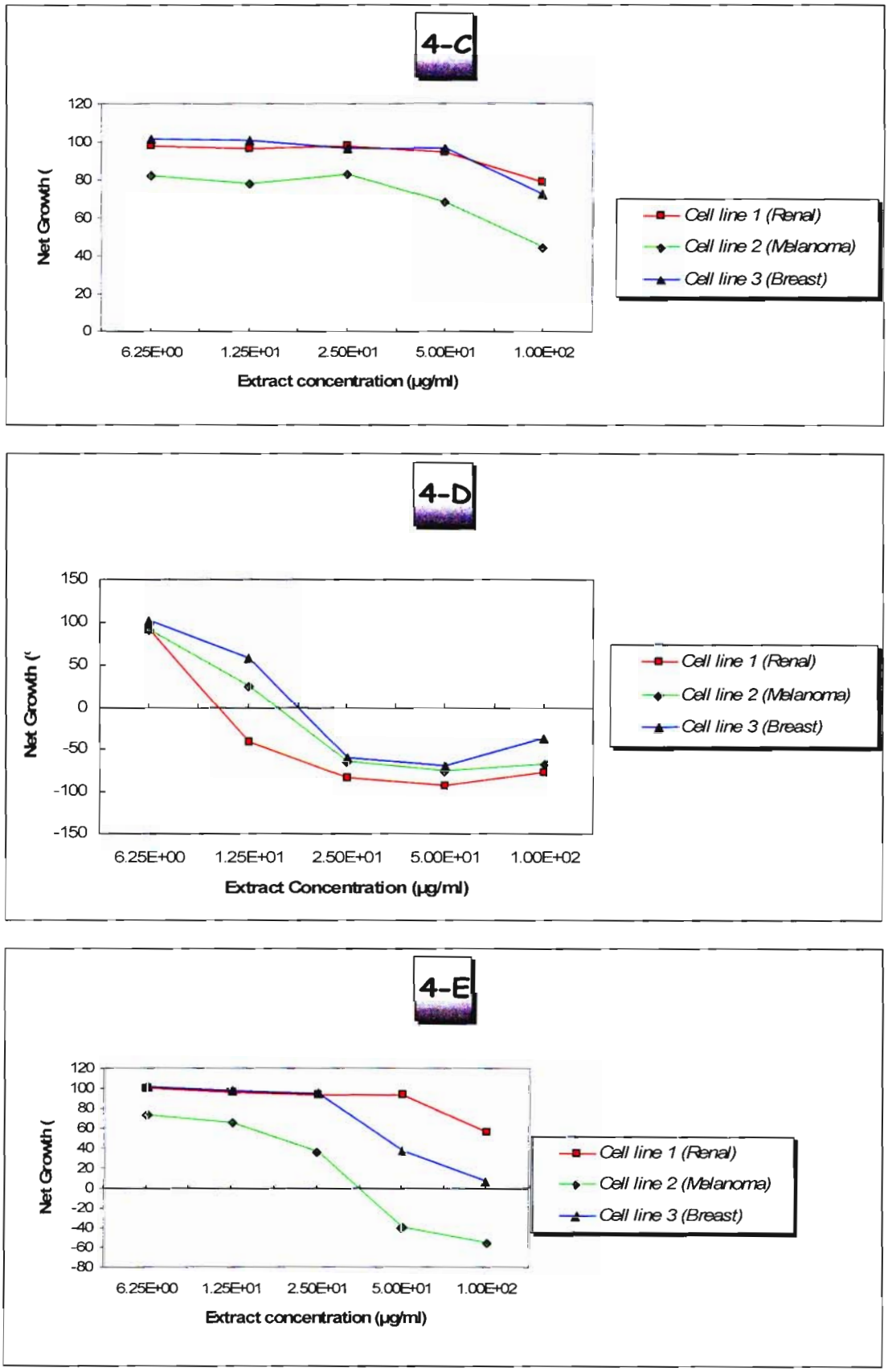


Figure 4.6: Graphical representation of the cytotoxic effect of the liquid-liquid partitioning fractions against cancer cells.

From the graphs it is evident that the non-polar hexane fraction **4-C** does not contribute to the anticancer activity. The highly polar fraction **4-E** showed low activity that could be attributed to the carry over of some compounds during the liquid partitioning. Only the dichloromethane fraction **4-D** gave an improved inhibition of the anticancer cells *in vitro* and a TGI concentration of less than 20.0 μg/ml for all three cell lines. This is more than half the concentration at which the active extract **4-B** showed the total inhibition. The dichloromethane fraction **4-D** was tested at NCI in the 60 cell line panel to confirm the observed increase in activity and it exhibited a mean log GI_{50} of 0.71 μg/ml together with a logTGI of 1.59 μg/ml which according to the criteria set is considered moderate. Of interest was the selectivity of the fraction in this cytotoxicity assay against the sixty cell lines as it was selective for the leukaemia, NSCLC, renal and PC-3 prostate. This is considered a significant improvement to the crude extract activity which was only moderate with no selectivity.

Further fractionation by repeated flash silica chromatography of the active dichloromethane fraction yielded ursolic acid (**4.5**) and α-amyrin (**4.6**). Both compounds were tested at five dose concentrations but at slightly lower concentrations than the extract and fractions test concentrations (Fig. 4.7). Only ursolic acid (**4.5**) shows activity that was found to be well below an extrapolated TGI less than 18.5 μg/ml (40.5 μM). The NCI calculated the logarithmic GI_{50} of 5.11 μg/ml obtained from the averaged sixty cell lines screening results.

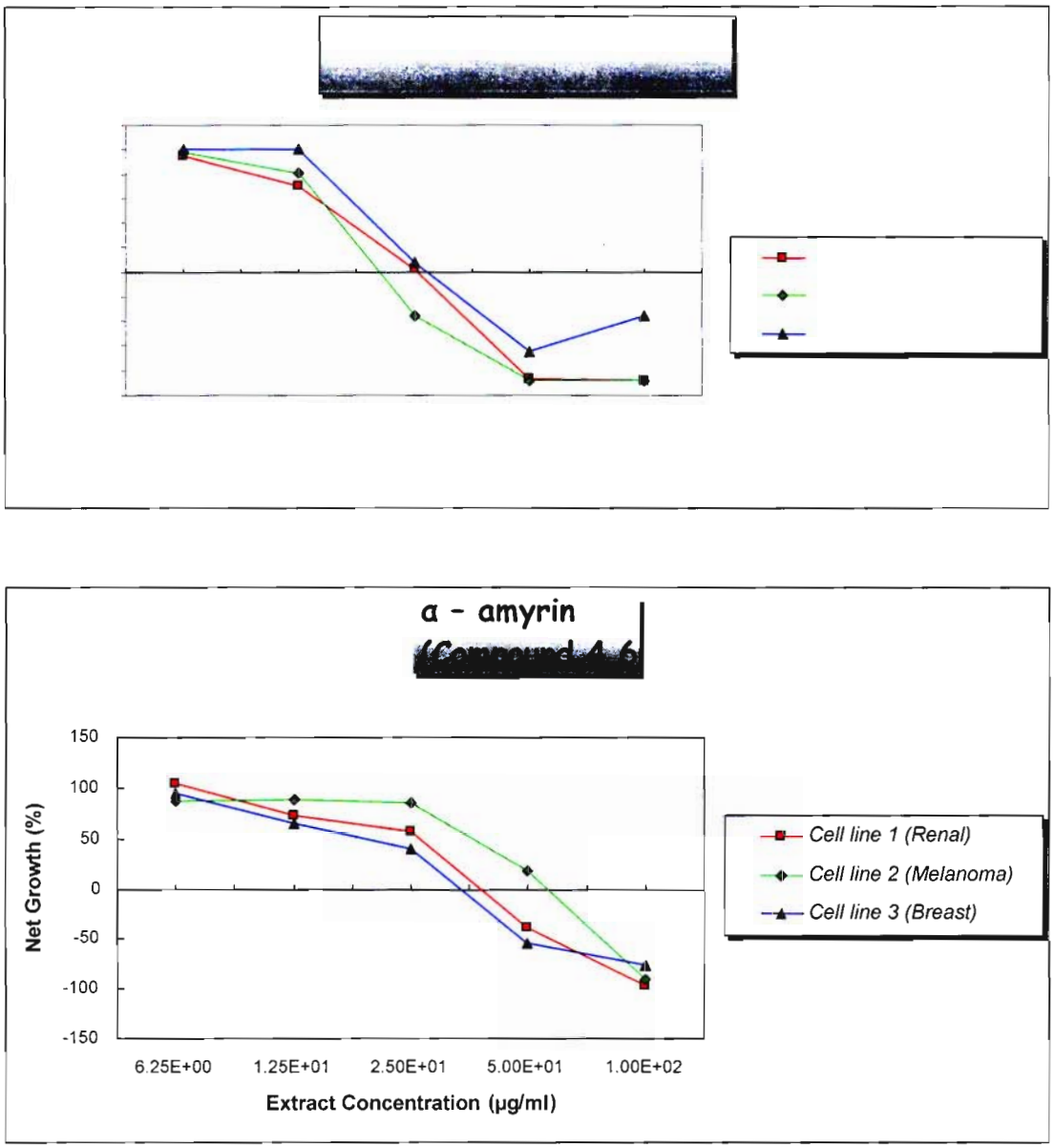


Figure 4.7: Anticancer activity of ursolic (**4.5**) acid and α-amyrin (**4.6**)

4.4.1. Reported biological activities of ursolic acid

Ursolic acid (**4.5**), also known as urson, prunol, micromerol and malol, is a pentacyclic triterpenoid that occurs naturally in a large number of vegetarian foods, medicinal herbs, and other plants. For a long time, it was considered to be pharmacologically inactive. However, it was found to be biologically active both topically and internally. Pharmacological effects reported include anti-tumor, hepatoprotective, anti-inflammatory (oral and topical), antiulcer, antimicrobial, anti-hyperlipidemic, antiviral and anti-HIV[17]

activity. Its anti-inflammatory,[18] antitumor (skin cancer)[19,20] and antimicrobial properties make it useful in cosmetic applications.[21]

This compound is also reported as an anti-infective agent, antineoplastic agent and phytogenic cyclooxygenase inhibitor.[22] *In vitro* the compound exhibits anticancer activity against the host cells with effective dose (ED_{50} which can be translated as same to GI_{50}) ranging from 3.2 to 4.6 μg/ml.[23,24] It was also reported that ursolic acid induces apoptosis in human melanoma (M4Beu) with ED_{50} of 20 μM.[25]

4.5. CONCLUSION

Ursolic acid (**4.5**) was isolated as the active constituent from *P. paniculata*. This compound and its derivatives have been patented for their anticancer properties.[26] The NCI published their own screen results of ursolic acid tested against their sixty cell lines as an averaged logarithm GI_{50} of 5.11, TGI of 4.78 and LC_{50} of 4.39 μg/ml.[27] Triterpenoids play a vital role as anticancer agents and structural modification of this class of compounds can result in the establishment of an innovative drug for the treatment of cancer. The downside of using triterpenoids in general is that their cytotoxicity is associated with their haemolytic and cytostatic properties and this necessitates the synthesis of analogues of low toxicity and improved potency.

4.6. EXPERIMENTAL

General experimental techniques are described in Chapter 3.

4.6.1. Plant material identification

A fresh herbarium plant sample was collected from Perseverance farm (GPS: 25°51'59S; 27°29'11E), R24 Marikana off ramp, Northwest Province, during September 1999 and identified by Mr. E Nienaber. A voucher specimen of the collected plant was deposited at the South African National Biodiversity Institute (SANBI) in Pretoria and the plant was identified as *Phylica paniculata* Willd. (Genspec No.: 4886000) belonging to the Rhamnaceae family.

4.6.2. Extraction and isolation of compounds

The fresh plant leaves (979.10 g) were oven dried at 45 °C. The dry plants were ground to a powder. The ground material was divided into two portions of unequal proportion. One portion (680.50g) was subjected to sequential extraction, first with purified water enough to cover the plant material and then the oven-dried residual plants were extracted with a 50% methanol-dichloromethane mixture covering the plant to afford the aqueous extract (**4-A**, 3.41 g) and organic extract (**4-B**, 20.24 g) extracts, respectively. The organic extract (**4-B**) exhibited a low activity whereas the water extract was inactive. Results from the 60 cell line screen at NCI confirmed the low and non-selective anticancer activity of the extract **4-B.** The remaining portion of plant material (298.60 g) was extracted with dichloromethane to afford the extract **4-G** (11.84 g, Fig. 4.4.).

Extract **4-B** (3.15 g) was subjected to liquid-liquid partitioning and afforded three fractions **4-C** (0.19 g, hexane extract), **4-D** (0.76 g, dichloromethane extract) and **4-E** (1.14 g, water extract) of which only the dichloromethane fraction (**4-D**) was active with improved anticancer activity as compared to the crude extract **4-B.** Fraction **4-D** was also tested at the NCI and showed moderate activity selective for leukaemia, NSCLC (Non-Small-Cell Lung Cancer) and PC-3 prostate cell lines. Fraction **4-D** was chromatographed on silica gel and elution with increasing polarities of dichloromethane-methanol generated 16 fractions of quantities ranging between 0.9 mg and 84.3 mg. The last eight fractions as eluted per

polarity range retained the anticancer activity and they were combined. The pooled (160 mg) fraction was purified on flash silica gel 60 (dichloromethane-2% methanol) to afford fractions **4-F1** (19 mg) and **4-F2,** a triterpenoid **4.5** (8.0 mg, R_f 0.18 using 1:4 ethyl acetate-hexane). The compound was not visible under ultraviolet light (254 nm), but it was visualised as a yellowish-pink spot after spraying the TLC plate with vanillin-sulfuric acid, the color changed to bright purple when heated. Increasing the polarity of the eluting solvent to dichloromethane-methanol (5%) resulted in the generation of seven fractions 4-F3 (17 mg), 4-F4 (15 mg), 4-F5 (6 mg), 4-F6 (7 mg), 4-F7 (7 mg), 4-F8 (23 mg) and 4-F9 (4 mg).

In order to identify other compounds that are present in the extract, semi purification of the methanol-dichloromethane extract (**4-B**, 11 g) by liquid-liquid partitioning was repeated. The generated dichloromethane fraction (**4-D,** 3.57 g) was combined with the dichloromethane extracts (**4-G,** 2.58 g) obtained by extracting the ground plant material.

Flash column chromatography of this combined material starting from chloroform and stripping the column with methanol resulted in the recovery of eight fractions with masses ranging between 45 mg and 1000 mg. Fraction **4-H1** was purified by flash chromatography and afforded seven semi-pure and pure compounds (see Fig. 4.7 & 4.8 for compounds isolated) and they were all sent for ^{1}H and ^{13}C NMR experiments. All of these compounds except one showed a very intense CH_2 peak which makes it difficult to identify the compounds by NMR. The compound was analyzed by GC-MS and no good match was obtained when compared to compounds in the library.

Fraction **4-H3** was further purified by flash chromatography and afforded more of compound **4.5** (ursolic acid) and another compound that was identified as α-amyrin (**4.6**, 7.3 mg).

The NCI published their own screen results of ursolic acid tested against their sixty cell lines as an averaged logarithm GI_{50} of 5.11 μg/ml.

4.6.3. LC-MS analysis of extracts

LC-MS experiments were performed using Alliance waters 2690 separations module system (HPLC) interfaced with a Micromass Quattro LC mass spectrometer. The mass spectrometer was operated in the positive mode using electrospray interface. The samples concentrations, 5000 ppm for the extracts and 2500 ppm for the fractions were prepared by dissolving 5-10 mg of the sample in 2 ml of methanol-acetonitrile (1:1) and injecting 25 µl. The mass spectra was set to scan from 50 to 2000 amu, cone volatage of 80 V, Capillary voltage of 3.0 kV, extractor at 5 V, desolvation and source block temperatures at 350 and 130 ^{O}C respectively. The variable PDA detector was scanning from 193 to 400 nm.

Table 4.3: Solvent gradient used to separate the metabolites in the extract

Time (minutes)	**% Water**	**% ACN**
0.00	90.0	10.0
61.00	20.0	80.0
64.00	0.00	100.0
75.00	0.00	100.0
75.10	90.0	10.0
90.00	90.0	10.0

4.7. PHYSICAL DATA

4.7.1 COMPOUND 4.5

Systematic name: 3β-Hydroxyurs-en-28-oic acid

Alternative name: Ursolic acid

Yield: 8.0 mg (0.25%)

Physical description: White amorphous solid

Optical rotation: $[\alpha]_D$ +66.3 (c = 0.33) in EtOH (Merck Index[28] reported $[\alpha]_D$ +67.5 at c = 1.0 in KOH).

Mass spectrum: *m/z* 456, $C_{30}H_{48}O_3$. base peak 248 $[M-208]^+$, 203 $[M-253]^+$, 133 $[M-323]^+$, 43 $[M-413]^+$.

^{1}H NMR data: See Table 4.1 ($CDCl_3/CD_3OD$)

^{13}C NMR data: See Table 4.1 ($CDCl_3/CD_3OD$)

4.7.2. COMPOUND 4.6

Systematic name:	Urs-12-en-3β-ol
Alternative name:	α-Amyrin
Yield:	7.3 mg (0.12%)
Physical description:	White amorphous solid
Optical rotation:	$[\alpha]_D$ +88.9 (c = 0.11) in $CHCl_3$+MeOH (Merck Index[28] reported $[\alpha]_D$ +96.5 at c = 1.0 in $CHCl_3$)
Mass spectrum:	*m/z* 426.72, $C_{30}H_{50}O$ with base peak 248.1769 $[M-208]^+$, 203.18 $[M-253]^+$, 133.09 $[M-323]^+$
^{1}H NMR data:	See Table 4.2 ($CDCl_3$)
^{13}C NMR data:	See Table 4.2 ($CDCl_3$)

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Chapter 5

ISOLATION AND BIOLOGICAL ACTIVITIES OF COMPOUNDS FROM *PERGULARIA DAEMIA*

5.1. INTRODUCTION

Information on a plant prescribed by traditional doctors to pregnant woman to prevent pain and birth difficulties was brought in by the late chairperson of the THC, TDr Solomon Mahlaba. The plant is traditionally known as Lushe (Tswana) or Mothuhu (Pedi). An infusion is prepared by boiling one tablespoon of dry ground plant powder in 1 L water for one and a half hours. A cup of the cold extract is taken per day. No side effects were reported for this plant except for the bitter taste of the infused tea. Evaluation of the plant involved the preparation of suitable organic and aqueous extracts of the plant according to pharmaceutical and traditional procedures.

These extracts were tested *in vitro* for cytotoxicity against three highly sensitive cancer cell lines available at the CSIR laboratories (melanoma UACC62, renal TK10 and breast MCF7). The organic extract (methanol–dichloromethane, 1:1, v/v) inhibited the growth of cancer cells and warranted further purification to isolate the active principle. This chapter is focused on the phytochemical investigation of the plant.

5.1.1. Botanical aspects of *Pergularia daemia*

Pergularia daemia (Forssk.) Chiovenda (Asclepiadaceae) is an herbaceous creeper with milky latex and hairy stems that smells like fish. The plant's latex is used as fish poison.[1] This plant grows up to 3 m in height and the heart-shaped leaves are pale-green and heart-shaped with hairy petioles (50 mm) always longer than the blade. The small, creamy flowers are borne auxiliary in drooping clusters. Flowers are bell-shaped with furry stems and petals, followed by paired, rough, horned fruits on long stems. Seeds are covered with long, silky floss. Flowering time is from late spring into summer.[5]

Pictures courtesy of 'Peoples Plants'[1]

Figure 5.1: *Pergularia daemia*

Synonyms for *P. daemia*. are *P. extensa* N.E.Br., *P. edulis* Thunb., *Daemia extensa* R.Br., *D. angolensis* Decne., *D. aethiopica* Decne., *D. cordifolia* K.Schum. ex Engl. and *Asclepias daemia* Forssk. Vernacular names used are Mothuho (Pedi), Lushe (Tswana), milkweed family (English) and bobbejaankambro (Afrikaans).

This plant species is widely distributed in tropical Africa in moist areas and is often cultivated as an ornamental. The genus *Pergularia* (synonym *Daemia*) contains toxins that make it useful as hunting or fishing poisons in remote areas. *P. daemia* is widely distributed in central Africa whereas *P. tomentosa* is distributed in western Africa and some other parts of southern Africa.[2]

5.1.2. Ethnopharmacology

Anecdotal information of *P. daemia* showed that the plant was widely used by people in rural areas for nausea, depilatory, abortion and treatment of skin diseases, amongst others.[3] *P. daemia* is also used in India as a folk medicine for the treatment of liver disorders.[4] It was shown that an ethanol extract from the aerial parts possessed significant hepatoprotective properties in rats after an oral dose of 200 mg/kg and no signs of acute toxicity were observed up to a dose level of 2000 mg/kg.[4]

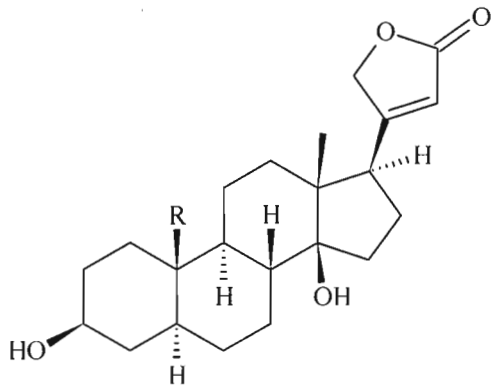
Root decoctions are taken for venereal diseases, arthritis, muscular pain, asthma and rheumatism. The leaves are eaten cooked as spinach.[1-5] The dried, pulverized roots are rubbed into stiff muscles and joint pains. In addition, cooked leaves of the plant are edible and work as an anthelmic, causing nausea.[6] Insects that fed on the plant were reported to have been intoxicated.[7] Sathish[8] reported that *P. daemia* has anti-inflammatory, anti-pyretic and analgesic activity while Wahi[9] reported on the anti-diabetic activity.

The roots of *P. daemia* have been used for fish poisoning and the subspecies *leiocarpa* was reported as having diuretic properties and was utilized with milk or water for the treatment of syphilis or blackwater fever. A related species, *P. tomentosa*, is applied for depilatory, poultice, abortive, laxative and for the treatment of some skin diseases.[10]

Watt and Breyer-Brandwijk[11] described the plant as a pot herb. In Ghana the leaf, which contains 5.6 mg/100 g vitamin C, has a peculiar mousy odour and is ingested by woman immediately after childbirth. The plant is claimed to be an emetic, has anthelmintic properties and works as an asthma remedy.

5.2. PREVIOUS PHYTOCHEMICAL STUDIES

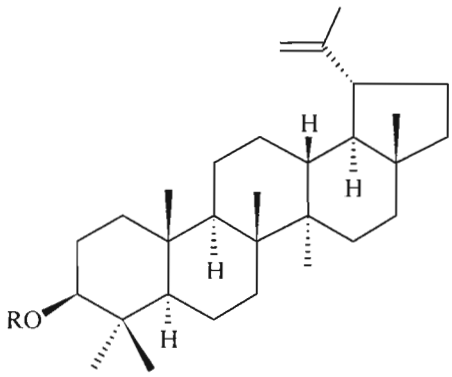
Dymock reported in 1891 that *P. extensa,* which is a synonym of *P. daemia,* contains an alkaloid daemine and a bitter glycoside. In 1962 Reichstein *et al.*[11] identified the cardenolides odorigenin B (uzarigenin) (**5.1**), coroglaucigenin (**5.2**), calotropin and corotoxigenin (**5.3**) from *P. extensa.*[12]



5.1: Uzarigenin R = CH_3

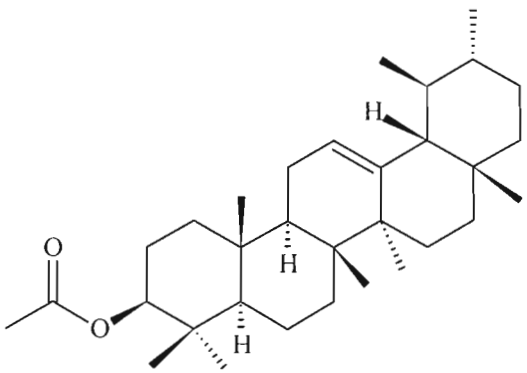
5.2: Coroglaucigenin R = CH_2OH

5.3: Corotoxigenin R = CHO



5.4: Lupeol R = H

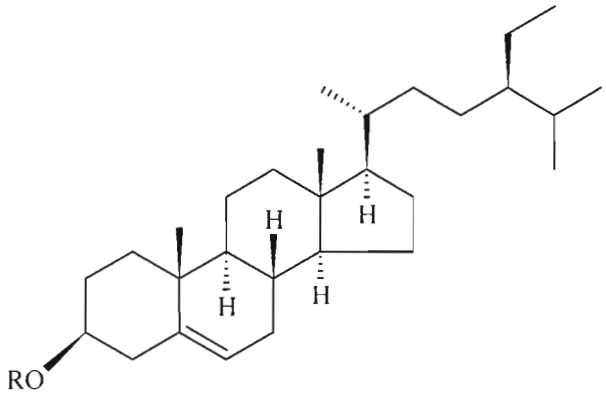
5.5: Lupeol acetate R = $COCH_3$



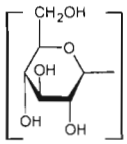
5.6: α–Amyrin acetate

Raman and Barua reported the presence of the triterpenoids lupeol (**5.4**), lupeol acetate (**5.5**), α-amyrin acetate (**5.6**) and β-sitosterol (**5.7**) in the leaves of *P. extensa*.[13,14] The roots contained less cardenolides and triterpenoids than the aerial parts.[15]

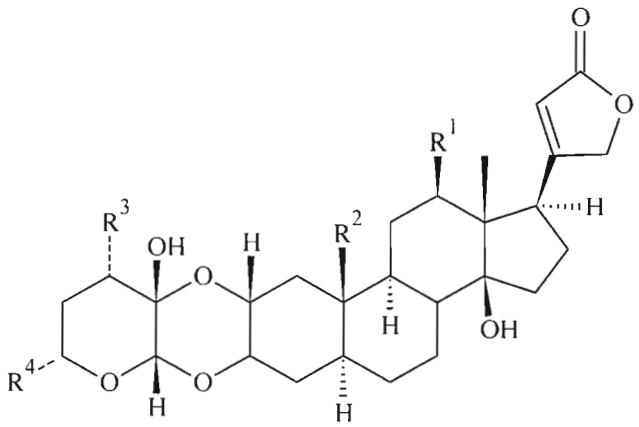
A literature search on the closely related species *P. tomentosa* showed that steroidal compounds, namely β–sitosterol (**5.7**), ghalakinoside (**5.10**) and calactine (**5.9**), as well as a nitrogenous base, choline were isolated. The cardenolide glycoside uzarigenin was shown to be present in the extracts of *P. tomentosa* along with the sugar moiety, glucose and digitoxose. Recently β–sitosterol glucoside (**5.8**) was added to the number of compounds isolated from the aerial parts of *P. tomentosa*.[16]



5.7: β-Sitosterol R = H

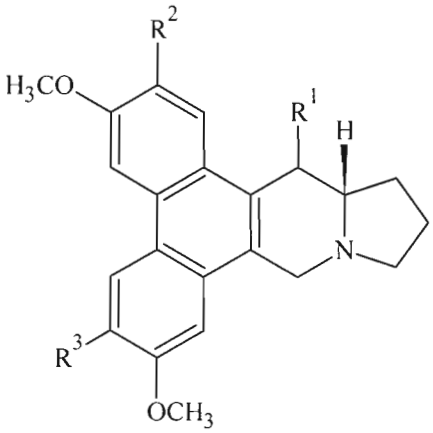
5.8: β-Sitosterol glucoside R = glucosyl 

Ghalakinoside (**5.10**) showed a strong inhibitory activity *in vitro* against cells derived from the human carcinoma of the pharynx ($ED_5 = 2.9x10^{-2}$ μg/ml).[10,17] An ED_{50} of 4 μg/ml for pure compounds is considered positive for antitumor screening. The extracts of the plant proved to be toxic to mice by intraperitoneal administration in 0.1 ml amounts.[18] From the roots of *P. tomentosa*, compounds **5.9** and **5.10** and other cardenolides caused apoptotic cell death of Kaposi's sarcoma cells as can be expected from their cytotoxic activity against a large range of cancer cell lines.[19]



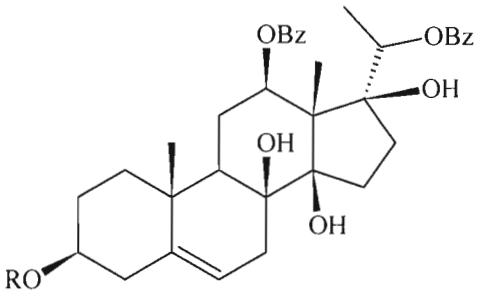
	R^1	R^2	R^3	R^4
5.9: Calactine	H	CHO	β-OH	CH_3
5.10: Ghalakinoside	OH	CH_2OH	α-OH	CH_2OH

Five phenanthrolizidine alkaloids pergularinine (**5.11**), tylophorinidine (**5.12**), tylophorine (**5.13**), deoxypergularinine (**5.14**)[20] and tylophorinicine (**5.15**) were isolated from the roots of the related species *Pergularia pallida*. Tylophorinicine was also isolated from *Tylophora asthmatica*.[21] Pergularinine and tylophorinidine exhibited significant antitumour activity *in vitro* by inhibiting the leukaemia leukocyte enzyme dihydrofolate reductase (EC 2.1.1.45),[22] which is a key target in cancer chemotherapy and was purified from *Lactobacillus leichmannii*.[23]

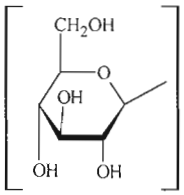


	R^1	R^2	R^3
5.11: Pergularinine	OH	H	OCH_3
5.12: Tylophorinidine	H	OH	OH
5.13: Tylophorine	H	OCH_3	OCH_3
5.14: Deoxypergularinine	H	H	OCH_3
5.15: Tylophorinicine	OH	OCH_3	OCH_3

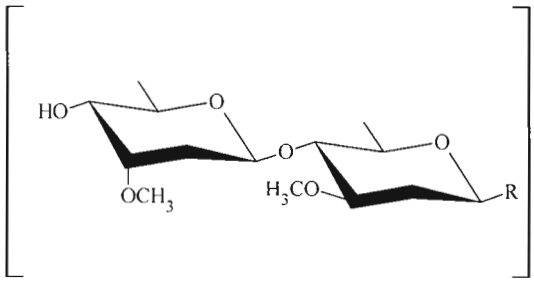
Two pregnane ester glycosides, pallidine (**5.16**) and pallinidine (**5.17**), were isolated from the twigs of *P. pallida* and their structures contain one glucosyl or two glucosyl units for pallidine and pallidinine, respectively.[24]



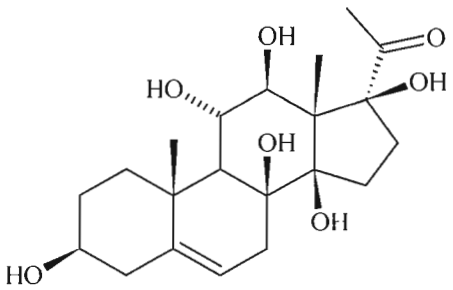
5.16: Pallidine R =



5.17: Pallidinine R =

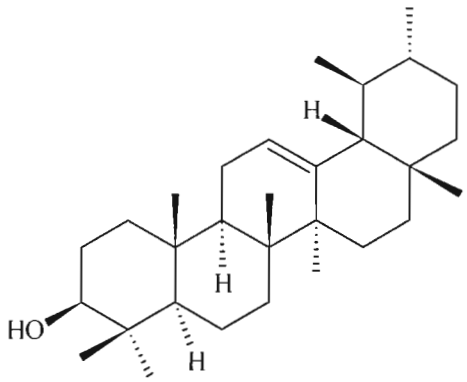


A pregnane genin was isolated from the chloroform extract of the dried twigs of *P. pallida* and earlier from *Sarcostemma brevistigma* and was identified as sarcogenin (3β,8β,11α,12β,14β,17β-hexahydropregn-5-en-20-one, **5.18**).[25]

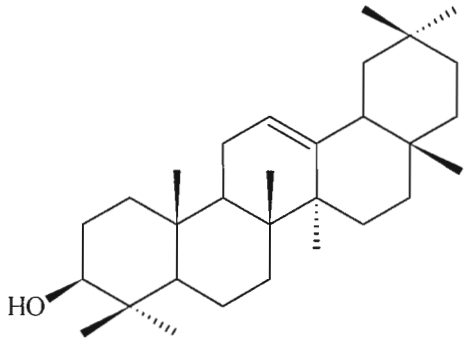


5.18: Sarcogenin

The present study followed after a chemical investigation of the dried twigs of *P. pallida* revealed the presence of α-amyrin (**5.19**), β-amyrin (**5.20**), sitosterol (**5.7**), drevogenin B and cissogenin.[26]



5.19: α-Amyrin



5.20: β-Amyrin

This study was aimed at isolating the compounds that were responsible for the cytotoxic effect of the plant extract on the three cancer cell lines that are used in the study. However, compounds without activity were also isolated to gain information on the secondary metabolites present in this plant. Most of the compounds reported in the literature were from related genera and only a few have been identified by analytical techniques.

5.3. ISOLATION AND STRUCTURAL ELUCIDATION OF ACTIVE COMPOUNDS

The methanol-dichloromethane extract of the oven-dried and ground plant of *Pergularia daemia* exhibited cytotoxic effects at a concentration of 100 μg/ml in the three cancer cell lines (breast, melanoma and renal). The aqueous extract did not show a significant inhibition of the growth of these cancerous cells at the same concentration, hence no further work was done on this extract. The fractionation procedure is outlined in Fig. 5.2 and it involved the primary purification of the organic methanol-dichloromethane extract to afford the partitioned hexane, dichloromethane and water fractions. Only the dichloromethane fraction showed a significant increase in anticancer activity.

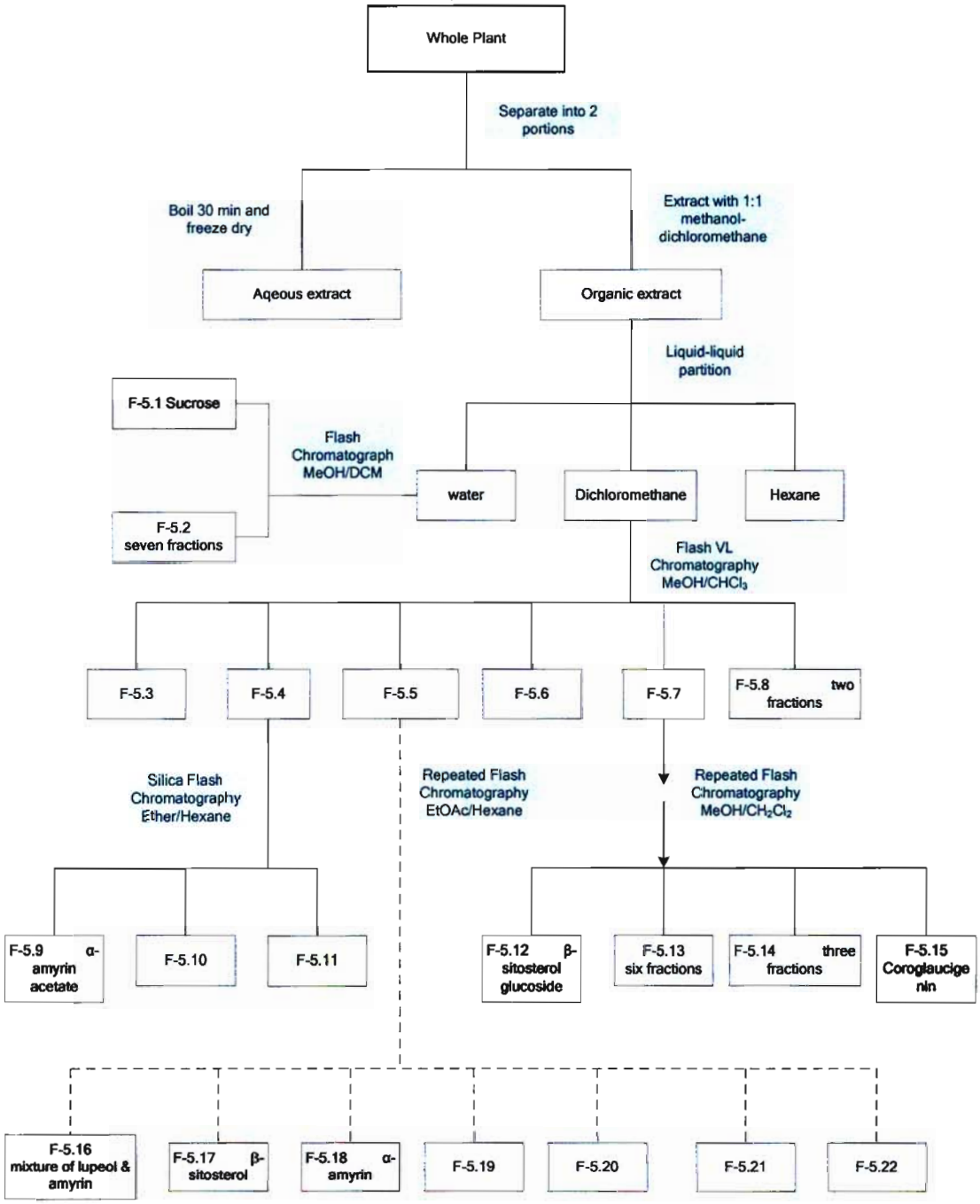
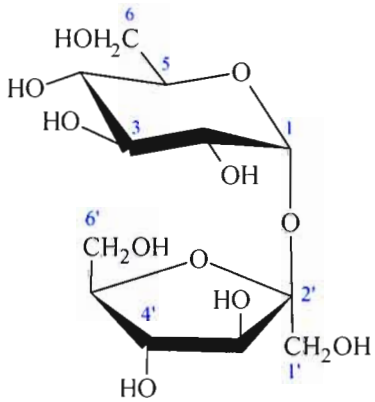


Figure 5.2: Schematic diagram of the fractionation and isolation of compounds from *Pergularia daemia*.

Column chromatography of the final dichloromethane fraction was done on silica gel by means of vacuum liquid chromatography (VLC) using various solvent systems. Seven fractions were generated and post purifications of three of these fractions as shown in Fig. 5.2 afforded five pure compounds. Although the water fraction obtained by liquid

partitioning did not show activity in the anticancer screen, fractionation of this resulted in the isolation of a carbohydrate that was identified as sucrose.

5.3.1. Characterization of sucrose



6.10: Sucrose

TLC indicated that this compound is highly polar. In the ^{13}C NMR spectrum twelve signals were observed in the region between δ_C 61 and δ_C 104. These chemical shifts are characteristic of carbon atoms attached to highly electronegative atoms such as oxygen, and indicate that the compound may be a carbohydrate.

The one anomeric carbon signal at δ_C 92.4 is characteristic of α-orientation of the hydroxyl group attached to position C-1 whereas the signal at δ_C 104.7 is corresponding to a quaternary carbon. The most common disaccharide with a quaternary acetal carbon is sucrose. NMR data of compound **5.21** were in close agreement with published data for sucrose (Table 5.1).

Mass spectral data that were determined by ESIMS$^-$ indicate a $[M]^-$ 341.2 which is in support of the molecular formula $C_{12}H_{22}O_{11} - [H^+]$. The specific optical rotation of the compound at 20 °C was determined as + 66.7 (c = 10.5 mg/10 ml) which is similar to the reported value for sucrose + 66.0 ± 1 (c = 1.2 g/100 ml in H_2O) obtained from literature.[27] the Merck Index.[28]

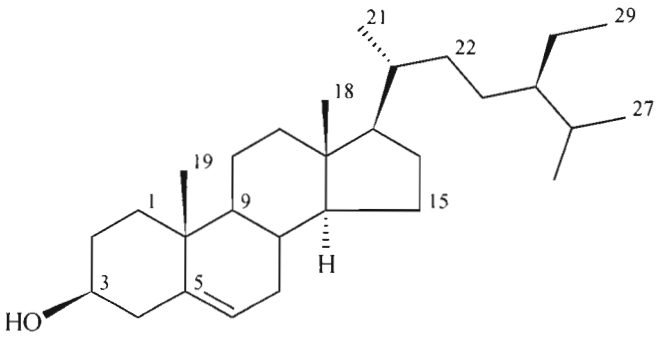
Table 5.1: NMR spectroscopic data for sucrose (**5.21**)

Sucrose				
	Isolated compound*		Literature[29#]	
Carbon	δ_C	δ_H (*J in Hz*)	δ_C	δ_H
1	92.4	5.12	93.20	5.41
2	72.3	3.13	72.14	3.56
3	73.5	3.61	73.68	3.76
4	70.5	3.07	70.31	3.86
5	73.4	3.42	73.44	3.47
6	61.1	3.58	61.24	3.81
1`	62.7	3.58, 3.35 (2H)	62.46	3.67
2`	104.7		104.71	
3`	77.7	3.83	77.51	4.21
4`	75.0	3.72	75.09	4.05
5`	83.2	3.52	82.42	3.89
6`	62.7	3.49	63.44	3.83

* Data obtained in DMSO, ^{13}C at 100.5 MHz and ^{1}H at 400 MHz
\# Data obtained in D_2O, ^{13}C at 100 MHz and ^{1}H at 400 MHz

Results obtained from SABS also confirmed the structure of the compound by using he analytical method published in the European Pharmacopoeia.[30]

5.3.2. Characterization of β-sitosterol



5.7: β-Sitosterol

β-Sitosterol was isolated from the dichloromethane fraction obtained from the liquid-liquid partitioning of the methanol-dichloromethane (1:1) extract of the plant.

This compound was compared to an authentic sample of β-sitosterol that was bought from Sigma-Aldrich. Thin-layer chromatography gave the same R_f for both samples and the same pink color after spraying the TLC plate with vanillin and baking in the oven. The

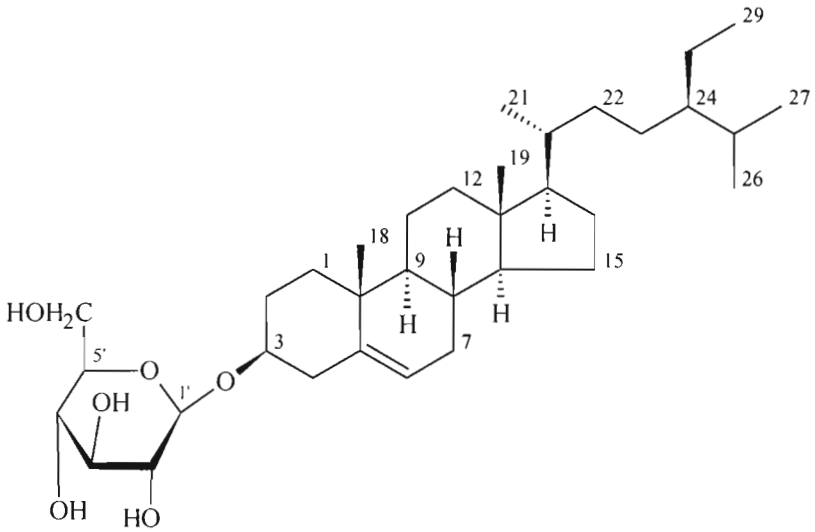
assignment was corroborated by NMR experiments, which showed that the commercial sample of β-sitosterol gave identical ^{13}C and ^{1}H spectra with $CDCl_3$ as solvent. Literature studies also revealed that the ^{13}C signal that is supposed to be seen for C-2 overlaps with the one in C-7 or C-8 and the peak signal for C-4 overlaps with the signal of the peak in C-13.[31] The ^{1}H NMR and ^{13}C NMR assignments were also correlated with previously published spectral data.[32] This evidence substantiated that the structure is β-sitosterol.

Table 5.2: NMR spectroscopic data for β-sitosterol (δ_H 400 MHz and δ_C 100 MHz in $CDCl_3$)

	β-sitosterol (**5.7**)				
	Observed			**Literature**	
C	δ_C	DEPT	δ_H (*J* in Hz)	δ_C	δ_H (*J* in Hz)
		CH_2			1.05 (m)
1	37.2		1.89	37.1	1.83 (m)
		CH_2			1.82 (m)
2	31.6		1.56	31.5	1.48 (m)
3	71.8	CH	3.49(m)	71.7	3.48 (m)
4	42.4	CH	2.28	42.2	2.28 (m)
5	140.7	C		140.8	
6	121.7	CH	5.32(d, 5.0)	121.7	5.32(d, 5)
7	31.8	CH_2	1.53	31.7	1.53 (m)
8	31.7	CH	1.93	31.7	1.90 (m)
9	50.1	CH	0.98	50.0	0.87 (m)
10	36.5	C		36.3	
11	21.1	CH_2	1.51	20.9	1.43 (m)
12	39.8	CH_2	2.03	39.6	1.13 (m) 1.99 (m)
13	42.3	C		42.7	
14	56.8	CH	0.99	56.7	0.94 (m)
15	24.3	CH_2	1.61	24.1	1.13 (m) 1.61 (m)
16	28.2	CH_2	1.85	28.1	1.35 (m) 1.86 (m)
17	56.1	CH	1.12	55.9	1.06 (m)
18	11.9	CH_3	0.64 (s)	11.7	0.65 (s)
19	19.4	CH_3	0.93 (s)	16.7	1.00 (s)
20	36.1	CH	1.37	36.0	1.32 (m)
21	19.4	CH_3	0.93 (d, 7.0)	19.2	0.92 (d, 7.0)
22	34.0	CH_2	1.35	33.8	0.99 (m) 1.26 (m)
23	26.1	CH_2	1.20	25.9	1.12 (m)
24	45.9	CH	0.96	45.7	0.95 (m)
25	29.2	CH	1.70	29.0	1.62 (m)
26	19.8	CH_3	0.83 (d, 7.0)	18.9	0.88 (d, 7.0)
27	19.0	CH_3	0.81 (d, 7.0)	18.6	0.77 (d, 7.0)
28	23.1	CH_2	1.27	22.9	1.20 (m)
29	12.0	CH_3	0.84 (t, 7.0)	11.8	0.82 (t, 7.0)

A molecular mass $[M]^+$ of *m/z* 414 in the mass spectrum is in agreement with the molecular formula of $C_{29}H_{50}O$. The molecular formula was in agreement with the successive loss of 85 units to *m/z* 329. The molecular ion peak underwent the loss of a water molecule to *m/z* 396 followed by a loss of *m/z* 15 attributed to loss of CH_3 to *m/z* 381. The side chain (C20 – C29) was cleaved and left an atomic mass unit of *m/z* 289.

5.3.3. Characterization of β-sitosterol-β-D-glucoside



5.8: β-Sitosterol-β-D-glucoside

β-Sitosterol β-D-glucoside was isolated from the dichloromethane fraction (Fig. 5.4) as a white amorphous substance. This compound is polar and could only be dissolved in methanol with heating or by a combination of solvents for example methanol, acetone and chloroform. Thus, NMR data were obtained by dissolving the sample in DMSO. The obtained proton and carbon spectral data were interpreted and the structure of a steroidal glycoside was evident.

The ^1H NMR spectra and ^{13}C NMR spectra were almost identical to those of β-sitosterol, except for the presence of the signals associated with a glycoside moiety. The ^{13}C NMR spectrum of the isolated compound showed 35 carbons. The chemical shifts of the sugar attached to the sitosteryl in Table 5.4 were compared to published data and was identified as β–D-glucosyl. The ^1H NMR spectral data showed three distinct singlet peaks of the methyl substituents at δ_H 0.64 (H-29), 0.81 (H-18) and 0.94 (H-19) as compared to those of

the published sitosteryl at δ_H 0.66, 0.89 and 0.93, respectively. Olefinic carbons were observed at δ_C 121.1 and 140.4, which correspond to the quaternary C-5 and the methine C-6, respectively. The ^{13}C NMR spectral assignments for the β-sitosterol fragment compared to previously published ^{13}C NMR data for the glycoside is given in Table 5.3.

Table 5.3: ^{13}C NMR and ^{1}H NMR chemical shifts of the isolated β-sitosterol glucoside **(5.8)** in DMSO (δ_C 100 MHz and δ_H 400 MHz).

	β-Sitosterol aglycone			
	Observed, **5.8**		Literature[33]	
C	δ_C	δ_H (*J* in Hz)	δ_C	δ_H
1	36.7	0.95	37.2	1.69; 0.94
2	29.2	2.12	29.4	2.12; 1.74
3	76.9	3.96	78.4	3.95
4	38.2	2.44	38.9	2.73; 2.46
5	140.4		140.8	
6	121.1	5.34 (d, 5.0)	121.9	5.35
7	31.3	1.88	31.9	1.87; 1.54
8	31.3	1.30	31.8	1.34
9	49.5	0.88	50.1	0.90
10	36.1		36.5	
11	20.5	1.40	21.1	1.40
12	39.7	1.14	39.7	1.98; 1.12
13	41.8		42.2	
14	56.1	0.91	56.7	0.91
15	23.7	1.07	24.2	1.54; 1.00
16	27.7	1.21	28.2	1.85; 1.23
17	55.3	1.09	56.0	1.09
18	11.6	0.66 (s)	11.9	0.64
19	19.0	0.95 (s)	19.5	0.94
20	35.4	1.35	36.1	1.38
21	18.5	0.86 (d, 6.4)	18.8	0.84
22	33.3	1.08	33.9	1.38; 1.10
23	25.4	1.24	26.0	1.25
24	45.1	0.94	45.8	0.94
25	28.6	1.67	29.1	1.67
26	19.6	0.86 (d, 6.4)	19.8	0.87
27	18.8	0.86 (d, 6.4)	19.0	0.87
28	22.5	1.22	23.0	1.20
29	11.7	0.87 (t, 6.4)	12.0	0.85

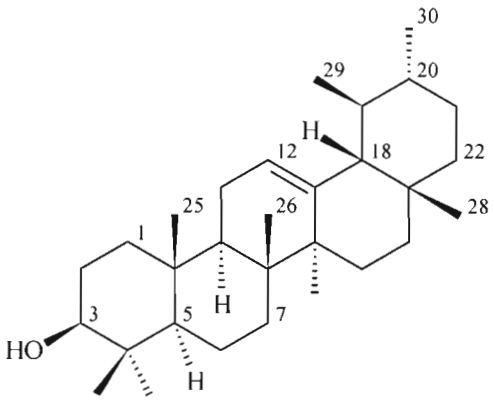
The compound has been assigned as β-sitosterol D-glucoside. The aglycone was positively identified as β-sitosterol and attached to C-3 of the aglycone is a beta D-glucosyl moiety. This data were confirmed by ^{13}C data of published sitosterol glucoside.[33,34] The spectral assignments for the aglycone and the glucoside moiety are given in Table 5.3 and 5.4 respectively and are compared to previously published data.

Table 5.4: ^{13}C NMR and ^{1}H NMR chemical shifts of the isolated β-sitosterol glucoside (**5.8**) in DMSO (δ_C 100 MHz and δ_H 400 MHz)

	Glycoside unit			
	Observed, **5.8**		Literature[34]	
C`	δ_C	δ_H (*J* in Hz)	δ_C	δ_H
1`	100.7	5.04 (d, 7.7)	102.6	5.05
2`	73.4	4.04, (t, 8.2)	75.4	4.06
3`	76.6	4.31, m	78.1	4.28
4`	70.0	4.31, m	71.7	4.28
5`	76.7	4.01 (t, 4.9)	78.6	3.97
6`	61.0	4.40 (d, 11.7)	62.8	4.40
		4.55 (d, 11.7)		4.57

In the mass spectrum, a molecular ion peak of *m/z* 576 was obtained for as β-sitosterol β-D-glucoside which is consistent with a molecular formula of $C_{35}H_{60}O_6$. The loss of the glucose moiety (*m/z* 180) resulted in the formation of the fragment with *m/z* 396, followed by the loss of a CH_3 (15) resulting in a fragment peak at *m/z* 381.

5.3.4. Characterization of α-amyrin (5.19)



5.19: α-Amyrin

This compound was isolated from subsequent dichloromethane fraction purification by silica column chromatography. It was obtained as white amorphous solids at room temperature that was soluble in chloroform. Based on NMR data (Table 5.5), the structure of the compound was assigned as α-amyrin. The structural elucidation of this compound was described in section 4.3.2.

Table 5.5: ^{13}C and ^{1}H NMR data of α-amyrin (**5.19**) observed at 100.5 MHz and 399.9 MHz respectively, in $CDCl_3$.

	α-amyrin			
	Observed, **5.19**			Literature[35]
C	δ_C	DEPT	δ_H (*J* in Hz)	δ_C
1	38.9	CH_2		38.7
2	23.4	CH_2	1.64 (m)	27.2
3	79.2	CH	4.53 (m)	78.3
4	37.1	C		38.7
5	55.4	CH		55.2
6	18.5	CH_2		18.3
7	33.1	CH_2		32.9
8	40.2	C		40.0
9	47.9	CH		47.7
10	37.1	C		36.9
11	23.5	CH_2	1.86 (m)	23.3
12	124.6	CH	5.09 (t, 3.6)	124.3
13	139.7	C		139.3
14	42.2	C		42.0
15	28.3	CH_2		28.7
16	26.8	CH_2	1.03	26.6
17	33.9	C		33.7
18	59.2	CH	1.21	58.9
19	39.8	CH		39.6
20	39.8	CH		39.6
21	31.4	CH_2		31.2
22	41.7	CH_2		41.5
23	28.9	CH_3	0.79 (s)	28.1
24	17.0	CH_3	0.83 (s)	15.6
25	15.8	CH_3	0.94 (s)	15.6
26	17.0	CH_3	0.87 (s)	16.8
27	23.4	CH_3	1.02 (s)	23.3
28	28.3	CH_3	0.83 (s)	28.1
29	17.6	CH_3	0.75 (d, 5.5)	17.4
30	21.5	CH_3	0.97 (d, 5.5)	21.3

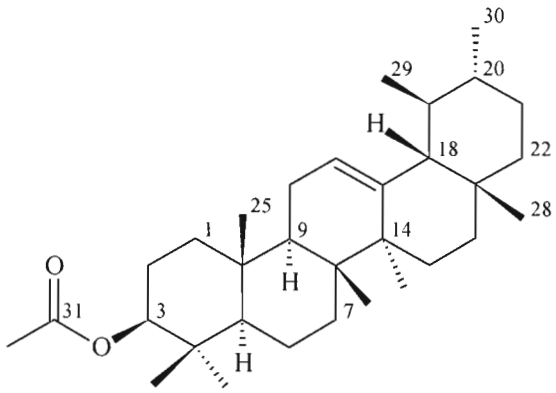
Confirmation for the structure of this compound was obtained by comparison of the NMR data with published data[35] in a review article of triterpenoids.[36]

5.3.5. Characterization of α-amyrin acetate (5.6)

Compound **5.6** exhibited six tertiary methyl singlet signals at δ_H 0.80, 0.83, 0.94, 0.97, 1.02 and 0.83 and two methyl doublet signals at δ_H 0.75 and 0.87 in the ^{1}H NMR spectra (Table 5.5). It further showed an olefinic proton resonating at δ_H 5.08 (1H, d, *J*=3.5 Hz, H-12) and a singlet at δ_H 2.00 (3H, s, H-32), which is characteristic of an acetate group. A one-proton

doublet at δ_H 1.27 characteristic of H-18 and a methine proton, geminal to an ester, that resonated at δ_H 4.46 was also identified.

The carbon-13 spectral data (Table 5.6) of compound **5.6** were compared to the spectroscopic data of α-amyrin (**5.19**) (Table 5.5) and it was observed that the only difference between the two compounds was the number of carbon signals. The peak at δ_C 81.1 was assigned to the acetate-containing carbon at position 3 with the carbonyl of the acetate resonating at δ_C 171.1 and its terminal methyl at δ_C 21.5. The presence of the acetate at C-3 was observed by the chemical shift of C-2 that resonated at δ_C 23.7 as compared to about δ_C 27.2 when an OH is attached to C-3. Two single protonated carbons had distinct signals at δ_C 55.4 and δ_C 59.2 for the carbons C-5 and C-18, respectively. The signal at δ_C 124.5 was ascribed to C-11 and that at δ_C 139.8 was attributed to C-12.



5.6: α–Amyrin acetate

Comparison of the chemical shifts of C-12 and C-13 gives evidence to the position and orientation of the methyl C-29 and C-30. This is provided by the resonance of the 19-β methyl at δ_C 39.7 in the equatorial position, which is in close proximity to the double bond. In oleanolic compounds a methylene group is at position 19, but if a β-OH is attached to C-19 of oleanolic derivatives, the double bond shifts will appear at the same chemical shift as those of ursolic derivatives. Further confirmation was obtained from the shift of C-18 that is shielded by approximately δ_C 11.5 in oleanolic derivatives as compared to ursolic derivatives and to the 20β axial methyl group at δ_C 39.8.

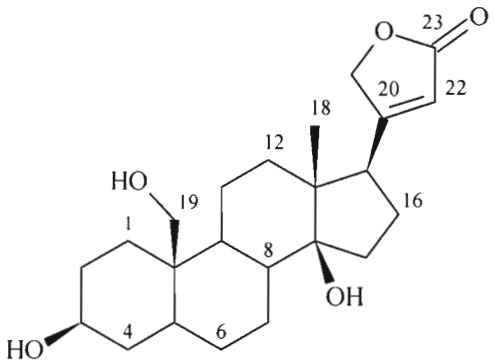
The mass spectral data obtained from the GC/MS matched that of urs-12-en-3-ol acetate which gave a molecular ion $[M]^+$ of *m/z* 468, which is in agreement with the assigned molecular formula of $C_{32}H_{52}O_2$. The base line peak was observed at m/z 218 $[C_{15}H_{22}O]^+$.

Table 5.6: The ^1H and ^{13}C NMR data of α-amyrin acetate in $CDCl_3$

	α-Amyrin acetate			
	Observed, **5.6**			Literature[36]
C	δ_C	DEPT	δ_H (*J* in Hz)	δ_C
1	38.6	CH_2	1.59	38.7
2	23.7	CH_2	1.59-1.64 (m)	27.2
3	81.1	CH	4.46 (m)	78.3
4	37.9	C		38.7
5	55.4	CH	0.80	55.2
6	18.4	CH_2	0.75-0.76	18.3
7	33.0	CH_2	0.83	32.9
8	40.2	C		40.0
9	47.8	CH	1.49	47.7
10	36.9	C		36.9
11	23.5	CH_2	1.86 (m)	23.3
12	124.5	CH	5.08(t, 3.6)	124.3
13	139.8	C		139.3
14	42.2	C		42.0
15	28.2	CH_2	0.76	28.7
16	26.7	CH_2	1.07	26.6
17	33.9	C		33.7
18	59.2	CH	1.27	58.9
19	39.7	CH		39.6
20	39.8	CH		39.6
21	31.4	CH_2		31.2
22	41.7	CH_2		41.5
23	28.9	CH_3	0.80	28.1
24	16.9	CH_3	0.83	15.6
25	15.9	CH_3	0.94	15.6
26	17.0	CH_3	0.97 (s)	16.8
27	23.4	CH_3	1.02 (s)	23.3
28	28.2	CH_3	0.83 (s)	28.1
29	17.6	CH_3	0.75	17.4
30	21.4	CH_3	0.87 (s)	21.3
31	171.1	C		
32	21.5	CH_3	2.00 (s)	

^{13}C observed at 100 MHz and ^1H observed at 400 MHz

5.3.6. Characterization of coroglaucigenin (5.2).



5.2: Coroglaucigenin

This compound was isolated as a white amorphous substance that showed up a violet color under short wave UV light (254 nm) on a TLC plate. The molecular ion peak was obtained by ESIMS operating in the negative mode resulting in *m/z* 434.9 that corresponds to $[M-H+HCO_2H]^-$ which is $[M+45]^-$. The calculated molecular weight was in accordance with the molecular formula $C_{23}H_{34}O_5$.

Examination of the ^1H NMR spectrum confirmed its steroidal nature by a series of multiplets from δ_H 0.7 to 2.4 and the presence of the characteristic α,β-unsatuated lactone of the cardenolide. The latter group was represented by a broadened singlet at δ_H 5.79 (1H, 22-H) and a pair of non-equivalent methylene protons at δ_H 4.94 and 4.76. Only one steroidal methyl was observed (δ_H 0.83, δ_C 15.6) and it was clear that a hydroxyl group is present on C-19 (δ_H 3.75 & 3.64 with J = 12 Hz, δ_C 59.0).

In the ^{13}C NMR spectrum of this compound there were 23 carbon signals. This number of carbons and the spectra pattern was viewed as typical of the steroids. The structure of this compound was further authenticated by comparison of the ^{13}C NMR data of **5.2** with that of coroglaucigenin which was previously isolated from *Pergularia extensa.*

Table 5.7: ^{13}C NMR and ^{1}H NMR chemical shifts of the cardenolide **5.2** in $CDCl_3$ and a few drops of CD_3OD (δ_C 125 MHz and δ_H 500 MHz).

	Coroglaucigenin			
	Observed, **5.2**		Literature[10,37]	
C	δ_C	δ_H	δ_C	δ_H
1	32.4		33.0	
2	31.7		32.7	
3	70.3	3.45	70.7	3.70 m
4	38.2		39.8	
5	44.3		42.5	
6	27.9		28.8	
7	31.3		28.1	
8	41.7		42.4	
9	49.8		50.8	
10	40.2		40.6	
11	22.6		23.4	
12	39.0		39.8	
13	49.7		50.2	
14	85.1		84.8	
15	31.2		32.0	
16	27.1		27.3	
17	50.7	2.70 (dd, 10, 5)	51.6	2.78 dd
18	15.6	0.83	16.3	0.92
19	59.0	3.75 (d, 12.0) 3.64 (d, 12.0)	59.2	3.85 dd
20	175.7		176.2	
21	73.7	4.94 (dd, 17.8, 1.4) 4.76 (dd, 17.8, 1.7)	73.4	4.80
22	117.0	5.79 (s)	117.6	5.88
23	175.3		174.6	

5.4. BIOLOGICAL ACTIVITY

The noted anecdotal information of the plant when it was delivered to the CSIR by a representative of the Traditional Healers Committee had no evidence of its efficacy as an anticancer candidate. A methanol-dichloromethane extract of the whole plant of *P. daemia* was prepared according to pharmaceutical preparations and the aqueous extract was prepared as done by traditional doctors.

The prepared extracts were evaluated for cytotoxic activity at a single dose concentration of 100 µg/ml against a panel of three cell lines (melanoma, renal and breast cell lines). The aqueous extract exhibited a TGI of greater than 100 µg/ml and the organic methanol-dichloromethane extract showed moderate activity. The methanol-dichloromethane extract

was then retested at five dilution concentrations ranging between 6.25 and 100 µg/ml and a TGI of 25 µg/ml obtained (results shown in Fig. 5.4) The extract has moderate activity with the melanoma cell lines being the most sensitive of the three cell line.

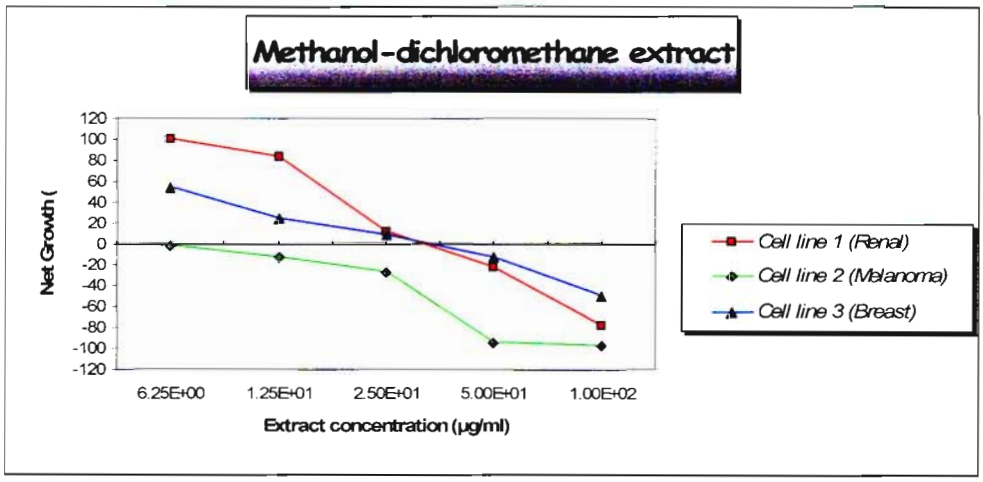


Figure 5.3: Dose-response curve of the anticancer activity of the organic extract.

The same extract was sent to the NCI laboratories for evaluation in the 60 cell line panel. The extract has a mean log GI_{50} of 1.11 µg/ml. The inhibition activity was specific for the NSCLC, renal and prostate DU145 cancer cell lines.

The extract was then partitioned to obtain the hexane, dichloromethane and water fractions. Screening of these fractions for anticancer activity at the CSIR showed that only the dichloromethane fraction had significant improved activity (see Fig. 5.4). The slight activity of the water fraction might be a false positive coming from the carry over from the dichloromethane–water partitioning.

Repeated column chromatography of the dichloromethane fraction resulted in the isolation of five compounds, two steroids β-sitosterol (**5.7**) and β-sitosterol glucoside (**5.8**), two triterpenoids of the ursane type α-amyrin (**5.19**) and α-amyrin acetate (**5.6**) and a cardenolide coroglaucigenin (**5.2**). Sucrose (**5.21**) was isolated from the water fraction obtained from liquid partitioning. In the CSIR laboratories, only the cardenolide **5.2** showed potent activity and a noteworthy activity was observed for α-amyrin, **5.19** (Fig. 5.5).

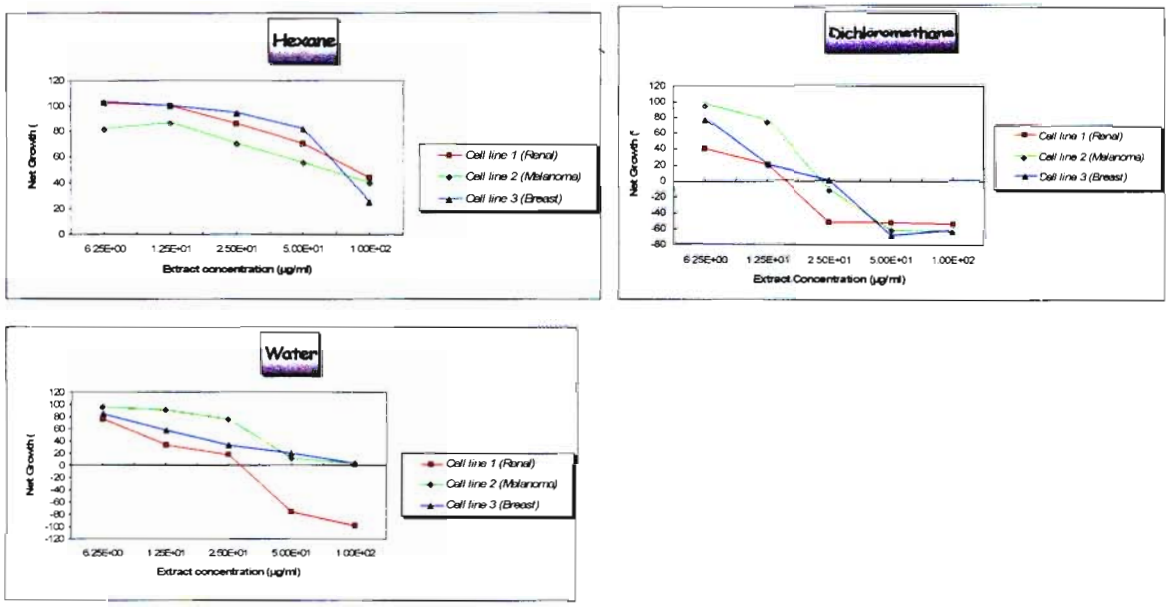


Figure 5.4: Anticancer activity of the hexane, dichloromethane and water fractions obtained from liquid-liquid partitioning of the methanol-dichloromethane extract.

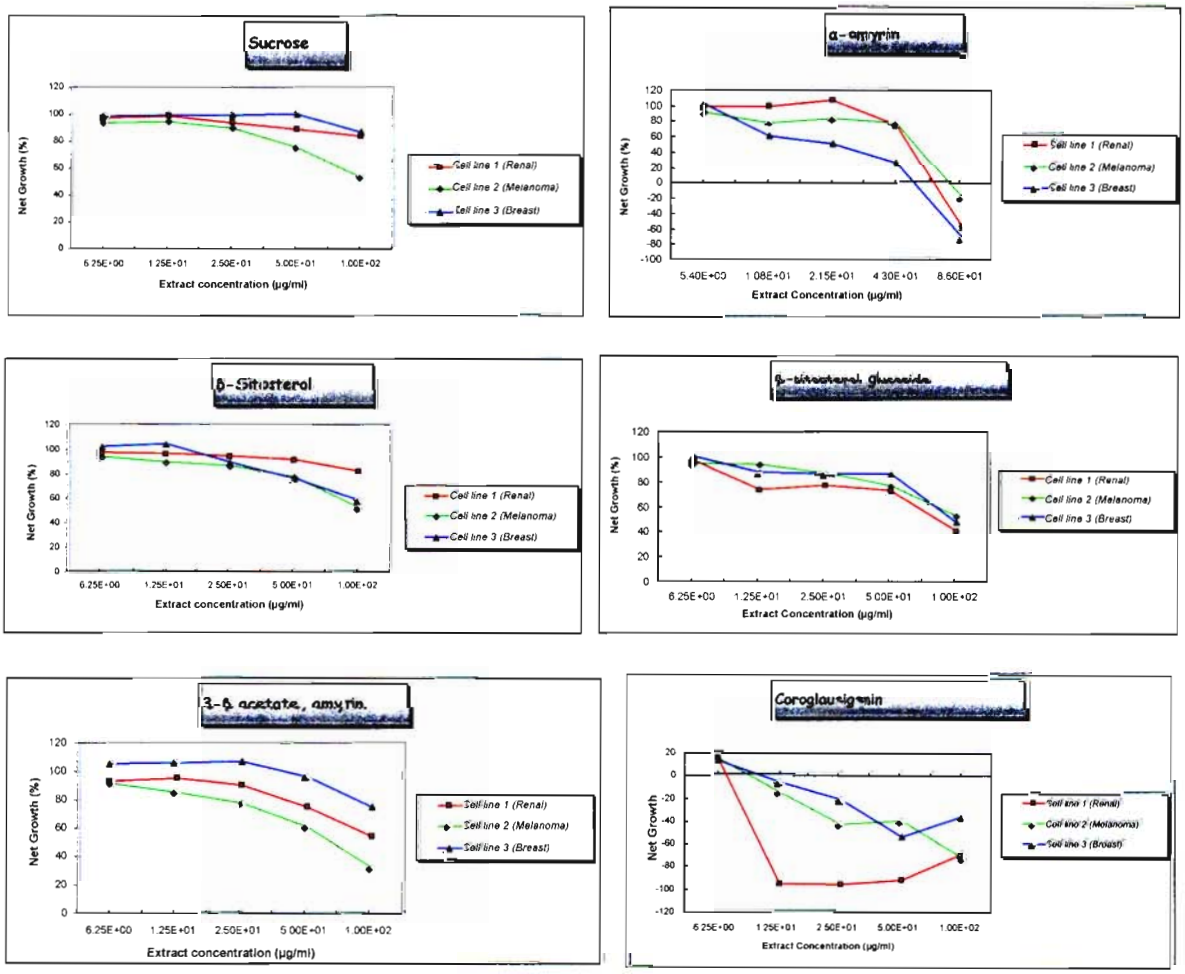


Figure 5.5: Dose-responds curves of the isolated compounds.

The amyrin-type compounds are not active except for the mild activity observed for α-amyrin. The observed activity can be attributed to the presence of the free hydroxyl group in position C-3, because replacement of this functionality with the acetate resulted in the total loss of activity. Coroglaucigenin exhibited the highest cytotoxic activity against the host cancer cell lines as compared to the activity of the crude dichloromethane fraction. It can be concluded that this is the active compound in the extract.

5.5. CONCLUSION

Six compounds, sucrose (**5.4**), β-sitosterol (**5.7**), β-sitosterol glucoside (**5.8**), α-amyrin (**5.19**), α-amyrin acetate (**5.6**) and coroglaucigenin (**5.2**) have been isolated from *P. daemia*. Compounds **5.7** and **5.19** have been isolated from this plant previously,[30] whereas **5.2**[14,15] and **5.6**[16,17] were isolated from the related species *P. extensa* and **5.8** from *P. tomentosa*.[19] The cytotoxic activity observed for the methanol-dichloromethane extract on the cancer cell lines can be attributed to the cardenolide coroglaucigenin (**5.2**) isolated from the organic extract. This is the first report on the cytotoxicity of coroglaucigenin.

5.6. EXPERIMENTAL

General experimental techniques are described in Chapter 3.

5.6.1. Plant material

The aerial parts of the plant were collected in Kwazulu-Natal near Ngwazuma village and the Pongola river during March. The plant was identified by SANBI in Pretoria and a voucher specimen has been deposited in the herbarium. The plant was identified as *Pergularia daemia* (Forssk.) Chiov. Var. *daemia*, Genspec Number 6917000.

5.6.2. Extraction and isolation

Fresh and uncrushed aerial parts (5.698 kg) of *P. daemia* var. *daemia* were stored in a cold room overnight before processing. Subsequently it was dried in an oven at 45 °C for three days. The dried plants (1.264 kg) were ground to a fine particle texture (1.022 kg) suitable

for tea bagging. This crushed material was extracted twice with methanol-dichloromethane (1:1, v/v). The solvent was filtered and evaporated to dryness under reduced pressure to produce 94.27 g of greenish extract and the residual plants were discarded. The aqueous extract was produced from ground material (0.142 kg) by boiling in water for one and half hour. The filtrate was freeze-dried to yield 23.68 g of dry brownish powder and the residual plant material was discarded. Evaluation of the cytotoxicity of the melanoma, renal, and breast cancer cell lines showed that only the methanol-dichloromethane extract was effective in inhibiting the growth of this cancer cell lines with a TGI of less than 25 μg/ml.

Primary fractionation of the methanol-dichloromethane extract involved liquid-liquid partitioning of the extract to yield hexane, dichloromethane and water fractions. The methanol-dichloromethane extract (88.58 g) was dissolved in 2 L of 90% aqueous methanol which was then extracted three times with 1 L of hexane. The combined hexane extracts were dried in vacuo to give 30.55 g extract. The methanol-water fraction was concentrated to yield a water aliquot which was enriched with more water to 2 L. This fraction was extracted with dichloromethane (3 x 1 L). The combined dichloromethane fractions were evaporated in vacuum and yielded a brownish dry extract (12.84 g). The residual water was freeze dried and amounted to a brownish powder weighing (18.11 g).

Chromatography of the water fraction (600 mg) on flash silica gel resulted in the isolation of a carbohydrate (**5.21**, 72.8 mg). The crystalline sugar was analyzed by SABS in Pretoria and confirmed to be sucrose.

The dichloromethane extract (11.78 g) was fractionated by VLC. The column was washed consecutively with 2 L each of hexane, hexane-chloroform (1:1 v/v), chloroform, chloroform-acetone (1:1 v/v), acetone and methanol. The collected fractions were pooled based on similarities as viewed on the TLC plate as follows;

2.93 g of fraction F-5.4 was column chromatographed (CC) on flash silica gel, eluting the column with diethyl ether-hexane of increasing polarity. Ten fractions were recovered and out of this one fraction was a pure compound. The compound was obtained as white amorphous solids. The compound was fully characterized by NMR data and in comparison with published data and it was assigned as β-acetate amyrin **5.6,** 53.1 mg.

Table 5.8: Fractions obtained from Vacuum Liquid Chromatography

Solvent	Weight (g)	Fraction number
Hexane	0.07	F-5.3
Hexane-chloroform	2.93	F-5.4
Chloroform	1.19	F.5.5
Chloroform-acetone	3.70	F-5.6
Chloroform-acetone	0.18	F-5.7
Acetone	1.06	F-5.8
Methanol	1.76	F-5.8

Further attempts to fractionate 1.19 g of fraction F-5.5 was done on flash CC eluting with 5% acetone in hexane. Five fractions were collected F-5.5A, 949.3 mg which contains most of the pink major compound and a few fluorescent compounds; F-5.5B which contained a dark spot below that of the pink major compound, 105.1 mg; F-5.5C, 210.1 mg; F-5.5D, 76.5 mg; F-5.5E; 13.5 mg. Purification of fraction F-5.5A by CC afforded the isolation of a triterpenoid that was characterized as α-amyrin (**5.19**, 21.3 mg, R_f of 0.25 in 1:9 ethyl acetate-hexane). Fractionation of F-5.5B afforded a mixture of the compound **5.19** and another compound. Silica CC of fraction F-5.5C afforded eight sub-fractions when eluting the column with 5% ethyl acetate-hexane. One fraction yielded 12.5 mg of β-sitosterol **5.7** which was isolated as a white amorphous solid.

Purification of fraction F-5.7, 0.18 g on flash silica gel column chromatography (2% MeOH/$CHCl_3$ increasing to 10%) was done and collecting fractions of 10 ml volume. The fractions were pooled together based on TLC profile and afforded the isolation of a compound characterized as β-sitosterol glucoside **5.8**, 30.1 mg that was obtained as greenish amorphous solids (R_f = 0.31 in 1:9 methanol-chloroform). The solids were then washed with acetone to remove coloured impurities. Six semi-pure fractions were collected along with this compound and their masses ranged from 3 to 76 mg. One of these fractions that showed moderate anticancer activity (TGI $\leq$ 10 μg/ml) was further purified on CC using 5% MeOH/CH_2Cl_2 and yielded 5 mg of a cardenolide coroglaucigenin (5.2).

5.7. PHYSICAL DATA

5.7.1. COMPOUND 5.7

Systematic name: 4-(3,4-Dihydroxy-10-hydroxymethyl-13-methyl-hexadecahydrocyclopenta[a]phenanthren-17-yl)-5H-furan-2-one

Alternative name: Coroglaucigenin

Yield: 5.0 mg (0.04%)

Physical description: White amorphous solid

Mass spectrum: *m/z* $[M\text{-}1H]^- + 2Na^+$ at 434.9, $C_{23}H_{34}O_5$

^{1}H NMR data: See Table 5.7 ($CDCl_3$)

^{13}C NMR data: See Table 5.7 ($CDCl_3$)

5.7.2. COMPOUND 5.6

Systematic name: Acetic acid 4,4,6a,6b,8a,11,12,14b-octamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-eicosahydro-picen-3-yl ester

Alternative name: β-Acetate, amyrin or α-Amyrin acetate

Yield: 53.1 mg (0.45%)

Physical description: White amorphous solid

Optical rotation: $[\alpha]_D^{20}$ +76.37 (c = 0.12, $CHCl_3$) [Lit. $[\alpha]_D^{20}$ +76.35 (c = 0.552, $CHCl_3$), Merck index[28]]

Mass spectrum: *m/z* 468, $C_{32}H_{52}O_2$

^{1}H NMR data: See Table 5.6 ($CDCl_3$)

^{13}C NMR data: See Table 5.6 ($CDCl_3$

5.7.3. COMPOUND 5.7

Systematic name: 17-(4-Ethyl-1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol

Alternative name: β-Sitosterol or (3β)-Stigmast-5-en-3-ol

Yield: 12.5 mg (0.10%)

Physical description: White amorphous solid

Optical rotation: $[\alpha]_D^{20}$ -38.9 (c = 0.11, $CHCl_3$) [Lit. $[\alpha]_D^{25}$ -37 (c = 2, $CHCl_3$), Merck index[28]]

Mass spectrum: *m/z* 414, $C_{29}H_{50}O$

^{1}H NMR data: See Table 5.2 ($CDCl_3$)

^{13}C NMR data: See Table 5.2 ($CDCl_3$)

5.7.4. COMPOUND 5.8

Systematic name: 2-[17-(4-Ethyl-1,5-dimethylhexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-yloxy]-6-hydroxymethyl-tetrahydro-pyran-3,4,5-triol

Alternative name: β-Sitosterol glucoside

Yield: 30.1 mg (0.25%)

Physical description: White amorphous solid

Optical rotation: $[\alpha]_D^{25}$ -38.9 (c = 0.11, $CHCl_3$+MeOH) [Lit. $[\alpha]_D^{25}$ -37 (c = 2, $CHCl_3$), Merck index[28]]

Mass spectrum: *m/z* 576, $C_{35}H_{60}O_6$

^{1}H NMR data: See Table 5.3 & 5.4 (DMSO)

^{13}C NMR data: See Table 5.3 & 5.4 (DMSO)

5.7.5. COMPOUND 5.19

Systematic name: 4,4,6a,6b,8a,11,12,14b-Octamethyl-1,2,3,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-eicosahydro-picen-3-ol

Alternative name: α-Amyrin or Urs-12-en-3β-ol

Yield: 21.3 mg (0.18%)

Physical description: White amorphous solid

Optical rotation: $[\alpha]_D^{20}$ +96.7 (c = 0.11 in $CHCl_3$) [Lit. $[\alpha]_D^{17}$ + 91.6 (c = 1.3 in benzene). Merck index[28]]

Mass spectrum: *m/z* 468, $C_{32}H_{50}O_2$

^{1}H NMR data: See Table 5.5 ($CDCl_3$)

^{13}C NMR data: See Table 5.5 ($CDCl_3$)

5.7.6. COMPOUND 5.21

Systematic name:	2-(3,4-Dihydroxy-2,5-bis-hydroxymethyl-tetrahydro-furan-2-yloxy)-6-hydroxymethyl-tetrahydro-pyran-3,4,5-triol **or** 13-O-Fructofuranosyl α-D-glucopyranoside
Alternative name:	Sucrose
Yield:	72.8 mg (12.1%)
Physical description:	White crystals
Optical rotation:	$[\alpha]_D^{20}$ +66.7 (c = 0.11, MeOH), [Lit +66.1 ± 1 (c = 1.3 in H_2O)]
Mass spectrum:	*m/z* 341.2, $C_{12}H_{22}O_{11}$
^{1}H NMR data:	See Table 5.1 (DMSO)
^{13}C NMR data:	See Table 5.1 (DMSO)

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Chapter 6

PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATIONS OF *MONSONIA ANGUSTIFOLIA*

6.1. INTRODUCTION

A traditional herb for the treatment of erectile dysfunction and for the enhancement of libido was brought to the CSIR by a traditional healer under the umbrella of the Traditional Healers Committee (THC) committee. The committee signed a material transfer agreement for scientific evaluation of their traditionally-used medicine. The traditional healers prescribe an herbal tea made from the plant to their patients for the treatment of erectile dysfunction problems. It was also observed by the traditional healers that the plant enhanced low sexual drive (libido) for both male and female patients. Upon using the herb in the form of tea, the patients reported improvement in both sexual dysfunction and libido. A tea is prepared by taking a handful of the plant material and boiling in a liter of water for thirty minutes.

Male patients who were diagnosed by traditional doctors to have an erectile dysfunction problem took an oral dose of the traditional preparations 1 – 2 hours in advance of sexual activity and reported to have normal erections during sexual intercourse with the effects lasting for up to 3 – 5 hours. For a stronger effect, patients were advised to take a cup of the cooled infusion of the same preparation twice a day for three consecutive days. It has been claimed that the effect lasts for three months before a repeat dose is necessary. No side effects were reported by the traditional doctors.

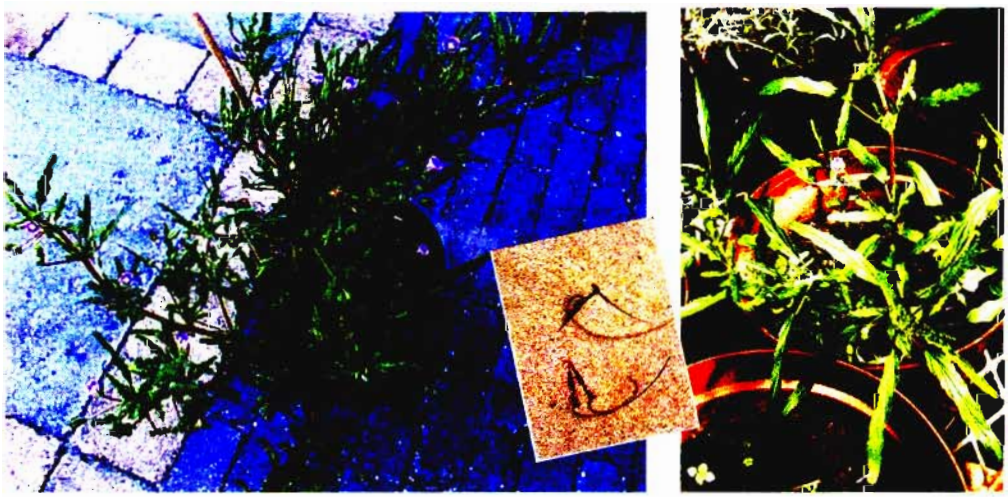
Aqueous and organic extracts of the aerial parts of the plant were tested for the *in vitro* relaxation of corpus cavernosum smooth muscle and the *in vitro* inhibition of the enzyme phosphodiesterase 5 (PDE 5). The same extracts were tested in the *in vivo* rat model to evaluate for the enhancement of sexual activity when compared to the control and an increase in the number of pregnancies of female rats.

The extracts were also screened for anticancer properties in the available anticancer screening technology at the CSIR Bioprospecting Platform hosting a panel of three cancer cell lines that are considered to be highly sensitive, *viz.* melanoma UACC62, renal TK10 and breast MCF7.

The aim of this investigation was to isolate the bioactive compounds by bioassay-guided fractionation.

6.1.1. Botanical aspects of *Monsonia angustifolia*.

The indigenous people who are using the plant and provided the information know the plant by its South Sotho name "*Mohlabakolobe*". SANBI identified the plant as *Monsonia angustifolia E.Mey. ex A. Rich.*, which belongs to the Geraniaceae family (Fig. 6.1). The *Monsonia* genus was named after Lady Anne Monson who, in the eighteenth century, investigated the use of the plant to treat dysentery.[1]



Picture on the left from an unknown electronic source and on the right was taken at the greenhouse at CSIR in Pretoria

Figure 6.1: *Monsonia angustifolia* in flowering state, inserted is a picture of the seeds

Known common names[1] of the plant are crane's bill (English), alsbossie, naaldbossie, teebosie, assegaaibos, rabas (Afrikaans), Mokorotswana (Sotho), Remarungana (Tswana) and Igqitha (Xhosa).

M. angustifolia is an annual herb with wavy leaves that is found in the open grassland throughout South Africa and it is often found by the roadside.[2] The plant grows very well

by seed germination. The species differs from other related species of the same genus by the colour of the flowers. Without the flower, the species look identical. *M. angustifolia* has a distinct bell-shaped purple flower that opens up during the early morning hours.

A literature study was conducted to establish prior art information before undertaking further validation of the plant. It was found that the plant was not well documented for its chemical composition and also for the therapeutic area under investigation.

6.1.2. Ethnopharmacology

Traditional doctors who provided the indigenous knowledge to the CSIR have described the plant to be used as a sexual stimulant, blood cleanser and for the treatment of STD's. The plant was also identified by an undisclosed source as the same as a plant used by the Zion Christian Church (ZCC) members, who use the plant as a blood cleanser under the common name *Special*.

We could not find any direct written record to support the information provided for the treatment of erectile dysfunction. Margaret Roberts[1] is the only source that records the use of this plant to treat other medical conditions:

- Heartburn - drink hot infusions of leaves, stems and fruits (tea)
- Anthrax - apply hot infusion of plant as a skin lotion
- Blackwater disease - use the plant leaves
- Diarrhoea - drink hot infusions of plant
- Eye infections - use weak infusion of plant
- Ophthalmia - wash infection with cooled infusion of plant
- Haemorrhoids - wash skin with cooled infusion of plant
- Snakebites - drink and apply hot infusion of plant (tea) as a skin lotion
- Sores (slow healing) - apply sap/juice from stems or leaves to affected area
- Ulcers - drink and apply cooled infusions of plant as skin lotion
- Dyspepsia - drink hot infusions of leaves, stems and fruits
- Flatulence - drink hot infusions of leaves, stems and fruits
- Digestive disturbances - drink hot infusions of leaves, stems and fruits
- Varicose veins - drink and apply cooled infusions of plant as skin lotion

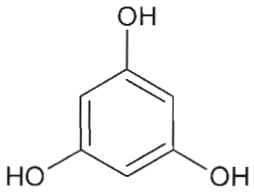
It was reported that the leaf and root extracts of the *M. biflora* DC are used in South Africa for diarrhea, dysentery and snakebite and an infusion of the whole plant is regarded as an abortifacient. Several other species of *Monsonia* are reported to have been used in the treatment of dysentery, typhoid fever, intestinal hemorrhage and diarrhea.[3]

M. emarginata is used by the Zulu's to treat snakebites and dysentery whereas the Xhosa and Mfengu use it the same way as *M. angustifolia* is used.[4] Decoctions are taken for colds and fever. The boiled roots of the plant species were drunk for rituals among the Central Kalahari Hunter Gatherers for the treatment of anodyne. A variety of species have been used for diarrhea, dysentery and gastric disturbances. The possible effects have been attributed to tannins.[6]

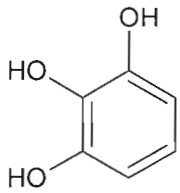
Monsonia species have been used as a snake bite antidote by the Zulu's. Decoctions are taken for colds and fever and whole plant infusions are taken as abortifacients. The plants are also part of ingredients in medicines taken for anthrax. The leaf sap is applied to the pustules associated with the diseases.[1]

6.2. PREVIOUS PHYTOCHEMICAL STUDIES

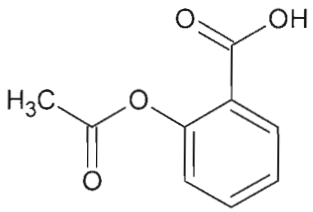
Phloroglucinol (**6.1**), which has antiprotozoal activity, tericin, and entericin (**6.3**)[5] were reported to be present in the plant genus.[6] Pyrogallol (**6.2**) was isolated from the alcoholic extract of *M. burkeana.* It was reported that *M. niveae* from the Arabian Gulf contain saponins, coumarins, flavonoids, sterols/triterpenes and tannins.[7] However, to our knowledge, there is no published phytochemical information on *M. angustifolia.*



6.1: Phloroglucinol



6.2: Pyrogallol

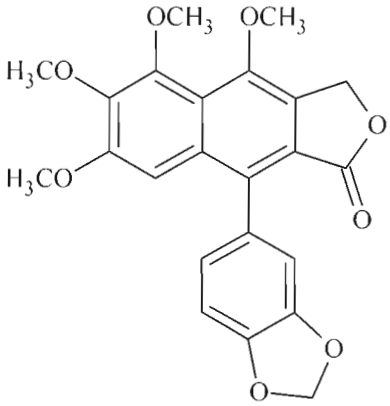


6.3: Entericin

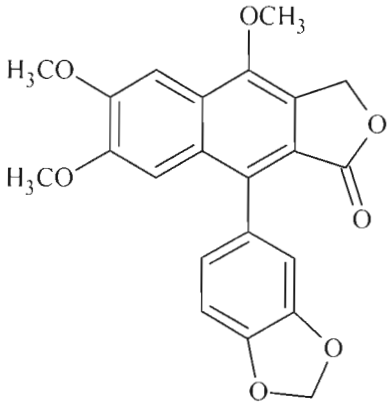
6.3. ISOLATION AND STRUCTURE ELUCIDATION OF THE ACTIVE COMPOUNDS

An aqueous extract mimicking the recorded traditional preparation method and an organic extract prepared according to the standardized pharmaceutical preparation that employed a (1:1, v/v) mixture of methanol and dichloromethane was obtained from the ground plant material. The efficacy was checked by testing both of the extracts for their potential to inhibit phosphodiesterase 5 (PDE 5), which limits the hydrolysis of cyclic guanosine monophosphate (cGMP) to 5' guanosine monophosphate (5'GMP) and thereby increases the intracellular concentrations of cGMP facilitating corpus cavernosum smooth muscle relaxation. The organic extract exhibited a much higher inhibition of PDE 5; henceforth we proceeded with purification of this extract (Fig 6.2).

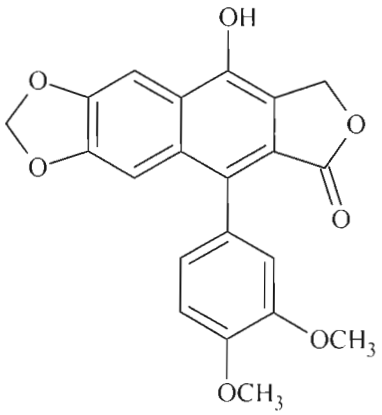
Initial liquid-liquid partitioning of the extract (see Chapter 3, liquid-liquid partition protocol) afforded hexane, dichloromethane and water fractions. Based on PDE 5 inhibition, the dichloromethane fraction showed a 20% improvement in activity compared to the original crude extract. Repeated column chromatography on flash silica gel afforded the isolation of five compounds that were identified as aryl naphthalene lignans **6.4** to **6.8**, which are formed biosynthetically from two phenyl propanyl (C6-C3) units. In addition one steroid, β-sitosterol (**6.9**), was also obtained.



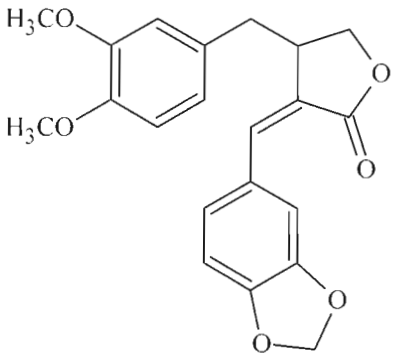
6.4: 5-Methoxyjusticidin A



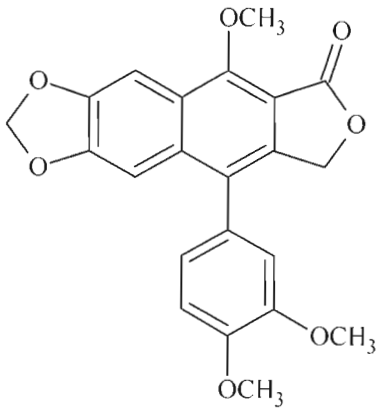
6.5: Justicidin A



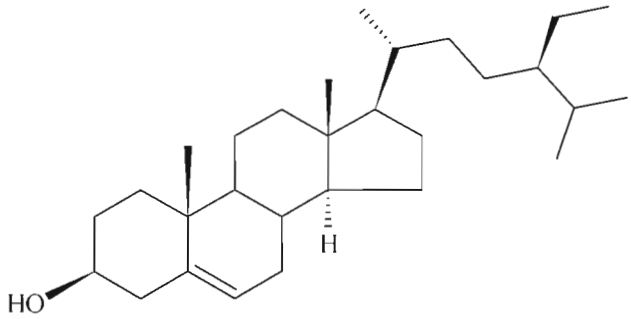
6.6: Chinensinaphthol



6.7: Suchilactone

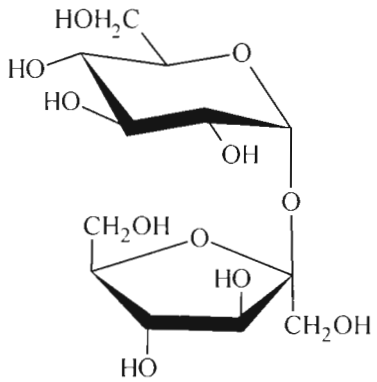


6.8: Retrochinensinaphthol methyl ether



6.9: β-Sitosterol

The aqueous extract had a less significant inhibition of PDE 5. Fractionation of this extract on silica gel afforded a carbohydrate disaccharide, sucrose (**6.10**). Sucrose has also been isolated from *Pergularia daemia* and the assignment of the structure is described in section 5.3.1.



6.10: Sucrose

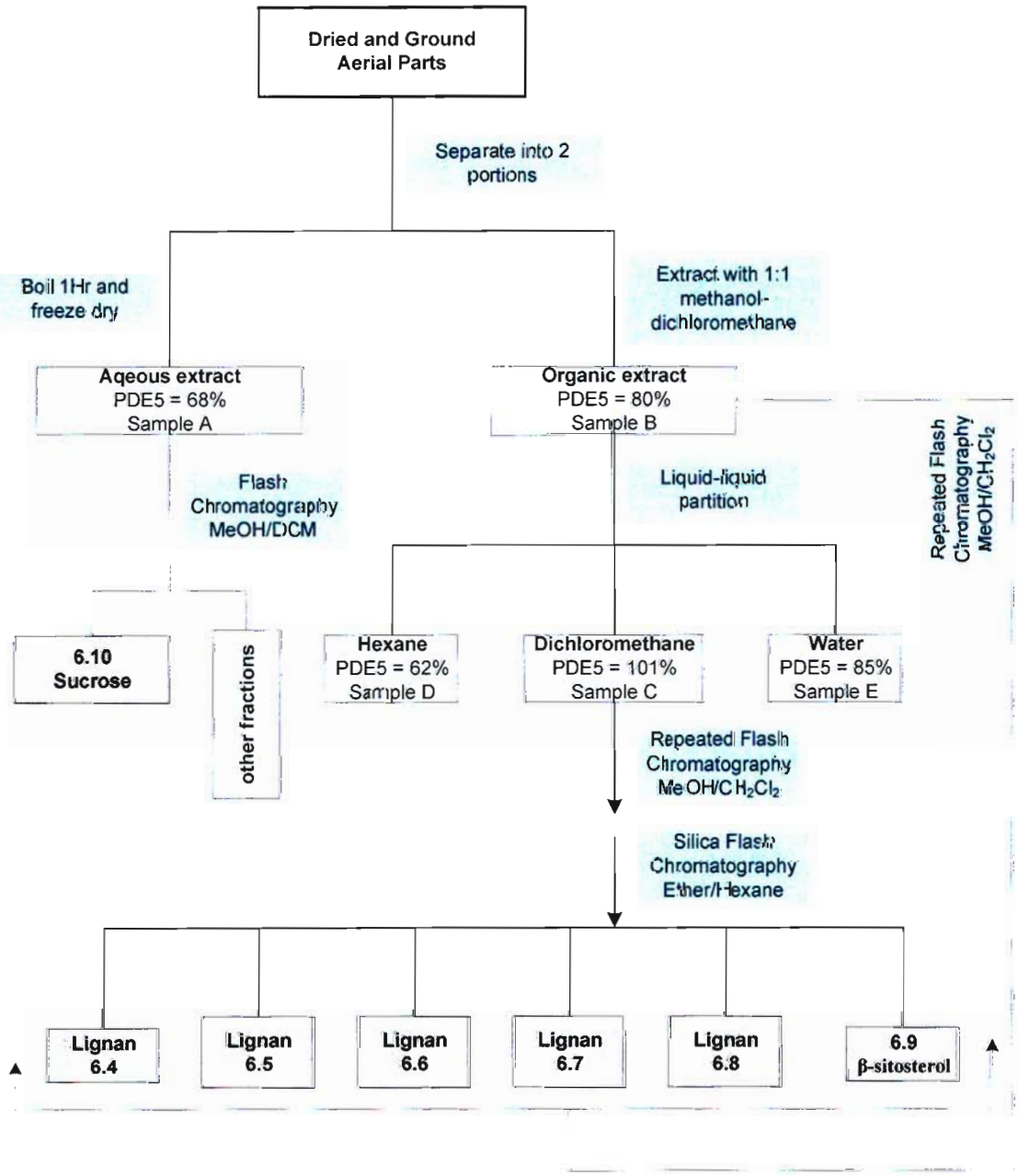


Figure 6.2: General extraction and isolation procedure

6.3.1 Characterization of 5-methoxyjusticidin A (6.4)

5-Methoxyjusticidin A (**6.4**) was isolated as a white amorphous solid. A molecular ion peak at *m/z* 425.4 corresponding to $[M+1]^+$ in the positive LRESIMS and at *m/z* 424 in AutoSpec ETOF EI^+ which are both pointing to the molecular formula $C_{23}H_{20}O_8$. The UV spectrum was obtained from a diode array detector (DAD) and absorptions were observed at λ 365, 263 and 237 nm.

In the ^1H NMR spectra of **6.4** (Table 6.1) the presence of a methylenedioxy group, δ_H 6.02 (1H, d) and δ_H 6.07 (1H, d) with a very small coupling of *J* 1.3 Hz was observed. The other methylene singlet observed at δ_H 5.41 is characteristic of the methylene group found in a penta-lactone ring of lignans. Four singlets assigned to the methoxyl groups were observed at δ_H 3.74, 3.95, 3.97 and 4.01. An aromatic proton singlet was observed for H-8 at δ_H 6.94. Three aromatic protons were found exhibiting the *ortho* coupling (*J* = 7.9 Hz) and *meta* coupling (*J* = 1.6 Hz) which are consistent with a 1,3,4 trisubstituted phenyl group.

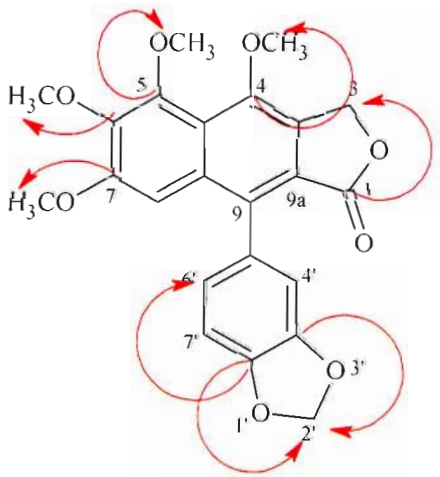
The ^{13}C NMR spectra showed 23 resonance signals. Four signals could immediately be assigned to the four methoxyl carbons resonating at δ_C 55.8, 61.4, 62.4, and 62.0. The multiplicity of the other carbon signals was obtained from DEPT spectra that showed 10 protonated carbon signals, two of them being CH_2 and four were CH, apart from the four that were already assigned as methoxy groups. There was also evidence of a carbonyl group at δ_C 169.5, which is characteristic of the lactone functionality in the strained five-membered ring. C-3 was also not found in the aromatic region and was assigned to the methylene group at δ_C 66.5 attached to the carbonyl forming a lactone. The structure was confirmed by the $^{>1}J_{CH}$ correlations observed in a HMBC experiment.

5-Methoxyjusticidin **6.4** was previously isolated by Siani *et al.* from the wood of *Protium unifoliolatum*[8]. In this article, some of the NMR signals were not assigned correctly (C-9, C-9a, C-8, and C-8a) and we were able to assign them with the aid of 2D experiments. The author acknowledged the wrong assignment and thus the assignment we propose can be taken as correct.

Table 6.1: NMR data* of 5-methoxyjusticidin A (**6.4**) in $CDCl_3$

C	δ_C	δ_H (*J* in Hz)	HMBC $^{>1}J_{CH}$ correlations
1	169.5		
3	66.5	5.41 (s)	C-1, C-4, C-9a
3a	120.7		
4	149.1		
4a	122.2		
5	148.1		
6	144.9		
7	153.1		
8	103.7	6.94(s)	C-4a, C-6, C-9
8a	133.5		
9	135.6		
9a	129.9		
4-OCH_3	62.0	3.97(s)	C-4
5-OCH_3	62.4	3.95(s)	C-5
6-OCH_3	61.4	4.01(s)	C-6
7-OCH_3	55.8	3.74(s)	C-7
2'	101.2	6.02, 6.07 (d, 1.4; d, 1.4)	C-7a', C-3a'
3a'	147.6		
4'	110.6	6.78 (d, 1.3)	C-6', C-9, C-7a'
5'	128.5		
6'	123.6	6.75, 6.76 (dd, 1.6, 7.9)	C-4', C-9, C-7a'
7'	108.3	6.92, 6.94 (d, 7.9)	5', C-3a'
7a'	147.5		

* ^{13}C NMR (125.7 MHz) and ^{1}H NMR (500 MHz)



6.4

Figure 6.3: Key correlations obtained from HMBC data

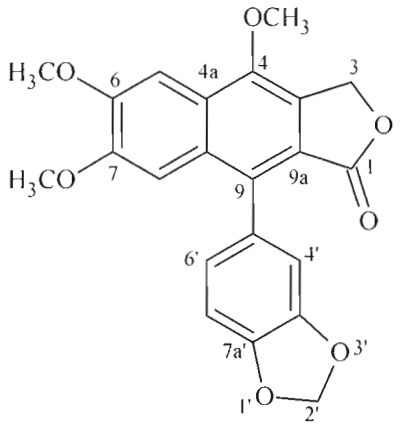
6.3.2 Characterization of justicidin A (6.5)

This compound was isolated as white amorphous flakes. The ^{1}H NMR spectrum of **6.5** was similar to that of **6.4**. The main difference was that the spectrum of **6.5** exhibits the presence of only three methoxy groups (δ_H 3.79, 4.04, 4.11) instead of four as for **6.4**. One methoxy at C-5 was replaced by a proton resonating at δ_H 7.52. The absence of observed coupling between the two protons resonating at δ_H 7.52 and 7.04 suggests a *para* relationship between them. The methylene signal at δ_H 5.51 which forms part of the furanone ring was still present as was the three aromatic protons that formed the 1, 3, 4-trisubstituted phenyl group. A pair of proton doublets with very weak coupling (δ_H 6.02 and 6.06 with a J_{HH} value of 0.9 Hz) was observed for the methylenedioxy substituent.

Twenty two ^{13}C NMR signals were observed which is in compliance with one less methoxy group. The three methoxy groups were assigned at δ_C 55.8, 56.1 and 59.7. A carbonyl signal was observed downfield at δ_C 169.5. The methylene group forming part of the furanone ring was assigned at δ_C 66.6. The methylenedioxy carbon signal was found at δ_C 101.2. The rest of the carbon signals were found to support the structural framework with the same substitution pattern as for 5-methoxyjusticidin A **(6.4).**

Justicidin A (**6.5**) showed a molecular ion peak of *m/z* 395.4 in ESIMS positive mode corresponding to $[M+1]^+$ and the molecular formula $C_{22}H_{18}O_7$. In the UV spectrum of this compound as obtained from a diode array detector has a strong absorption maximum at 261.7 nm. This compound is widely distributed within the different species of *Justicia* and it has been widely studied before for antiviral[9], anti platelet[10,11] and cytotoxic[12] activity but not for the activity reported here.

Published NMR data for justicidin A[13,14] supported the structural assignment of justicidin A **(6.5)**.



6.5

Table 6.2: NMR data of justicidin A (6.5) in $CDCl_3$

C	δ_C	Dept & HMQC	δ_H (J_{HH} in Hz)	COSY	HMBC $^{>1}J_{CH}$ correlations
1	169.5	C=O			
3	66.6	CH_2	5.51		C-9a, C-4, C-1
3a	119.3	C			
4	147.8	C			
4a	126.0	C			
5	100.6	C	7.52	6-OCH_3	
6	151.6	C			
7	150.4	C			
8	106.2	CH	7.04	7-OCH_3	C-4a, C-9, C-6
8a	130.7	C			
9	134.4	C			
9a	124.5	C			
4-OCH_3	59.7	OCH_3	4.11		C-4
6-OCH_3	56.1	OCH_3	4.04		C-6
7-OCH_3	55.8	OCH_3	3.79		C-7
2'	101.2	CH_2	6.02, 6.06 (d, 1.0)		C-7a', C-3a'
3a'	147.5	C			
4'	110.8	CH	6.8 (s)		C-6', C-9, C-7a'
5'	128.5	C			
6'	123.6	CH	6.76, 6.78(dd, 7.9, 1.4)	H-7'	C-4', C-9, C-7a'
7'	108.2	CH	6.92, 6.94 (d, 7.9)	H-6'	C-5', C-3a'
7a'	147.4	C			

* ^{13}C NMR (125.7 MHz) and ^{1}H NMR (500 MHz)

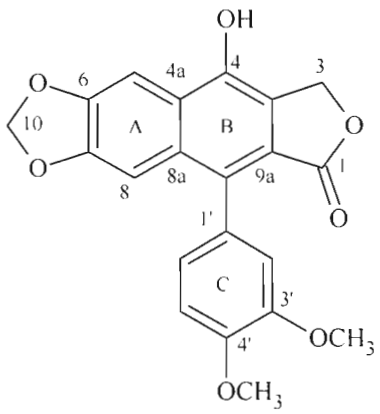
6.3.3. Characterization of chinensinaphthol (6.6)

Compound **6.6** is commonly known as chinensinaphthol and it was previously isolated from *Justicia procumbens* and tested for antiplatelet properties as documented by Z. Horii et al 1968.[15]

A mass to charge ratio (*m/z*) of 381.4 observed in LRESIMS in positive mode was assigned to the molecular ion peak $[M+1]^+$, which is in agreement with the formula $C_{21}H_{16}O_7$. The UV maximum plot of this compound from the DAD spectrum showed absorptions at 322.0, 266.4 and 228.8 nm, which is characteristic of aryl naphthalene molecules.

Compound **6.6** was purified by flash silica gel chromatography and visualization under long range UV 254 nm gave a purple coloured spot and a very intense blue fluorescent color when viewed at 366 nm. It was obtained as a white amorphous substance that could not be re-dissolved in a single solvent only. Thus, it was dissolved in a mixture of $CDCl_3$ and MeOH-d_4 for NMR experiments and the obtained data are collated in Table 6.3.

^{1}H NMR data showed a singlet at δ_H 5.35 which can be assigned to the lactone methylene group and a resonance at δ_H 6.15 (s, H-10) that is characteristic of a methylenedioxy group, and two methoxyl groups appeared as singlets at δ_H 3.71 and 3.84. In the previous two structures (**6.4** and **6.5**), the methylenedioxy groups were attached to the C ring and the methoxy groups to the A ring. However, $^{>1}J_{CH}$ correlations observed in the HMBC experiment, indicated that in this compound, the methylene group is on the A ring and the methoxy substitutents on the B ring. The three aromatic C ring protons formed an ABX system characteristic of trisubstituted phenyl as it was observed for the lignans **6.4** and **6.5**. These protons resonated at δ_H 6.83 (H-2', d, 8.24), 6.77 (H-6', dd, 2.07 & 8.03), 7.05 (H-5', d, 8.24) and their coupling was supported by the COSY 2D correlations.



6.6

Table 6.3: NMR data* of Chinensinaphthol (**6.6**) in $CDCl_3$ & CD_3OD

C	δ_C	Dept & HMQC	δ_H (J in Hz)	COSY	HMBC $^{>1}J_{CH}$ correlations
1	169.4	C=O			
3	66.4	CH_2	5.35 (s)		C-1, C-9a , C-4
3a	119.0	C			
4(-OH)	145.1	C			
4a	124.6	C			
5	97.9	CH	7.61 (s)		C-8a, C-4, C-7
6	148.6	C			
7	148.0	C			
8	102.5	CH	6.85 (s)		C-4a, C-6, C-9
8a	131.0	C			
9	130.3	C			
9a	122.3	C			
10	101.9	CH_2	6.15 (s)		C-6, C-7
1'	127.5	C			
2'	114.2	CH	6.83 (d, 2.1)	H-6'	C-4', C-6', C-9
3'	148.2	C			
4'	148.3	C			
5'	111.2	CH	7.05 (d, 8.2)	H-6'	C-1', C-3'
6'	122.5	CH	6.77 (dd, 2.1 & 8.0)	H-5', H-2'	C-2', C-3', C-9
3'-OCH_3	55.5	OCH_3	3.71 (s)		C-3'
4'-OCH_3	55.4	OCH_3	3.84 (s)		C-4'

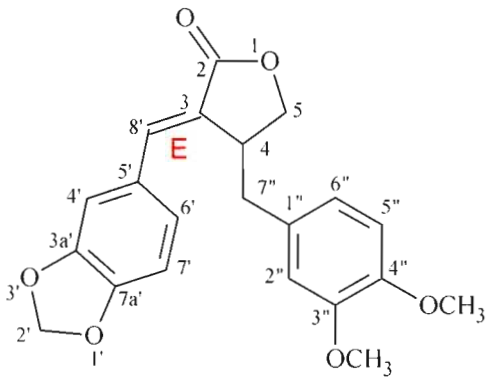
* ^{13}C NMR (100.6 MHz) and ^{1}H NMR (400 MHz)

^{13}C NMR spectra data with 21 carbons supported the proposed structure **6.6** and the mass spectrum data obtained. The carbonyl C-1 of the lactone ring and the methylene group C-3 in the same ring were assigned resonances at δ_C 169.4 and 66.4 respectively. Other important signals were the two methoxy groups that resonated at δ_C 55.4 and 55.5 and the

methylenedioxy C-10 that resonated at δ_C 101.9.

On the basis of the spectral data and the supporting correlations observed in 2D NMR experiments, compound **6.6** was identified as chinensinaphthol.

6.3.4. Characterization of suchilactone, 6.7

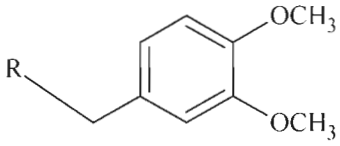


6.7

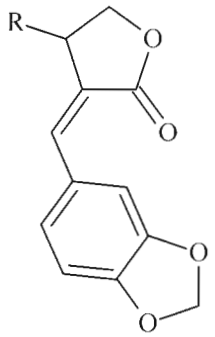
Crystalline and yellowish **6.7** was isolated from the methanol-dichloromethane extract of *M. angustifolia* and its spectral data is collated in Table 6.4. The structure was deduced with the help of the 2D NMR correlations that provided information to the C-H connectivity pattern. The proposed structure was also confirmed by comparison to literature data.[16]

ESIMS (+) of compound **6.7** showed a molecular ion peak of *m/z* 368 which agrees with a molecular formula of $C_{21}H_{20}O_6$. In the mass spectrum, fragments were observed at m/z 151, which was assigned to the fragment **6.7.1** and at *m/z* 217 assigned to the fragment **6.7.2**.

Two methoxy groups were observed in the ^{1}H NMR spectrum at δ_H 3.84 and 3.86 as singlets. In contrast to the other compounds where only one 1,3,4-trisubstituted phenyl ring was observed, in the ^{1}H NMR spectrum of **6.7,** a second set of ABX protons were present. Additionally, an olefinic proton was observed at δ_H 7.50.



6.7.1



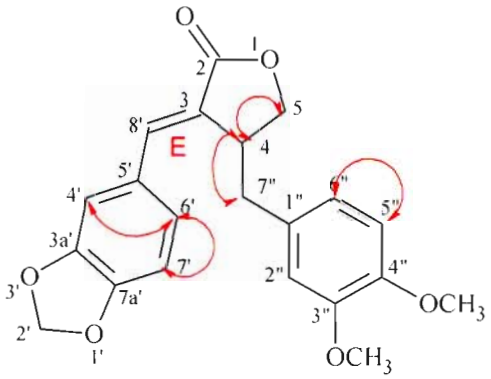
6.7.2

Table 6.4: NMR data* of Suchilactone (**6.7**) in $CDCl_3$

C	δ_C	δ_H (J in Hz)	DEPT & HMQC	COSY	HMBC $^{>1}J_{CH}$ correlations
2	172.5		C=O		
3	126.1		C		
4	40.1	3.75 (m)	CH	H-7", H-5	C-8', C-2, C-1"
5	69.8	4.26 (m)	CH_2	H-4	C-7", C-3, C-2
2'	101.7	6.02 (d, 1.9)	CH_2		C-3a', C-7a'
3a'	148.4		C		
4'	126.3	7.08 (s)	CH		C-6', C-7a', C-8'
5'	128.3		C	H-6'	
6'	108.5	7.06 (s)	CH	H-7', H-4'	C-4', C-8', C-7a'
7'	108.8	6.87 (dd, 8.5, 1.9)	CH	H-6'	C-5', C-3a'
7a'	149.2		C		
8'	137.2	7.50 (d, 1.9)	CH		C-4', C-6', C-2, C-4
1"	130.4		C		
2"	120.9	6.73 (dd, 2.0, 8.2)	CH		C-4", C-6", C-7"
3"	149.2		C		
4"	148.1		C		
5"	111.5	6.85 (d, 8.1)	CH	H-6"	C-1", C-3"
6"	112.2	6.69 (d, 2.0)	CH		C-2", C-4", C-7"
7"	37.7	2.63 (dd, 14.13, 4.31); 3.01 (dd 14.12, 10.02)	CH_2	H-4	C-3, C-5, C-2", C-6"
4"-OCH_3	55.9	3.86 (s)	OCH_3		C-4"
3"-OCH_3	55.9	3.84 (s)	OCH_3		C-3"

* ^{13}C NMR (100.6 MHz), ^{1}H NMR (400 MHz)

From the COSY correlations it was observed that the protons at H-4 couples with those protons at positions H- 5 and H-7". These key correlations are shown in Fig 6.4 and they are represented by arrows in red. The other correlations observed were H-6' to H-4' and H-7' and also in the C ring there was a strong correlation between H-5" and H6".



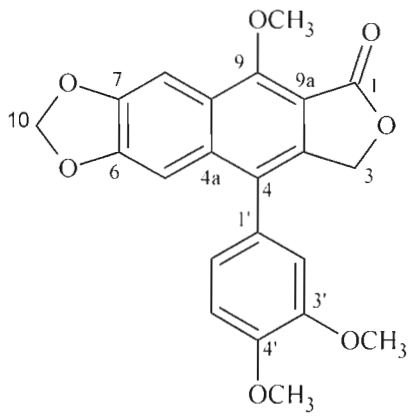
6.7

Figure 6.4: Key COSY correlation of compound **6.7**

The ^{13}C NMR of compound **6.7** showed 21 carbon signals with two methoxy groups overlapping at δ_C 55.9. The carbonyl in the lactone ring resonates at δ_C 172.5 and the methylene group in the same ring appears at δ_C 69.8. The exocyclic double bond of the lactone ring showed resonance peaks at δ_C 126.1 and 137.2 for the carbons at C-3 and C-8', respectively. The methylene carbon at C-7" was assigned to the signal at δ_C 37.7. The methylenedioxy group at C-2' showed its resonance peak at δ_C 101.7. The DEPT spectrum was used to assign the multiplicity of the carbon resonances. Two overlapping methoxy peaks, three CH_2 and eight CH peaks, leaving the remaining eight carbon signals to be assigned as quaternary carbon atoms.

6.3.5. Characterization of retrochinesinaphthol methyl ether, 6.8

Compound **6.8** ($C_{22}H_{18}O_7$) was identified as retrochinesinaphthol methyl ether.[17] A mass-charge ratio *m/z* 395.4 $[M+1]^+$ (ESIMS) was obtained which supports the molecular formula of $C_{22}H_{18}O_7$. The structure of the compound is similar to that of **6.6** with the difference that the methylene and the carbonyl groups of the lactone ring are swopped around and that the hydroxyl group in position C-4 was now replaced by a methoxy group.



6.8

Table 6.5: NMR data* of compound **6.8** in $CDCl_3$

C	δ_C	Dept & HMQC	δ_H (*J* in Hz)	COSY	HMBC $^{>1}J_{C-H}$ correlations
1	169.1	C=O			
3	68.8	CH_2	5.11 (d,15.0; d, 15.0)		C-1, C-4, C-9a
3a	139.4	C			
4	127.5	C			
4a	135.3	C			
5	102.2	CH	6.98 (s)	H-8	C-7, C-8a, C-4
6	148.2	C			
7	150.8	C			
8	100.2	CH	7.71 (s)		C-4a, C-6, C-9
8a	125.3	C			
9	155.7	C			
9a	110.1	C			
10	101.8	CH_2	6.06 (s)		C-6, C-7
9-OCH_3	63.5	OCH_3	4.31 (s)		C-9
1'	128.5	C			
2'	112.6	CH	6.79 (d, 2.0)	H-6'	C-4', C-6', C-4
3'	149.4	C			
4'	149.0	C			
5'	111.8	CH	6.99 (d, 8.0)	H-6'	C-1', C-3'
6'	121.9	CH	6.84, 6.86 (dd, 2.0, 8.0)	H-5', H-2'	C-2', C-4', C-4
3'-OCH_3	56.1	OCH_3	3.86 (s)		C-3'
4'-OCH_3	56.0	OCH_3	3.96 (s)		C-4'

* ^{13}C NMR (100.6 MHz) and ^{1}H NMR (400 MHz)

Comparison of the ^{1}H and ^{13}C NMR data of this compound (**6.8**) with those reported in the literature[17] and observed for **6.6** led to the assignment of compound **6.8** as retrochinesinaphthol methyl ether.

6.3.6. Characterization of β-sitosterol (6.9)

The structure of compound **6.9** was assigned as β-sitosterol by comparison (TLC, GC, MS and NMR) with the compound isolated from *Pergularia daemia* (section 5.3.2) and with a commercial sample of β-sitosterol obtained from Sigma Aldrich.

Table 6.6: NMR data* of β-sitosterol (**6.9**) in $CDCl_3$

	Observed			Literature	
C	δ_C	Dept	δ_H (J in Hz)	δ_C	δ_H (J_{HH} in Hz)
1	37.2	CH_2	1.89	37.1	1.05m; 1.83m
2	31.6	CH_2	1.56	31.5	1.82m; 1.48m
3	71.8	CH	3.50(1H,m)	71.7	3.48m
4	42.4	CH	2.26	42.2	2.28m
5	140.7	C		140.8	
6	121.7	CH	5.32(1H, d, 5.0)	121.7	5.32(d, 5.0)
7	31.8	CH_2	1.53	31.7	1.53m
8	31.7	CH	1.93	31.7	1.90m
9	50.1	CH	0.98	50.0	0.87m
10	36.5	C		36.3	
11	21.1	CH_2	1.52	20.9	1.43m
12	39.8	CH_2	2.03	39.6	1.13m; 1.99m
13	42.3	C		42.7	
14	56.8	CH	0.99	56.7	0.94m
15	24.3	CH_2	1.61	24.1	1.13m; 1.61m
16	28.2	CH_2	1.85	28.1	1.35m; 1.86m
17	56.1	CH	1.12	55.9	1.06m
18	11.8	CH_3	0.64 (s)	11.7	0.65s
19	19.3	CH_3	0.93 (s)	16.7	1.00s
20	36.1	CH	1.37	36.0	1.32m
21	19.3	CH_3	0.90 (d, 7.0)	19.2	0.92 (d, 7.0)
22	33.9	CH_2	1.35	33.8	0.99m; 1.26m
23	26.1	CH_2	1.23	25.9	1.12m
24	45.8	CH	0.96	45.7	0.95m
25	29.2	CH	1.70	29.0	1.62m
26	19.8	CH_3	0.82 (d, 7.0)	18.9	0.88 (d, 7.0)
27	19.0	CH_3	0.81 (d, 7.0)	18.6	0.77 (d, 7.0)
28	23.1	CH_2	1.27	22.9	1.20m
29	11.9	CH_3	0.84 (t, 7.0)	11.8	0.82 (t, 7.0)

* 500MHz for ^{1}H NMR and 125MHz

The mass spectra data was observed giving the molecular ion peak *m/z* at 415 and a base line peak at *m/z* 414 $[M-H]^+$ as determined by the AutoSpecETOF operating at EI^+. The observed molecular ion is in agreement with the proposed molecular formula of $C_{29}H_{30}O$. Further confirmation of the mass spectral data was obtained for the $ESIMS^-$ operating in the negative ion mode which gave a molecular ion peak of 459.2 that was attributed to the chelating of this molecule with two sodium adducts $[M+2Na]^-$.

6.3.7. Characterization of sucrose (6.10)

A disaccharide **6.10** was obtained as clear and crystalline compound and by comparison with the compound from *Pergularia daemia* (section 5.3.1) and commercial material, identified as sucrose.[18] The mass spectra data supported the proposed structural formula of $C_{12}H_{22}O_{11}$ with a base peak of *m/z* 341 $[M-H]^+$ obtained in the negative ion mode on ESIMS.

Table 6.7: NMR data of the sucrose (**6.10**)

	Sucrose (6.10)			
	Isolated compound*		Literature[19#]	
C	δ_C	δ_H (J_{HH}, in Hz)	δ_C	δ_H
1	92.4	5.12	93.20	5.41
2	72.3	3.13	72.14	3.56
3	73.5	3.61	73.68	3.76
4	70.5	3.07	70.31	3.86
5	73.4	3.42	73.44	3.47
6	61.1	3.58	61.24	3.81
1`	62.7	3.58, 3.35 (2H)	62.46	3.67
2`	104.7		104.71	
3`	77.7	3.83	77.51	4.21
4`	75.0	3.72	75.09	4.05
5`	83.2	3.52	82.42	3.89
6`	62.7	3.49	63.44	3.83

* Data obtained in DMSO, ^{13}C at 100.5 MHz and ^1H at 400 MHz
\# Data obtained in D_2O, ^{13}C at 100 MHz and ^1H at 400 MHz

6.4. QUANTIFICATION OF *M. ANGUSTIFOLIA* EXTRACTS BY HPLC-MS

As part of the investigation on this plant for its use as a treatment for erectile dysfunction and enhancing sexual drive, analytical methods for the validation of the extract were developed.

The extract was chromatographed on reverse phase C18 column in order to obtain a chemical profile on the LCMS. The solvent gradient used was composed of a mixture of acetonitrile and water as tabulated in Table 6.8.

Table 6.8: Alliance 2690 HPLC pump gradient and UV/MS event conditions

Time (min)	% Water	% ACN	Flow Rate
0.00	90.0	10.0	1.000
61.00	20.0	80.0	1.000
64.00	0.0	100.0	1.000
75.00	0.0	100.0	1.000
75.10	90.0	10.0	1.000
90.00	90.0	10.0	1.000
Column temperature		$40^{O}C$	
Mass range scan		50 to 2000	
Wavelength scan (nm)		193.00 to 400.00	

The extract was chromatographed at concentrations of 5000 ppm and 25 µl was injected. The chemical fingerprint obtained for the methanol-dichloromethane extract is shown in Fig. 6.5.

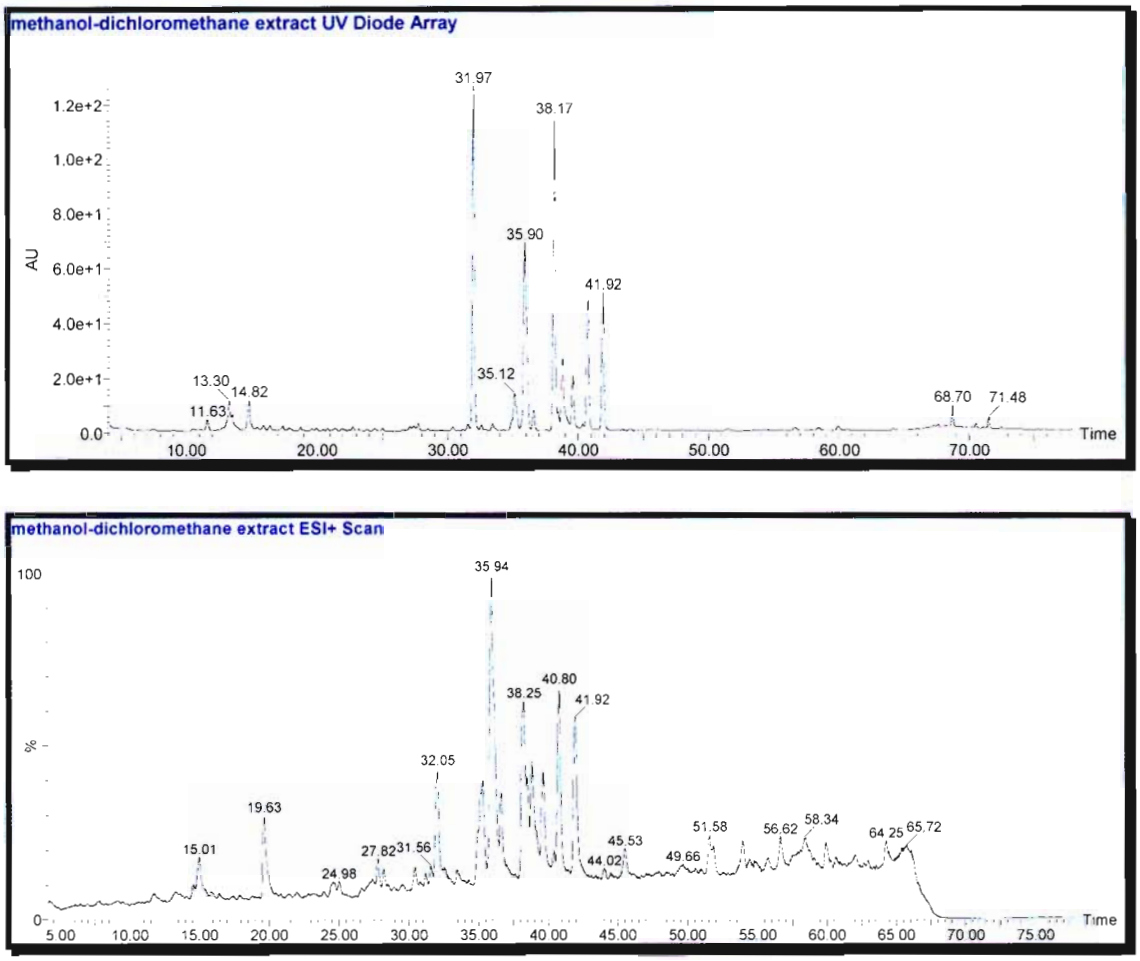


Figure 6.5: UV and ESIMS$^+$ chromatogram of the methanol-dichloromethane extract

The extract demonstrated the presence of several compounds but the most UV absorbing compounds are concentrated in the region between 30 and 45 minutes. In light of the obtained results, continuation of this work in order to identify the active ingredients was done by means of liquid-liquid partition, column chromatography purification and characterization by NMR spectroscopy experiments. Five lignan compounds, **6.4** to **6.8** were isolated and they were shown to be present in the original methanol-dichloromethane extract by HPLCMS chromatography experiments. Figure 6.6 lists the five chromatograms as obtained for the five compounds.

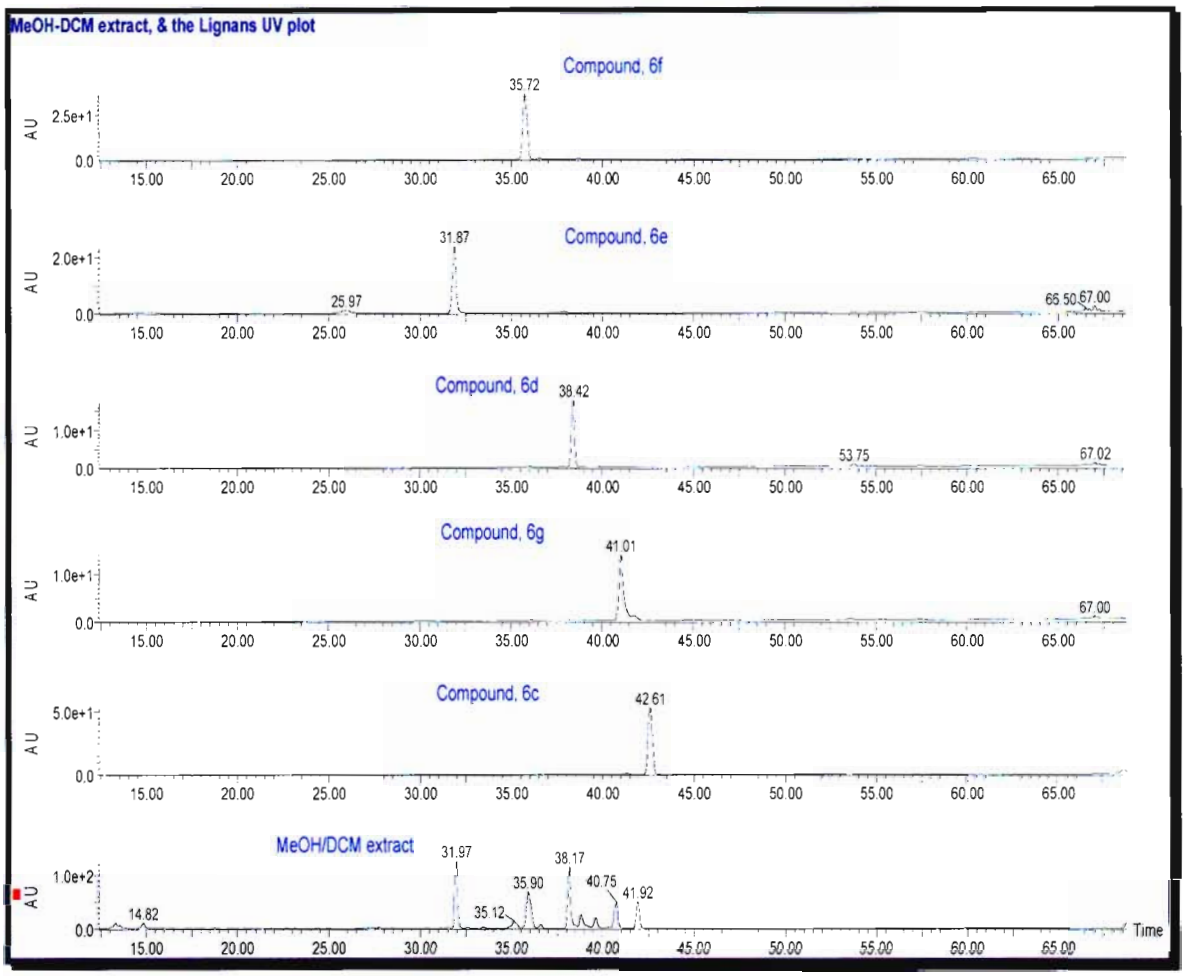
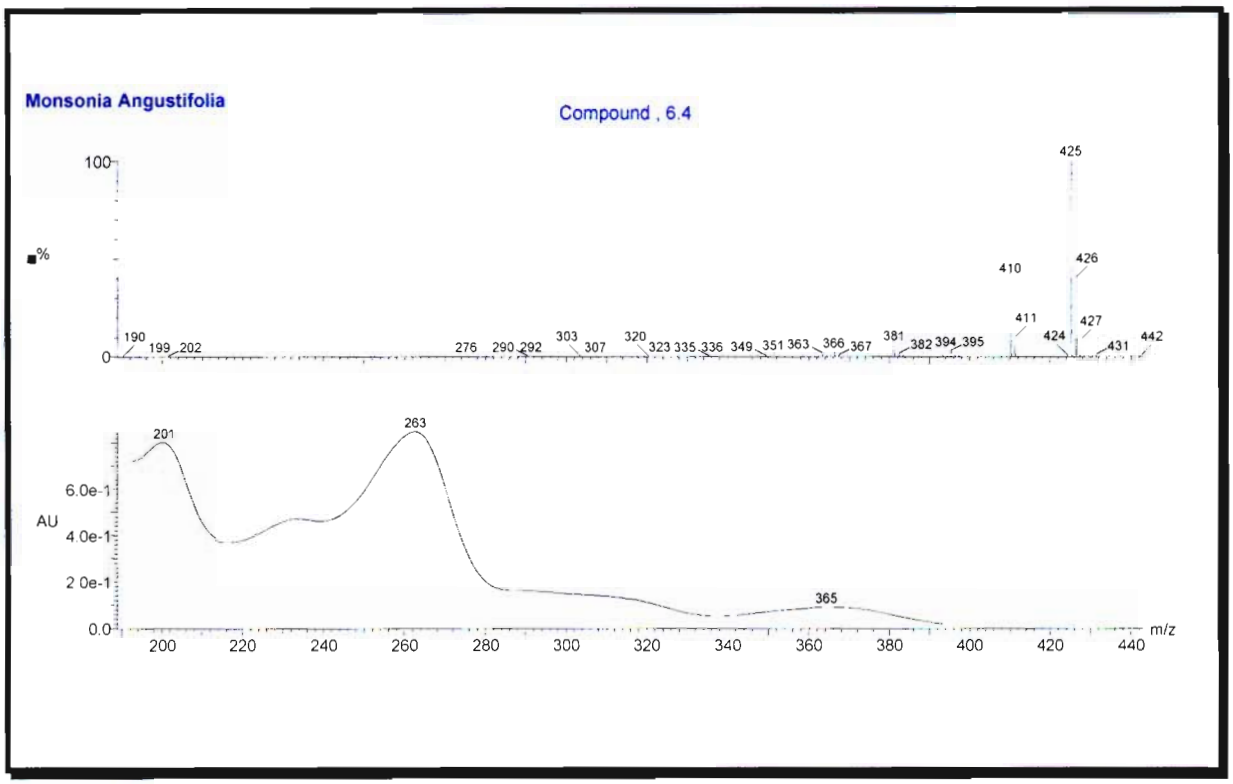


Figure 6.6: UV chromatograms of the five lignans isolated from the aerial parts of *M. angustifolia*. Compounds 6c = **6.4**, 6d = **6.5**, 6e = **6.6**, 6f = **6.7** and 6g = **6.8**.

It was very clear by looking at the UV plots of the compounds against the extract in Figure 6.6 that the extract contained all of the compounds. The chromatogram also shows that there are still a few UV absorbing compounds that were not yet isolated, but the scope of this kind of work does not form part of this study and will be reported elsewhere. The UV chromophores and the mass spectra of each of the compounds are pasted in Figure 6.7, confirming the presence of the compounds. It is worth noting that each compound is shown to absorb at a high UV range which is the long range spectra visible at 366 nm. These compounds appeared as blue spots of varying intensities at this wavelength. The UV chromophores also show that the compounds are UV absorbing at the UV Visible region at wavelength of 254 nm. The compounds appear purple-pink at this wavelength. All compounds are shown to absorb from between 257 to 263 nm. Their molecular weights ranged between 368 and 424 amu as determined by the $[ESI\text{-}MS]^{+}$.



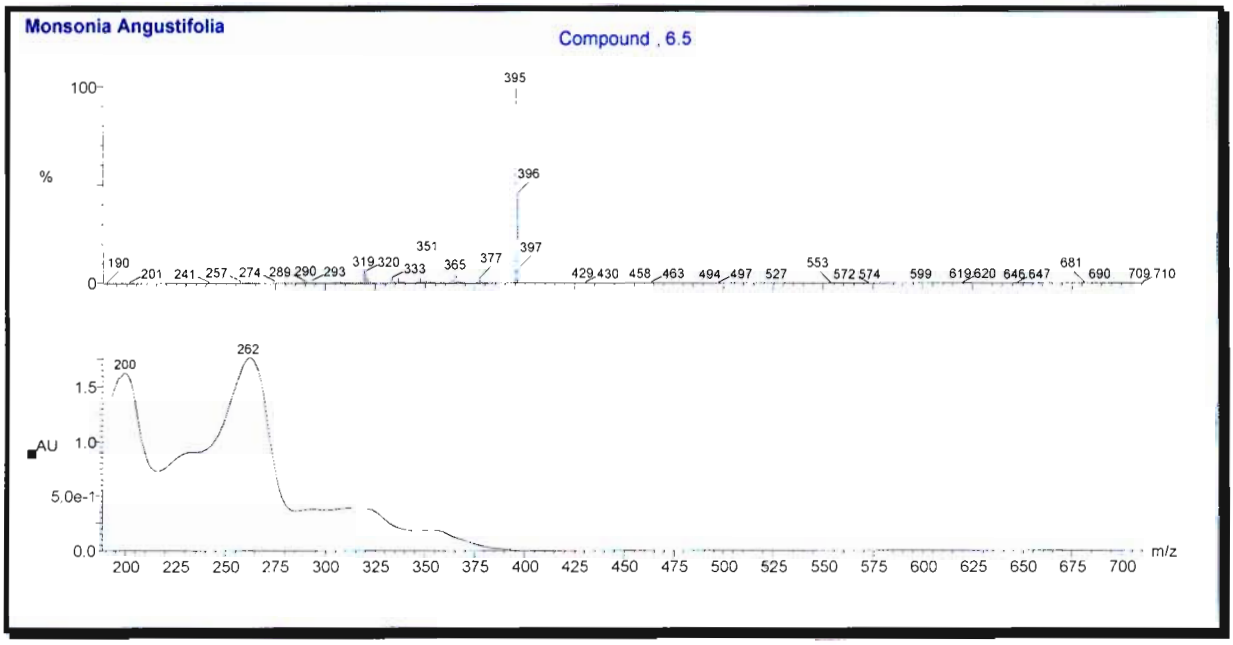
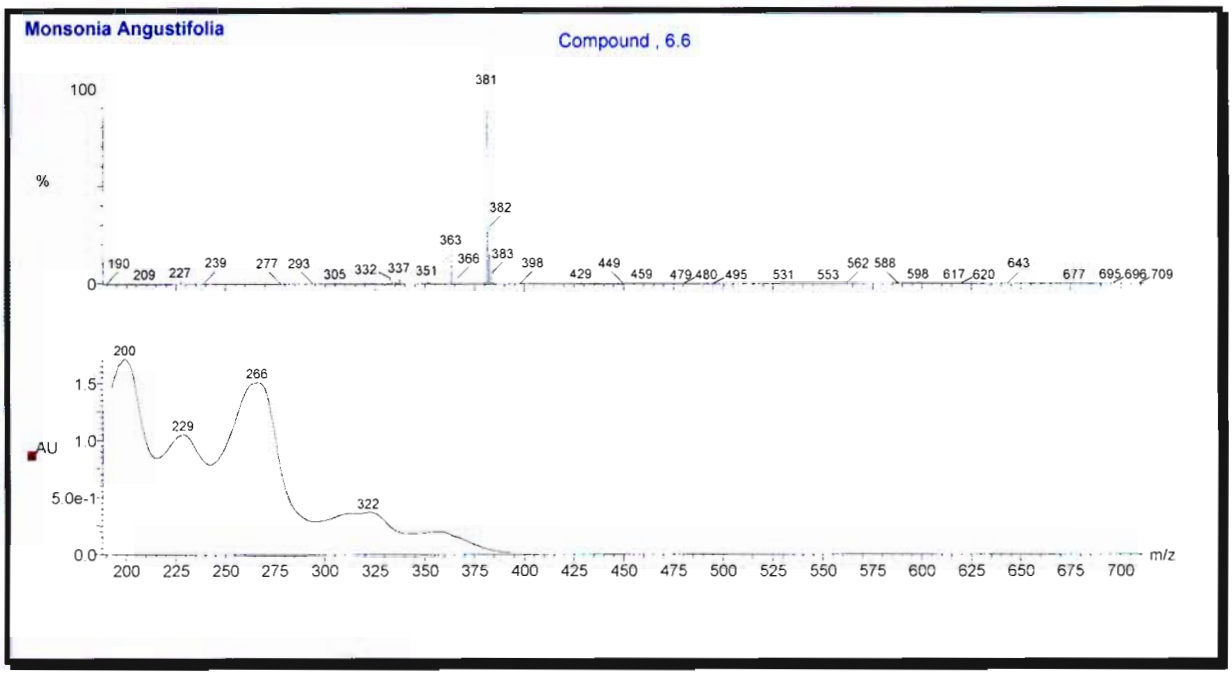


Figure 6.7: UV/MS chromophores of the isolated lignans **6.4** and **6.5** as obtained from the LCMS



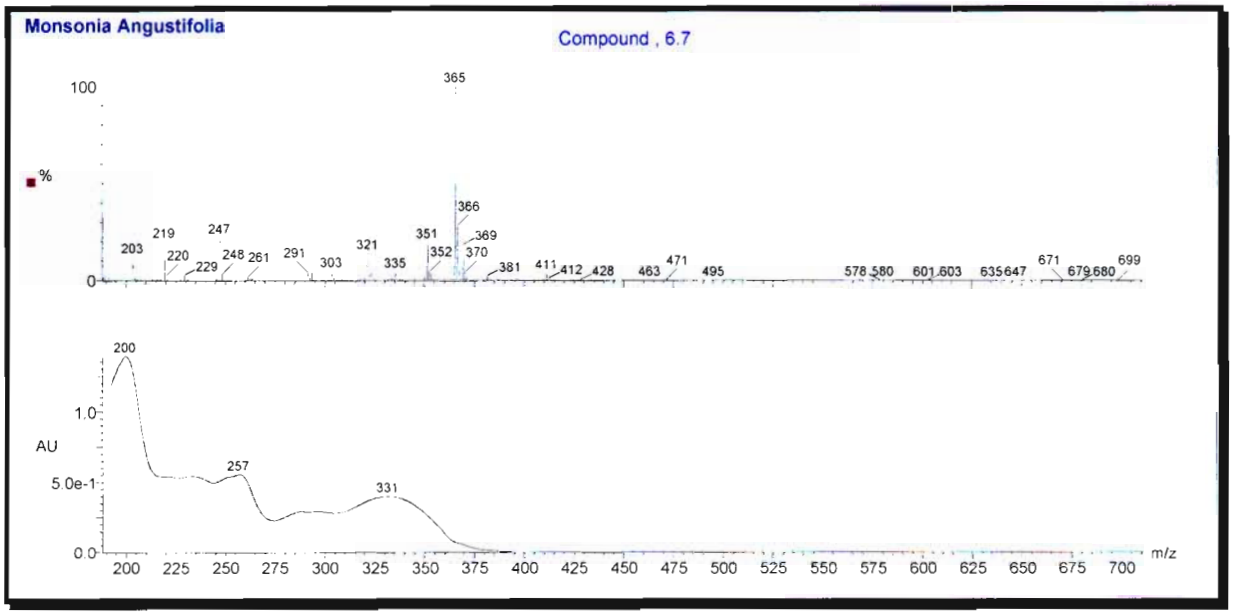


Figure 6.7 continues: UV/MS chromophores of the isolated lignans **6.6** and **6.7** as obtained from the LCMS

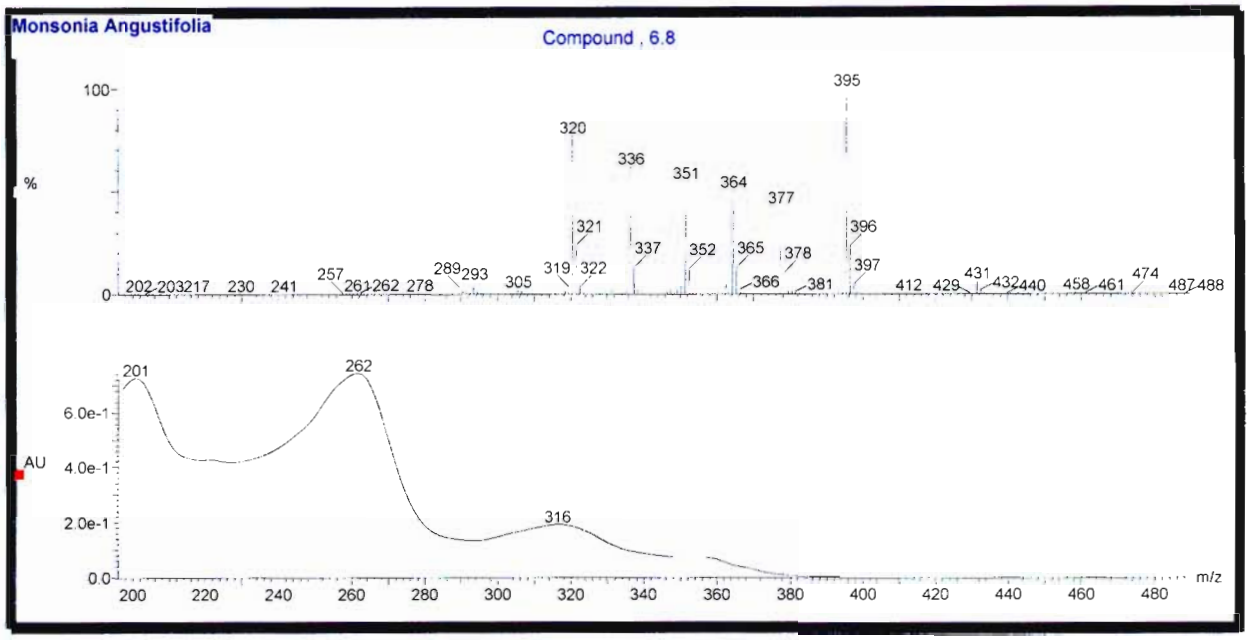


Figure 6.7 continues: UV/MS chromophores of the isolated lignans **6.8** as obtained from the LCMS

6.4.1. Development of the standard curves

In order to quantify *M. angustifolia* extracts, standard curves were developed for each of the five lignan compounds (Fig. 6.8). This was done by the chromatography of each of the compounds at five concentrations representing five serial dilutions ranging from 50 to 250 ppm. The chromatograms were then integrated to obtain the area percentage of each peak. To demonstrate this result, the methanol-dichloromethane extract was integrated and the chromatogram is shown in Fig. 6.9. One of the chromatograms is showing the area percentage table based on all the detected peaks. The result from this type of table for each of the compounds is collated and plotted into a graph to produce the standard curves.

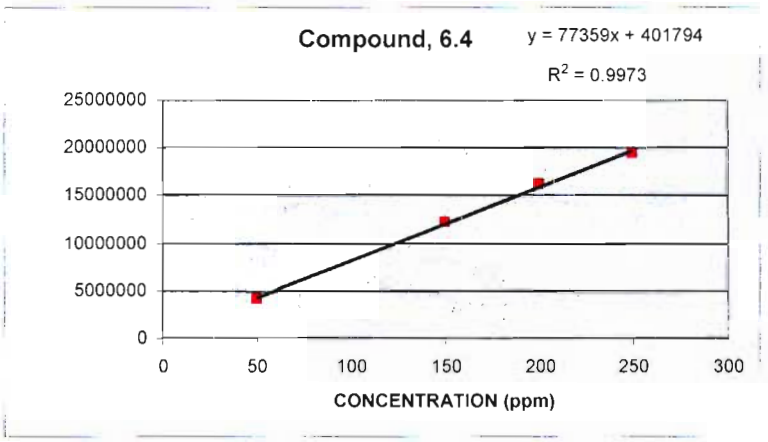
Standard curves were developed for each of the compounds and are displayed. The result of these curves is the opportunity to calculate the quantity of each compound in the original extract based on the peak area of the compound from the integration of the chromatogram.

Note that one of the values for a concentration at 100 ppm for compound **6.4** was not included in the plotting of the standard curve because its peak area lies out of the confidence limit. This set back must be due to an experimental error on sample preparations.

The compound was analyzed at three concentrations only; there was a problem experienced with the solubility of the extract which required the addition of a few drops of acetone when dissolving the compound.

Standard curve for 5-methoxyjusticidin A (**6.4**)

Compound, 6.4	
Concentration (ppm)	Peak Area
50	4089242
100	
150	12171114
200	16264987
250	19365486



Standard curve for justicidin A (**6.5**)

Compound, 6.5	
Concentration (ppm)	Peak Area
50	3523679
100	8476433
150	12408889
200	15772503
250	19655240

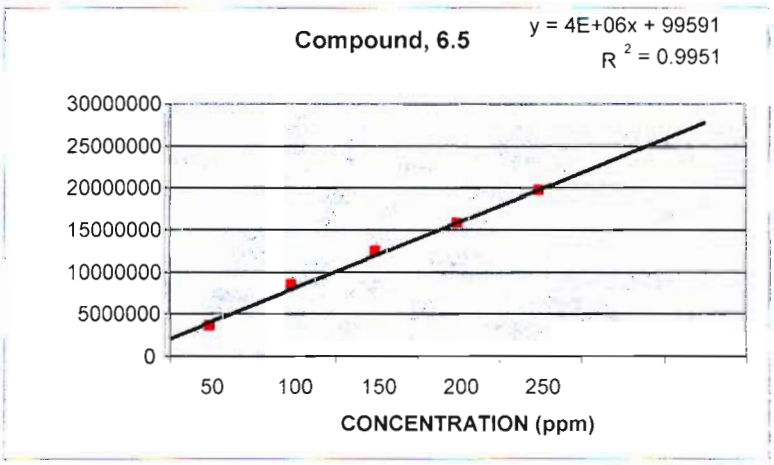
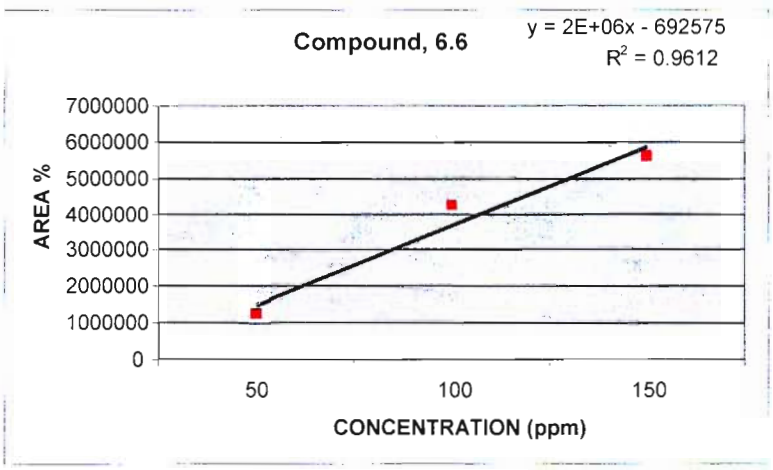


Figure 6.8: Standard curves of the compounds

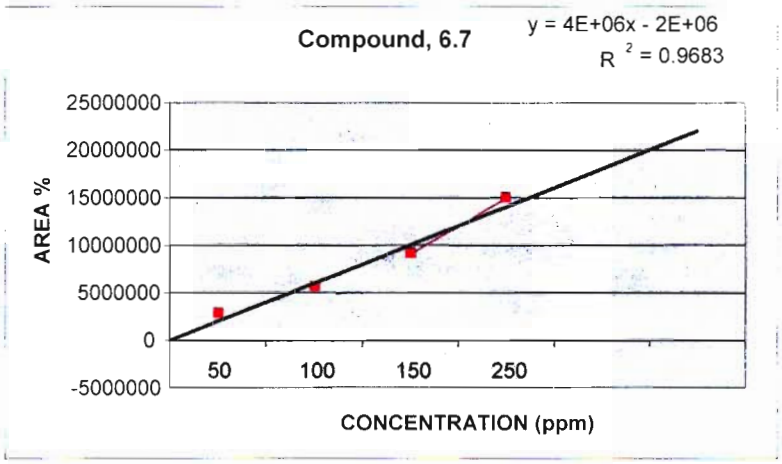
Standard curve for chinensinaphthol (**6.6**)

Compound 6.6	
Concentration (ppm)	Peak Area
50	1239714
100	4186642
150	5611611
200	
250	



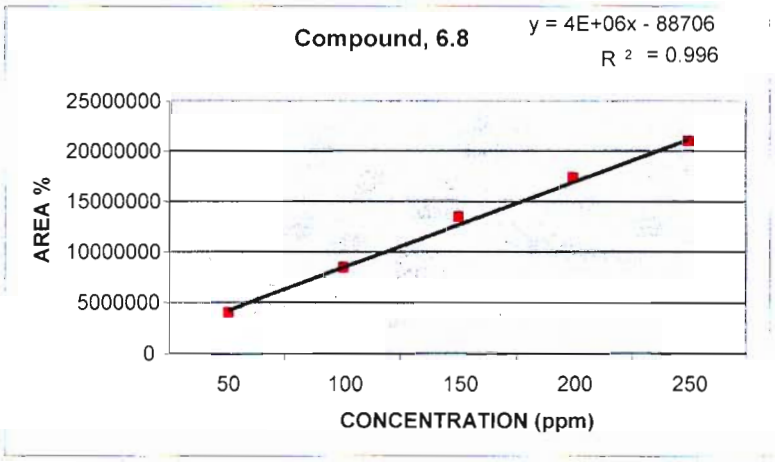
Standard curve for compound suchilactone (**6.7**)

Compound 6.7	
Concentration (ppm)	Peak Area
50	2700968
100	5500171
150	8972516
200	16219790
250	14916583



Standard curve of compound retrochinensinaphthol (**6.8**)

Compound 6.8	
Concentration (ppm)	Peak Area
50	3922012
100	8236955
150	13281764
200	17156990
250	20713618



Continue Figure 6.8: Standard curves of the compounds

Determination of the concentration of each compound in the extract

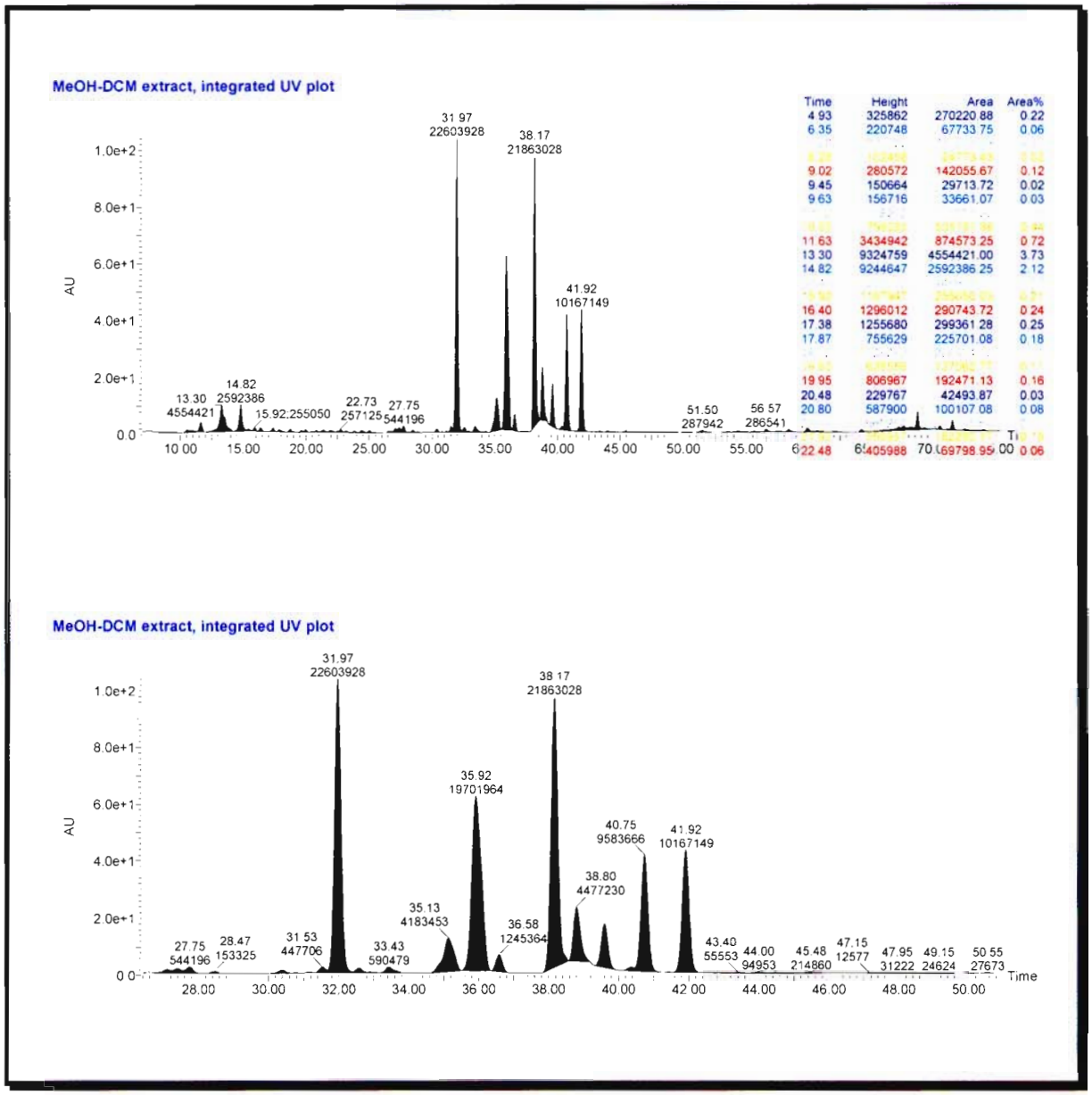


Figure 6.9: Integrated UV plot of the methanol-dichloromethane extract

6.5. BIOLOGICAL ACTIVITY

In order to validate the use of the plant for the treatment of erectile dysfunction and the enhancing of libido, the prepared organic and aqueous plant extracts were evaluated in the available and accessible biological assays. These extracts were prepared as illustrated in Fig 6.3.

6.5.1. *In vivo* mating frequency and pregnancy model (MFM)

In vivo assaying of the combined aqueous (sample A) and organic 1:1 methanol-dichloromethane (sample B) extracts (4:6 w/w mixture, referred to as CIR 40 in Tables 6.9 and 6.10) in a sexual activity, mating frequency/pregnancy model (MFM) provided the first scientific evidence for the efficacy of the plant. The extract mixture was administered orally to 3 male rats at a dose of 300 mg/kg for 8 consecutive days. Three dosed rats and three placebo-fed male rats were each paired with six female rats. Sexual activity was recorded by video surveillance during the nocturnal period *i.e.* from 17h00 to midnight. Differences are considered significant at $P<0.05$. The vehicle control substance (water) was dosed orally at 10 ml/kg per animal. The extract mixture exhibited a significant increase in mating frequency and the number of pregnancies. The results obtained from the bioassay are shown in Tables 6.9 and 6.10.

Table 6.9: Results obtained in the sexual activity, mating frequency assay

Treatment	Group Number	Mating frequency (individual groups)							Total	Mean±SEM
		Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8		
Vehicle (Distilled water)	1	13	0	0	0	43	11	1	**68**	82.0 ±10.7
	2	42	0	31	1	0	0	1	**75**	
	3	38	10	5	0	17	11	22	**103**	
Test substance (CIR 40)	1	39	75	50	48	87	2	0	**301**	243.0 ±55.5
	2	6	118	56	74	41	0	1	**296**	
	3	10	9	11	55	12	17	18	**132**	

The extract mixture demonstrated an increased mating frequency as shown by the high mean number of mounts 243 as compared to the vehicle control where only 82 mounts were counted. The difference in the number of mounts between the extract fed rats and those given a placebo is almost three fold. This result proves the capability of the extract to enhance the libido of the male rats that were fed with the extract mixture. The model is set

up such that after 8 days the female rats are inspected for the following two weeks for pregnancy and the result are collated in Table 6.10. A much higher number of pregnancies were observed in the groups where the males received the plant extract.

Table 6.10: Results obtained from the sexual activity, pregnancy assay

Treatment	Group Number	Individual Number of pregnancies	Total number of pregnancies	Mean±SEM
Vehicle (Distilled water)	1	3	8	2.7 ± 0.3
	2	2		
	3	3		
Test substance (CIR 40)	1	5	14	4.7 ± 0.3
	2	5		
	3	4		

6.5.2 *In vitro* phosphodiesterase 5 inhibition

In order to establish the possible mode of action of the plant for the activity observed in the rat model, the extracts were screened *in vitro* for inhibition of the enzyme phosphodiesterase 5 (PDE 5). Inhibition of PDE 5 limits the hydrolysis of cGMP to 5'GMP and thereby increases the intracellular concentrations of cGMP which then concomitantly facilitates the relaxation of the corpus cavernosum smooth muscle. A combination of the aqueous (sample A) and organic (sample B) extracts in a 1:1 (w/w) ratio was assayed for the *in vitro* inhibition of PDE 5. The mixture exhibited a significant inhibition (80%) at 100 ppm (Table 6.11).

When the organic (sample B) and the aqueous extracts (sample A) were assayed separately, the organic sample B exhibiting the highest inhibition PDE 5 (96%). Partitioning of this extract yielded three fractions of different polarities, the hexane (Sample D), dichloromethane (Sample C) and water (Sample E) fractions. All three samples showed a significant inhibition of the enzyme with the highest inhibition observed for the dichloromethane fraction (sample C).

Table 6.11: Summary of results demonstrating PDE 5 inhibition

Test sample		Concentration µg/ml	% Inhibition of PDE 5
Sample A	Aqueous	100	68
Sample B	1:1 MeOH/DCM	100	96
Sample A + B (1:1)	Aqueous + organic	100	80
Sample C	DCM	100	101
Sample D	Hexane	100	62
Sample E	Water	100	85

6.5.3. *In vitro* relaxation of the pre-contracted rabbit cavernosum smooth muscle

Since the inhibition of the PDE 5 concurrently induces the relaxation of the smooth muscles, samples A and B were also tested for their relaxation effect on the strips of the rabbit corpus cavernosum smooth muscle pre-contracted with 2 ml of Krebs-PSS (17.8 mg/ml). The contraction/relaxation is reported relative to sildenafil tested at 78 ng/ml.

Both extracts were observed to relax the rabbit cavernosum smooth muscle significantly and the results are shown in Table 6.12. The extracts tested at 2.6 mg/ml showed a 50% relaxation but at this point it was not clear whether the activity is due to cytotoxicity. Viagra (sildenafil) was reported to facilitate 100% relaxation of the same smooth muscle at a concentration of 1.8×10^{-5} mg/ml which is a thousand fold more potent than the test extract. This result suggests that the extracts act by inhibiting the PDE 5 enzyme thereby decreasing the intracellular hydrolysis of cGMP to 5'GMP and this in turn stimulates the relaxation of the smooth muscles thereby allowing the easy flow of blood into the relaxed penile.

Table 6.12: The rabbit corpus carvernosum smooth muscle relation and cytotoxicity results of the extracts.

Test sample	**Concentration of sample** mg/ml	**Relax (R) / Contraction (C)%** (standard deviation in brackets)	**Cytotoxicity, CHO assay IC_{50}** µg/ml
Sample A	2.6	50 (0) R	>100
Sample B	2.6	**46 (7.1) R**	18.6

The samples were tested with reference to Sildenafil or Viagra that showed 100% smooth muscle relaxation at $1.8X10^{-5}$ mg ml

6.5.4. Activity of isolated compounds

The five lignans (**6.4** to **6.8**) isolated from the organic extract were tested for relaxation of strips from the basal area of the rabbit corpus cavernosal precontracted by phenylephrine (3 µM). A test substance (30 µM) that induces relaxation by 50 percent or more (≥50%) within 5 minutes, relative to the control 0.3 µM sodium nitroprusside response, indicates significant relaxation. These compounds were also screened for PDE 5 inhibition at a concentration of 10µM (Table 6.13).

Table 6.13: Compounds isolated and assaying results

Compound No.	% PDE5 [b,c] inhibition at 10 μM	Smooth muscle relaxation at 30 μM	[a]Cytotoxicity (CHO) IC_{50} (μg/ml)	Molecular formula
6.4	11	62	9.0	$C_{23}H_{20}O_8$
6.5	22	0	1.0	$C_{22}H_{18}O_7$
6.6	29	75	28.5	$C_{21}H_{16}O_7$
6.7	35	71	>100	$C_{21}H_{20}O_6$
6.8	53	57	>100	$C_{22}H_{18}O_7$

[b] *Viagra showed 100% relaxation at 1.8 x 10^{-2} μg/ml or an IC_{50} value of 4.1 x 10^{-3} μM for PDE 5 inhibition*
[a] *IC_{50} values ≤1.0 are considered to be cytotoxic in CHO tests*
[c] *PDE 5 inhibition and smooth muscle relaxation were considered significant at ≥50% values*

Compared to the synthetic sildenafil that showed 100% relaxation at 1.8 x 10^{-2} μg/ml and an IC_{50} value of 4.1 x 10^{-3} μM for PDE 5 inhibition, these compounds are at least a thousand fold less active. The most significant inhibition of PDE5 was displayed by retrochinensinaphthol methyl ether (**6.8**). Four of the compounds, **6.4**, **6.6**, **6.7** and **6.8,** exhibited a significant relaxation of the smooth muscle at concentrations of 30 μM for each compound.

Rabbit corpus cavernosum smooth muscle relaxation was observed for compounds **6.4**, **6.6**, **6.7** and **6.8** with the lowest percentage of the smooth muscle relaxation of 57% and the highest being 75%. Only one compound, justicidin A (**6.5**), exhibited minimal PDE 5 inhibition and nil efficacies for smooth muscle relaxation at the test concentrations. Three compounds **6.4**, **6.6** and **6.7** showed a significant smooth muscle relaxation alongside a weak inhibition of the PDE 5. Retrochinesinaphthol methyl ether (**6.8**) is the only compound with a significant result of both the inhibition of PDE 5 and relaxation of the smooth muscle that is more than fifty percent. There are no obvious structural features in the lignans **6.4** – **6.8** that can be associated with the activities of the compounds.

These findings suggest that the extract act by synergistic effect of its component compounds through the mechanism of the blocking of the PDE 5 thereby causing the relaxation of the corpus cavernosum smooth muscle.

6.5.5. Cytotoxicity of compounds and extracts on CHO cells

The compounds and the extracts were tested for toxicity against the mammalian Chinese Hamster Ovarian cells (CHO) and the results are collated in Tables 6.12 and 6.13. A test substance was considered cytotoxic if it exhibits an IC_{50} values ≤1.0 μg/ml. Emetine with an IC_{50} value of 0.05 μg/ml was used as a standard.

The aqueous extract was tested to evaluate whether this form of formulation used traditionally by the traditional healers is not toxic. The organic extract was prepared as an innovative step to extract more of the active compound(s). The organic extract did not exceed the limit set for cytotoxicity, but with an IC_{50} of 18.6 μg/ml, may contain some cytotoxic compounds. No toxicity was observed for the aqueous extract.

Two compounds suchilactone (**6.7**) and retrochinesinaphthol methyl ether (**6.8**) were non-cytotoxic at a concentration of 100 μg/ml. The most cytotoxic compound, justicidin A (**6.5**), did not display any relaxation of the rabbit smooth muscle or a significant inhibition of the PDE 5 enzyme at the test concentrations (Table 6.13). Looking at the structures of the compounds, the cytotoxicity of **6.5** might be due to the substitution of one of the methoxy group by hydrogen atom. The toxicity of this compound also proves that the activity observed for the compounds in the inhibition of the PDE 5 and relaxation of the smooth muscle might not be due to toxicity since justicidin A (**6.5**) exhibited no activity in these two assays.

6.5.6. Anticancer activity of extracts and compounds

The plant aqueous sample A and organic sample B extracts were also evaluated for anticancer activity in the available CSIR in-house cancer screen against the melanoma, renal and breast cancer cell lines (Table 6.14). Only the sample B exhibited a moderate activity with selectivity for the melanoma cell line. This extract was sent to the NCI for further evaluation in their panel of 60 cell lines.

Table 6.14: Results obtained in anticancer screening of the extracts and compounds

Compound /Sample	**Result evaluation**	**Effective concentration, TGI** ($x\ 10^{+01}$ µg/ml or ppm)
Sample A	Weak	5.00
Sample B	Moderate	1.25
6.4	Moderate	4.20
6.5	Moderate	5.40
6.6	Inactive	8.00
6.7	Inactive	6.78
6.8	Inactive	6.71

Activity criteria: *Total growth inhibition (TGI) > 15 ppm < 50 ppm (weakly active): TGI >6.25 to 15 ppm for two to three cell lines (moderate activity): TGI < 6.25 for two to three cell lines (potent activity)*

The two compounds **6.4** and **6.5** showed moderate to potent selective activity for the melanoma cell line. Compound **6.4** passed the crucial evaluation against the NCI database of compounds that have been tested for antitumour activity. This compound was tested in the *in vitro* 60 cell lines at a minimum of five concentrations at 10 fold dilutions (Fig. 6.9, 6.10). A 48 hour continues drug exposure protocol was used and a sulforhodamine B (SRB) protein assay was used to estimate the cell viability or growth. The NCI finding was that the compound did not exhibit the anticancer activity at the minimum potency concentration range they use for their screening protocol. This minimum concentration is based on some of the drugs already available and patented for use in chemotherapy. Thus no further work is to be done on this compound unless structural modifications to enhance the cytotoxic activity are considered.

5-Methoxyjusticidin (**6.4**) exhibited an average log TGI of - 4.28 (i.e. TGI = 5.248×10^{-5} M). The NCI clinically tested the compound etoposide[20] and they found an average TGI of 3.45×10^{-5} M.

In regard to cytotoxicity, no further work will be done on this compound. Structural modifications to enhance the cytotoxic activity may be considered.

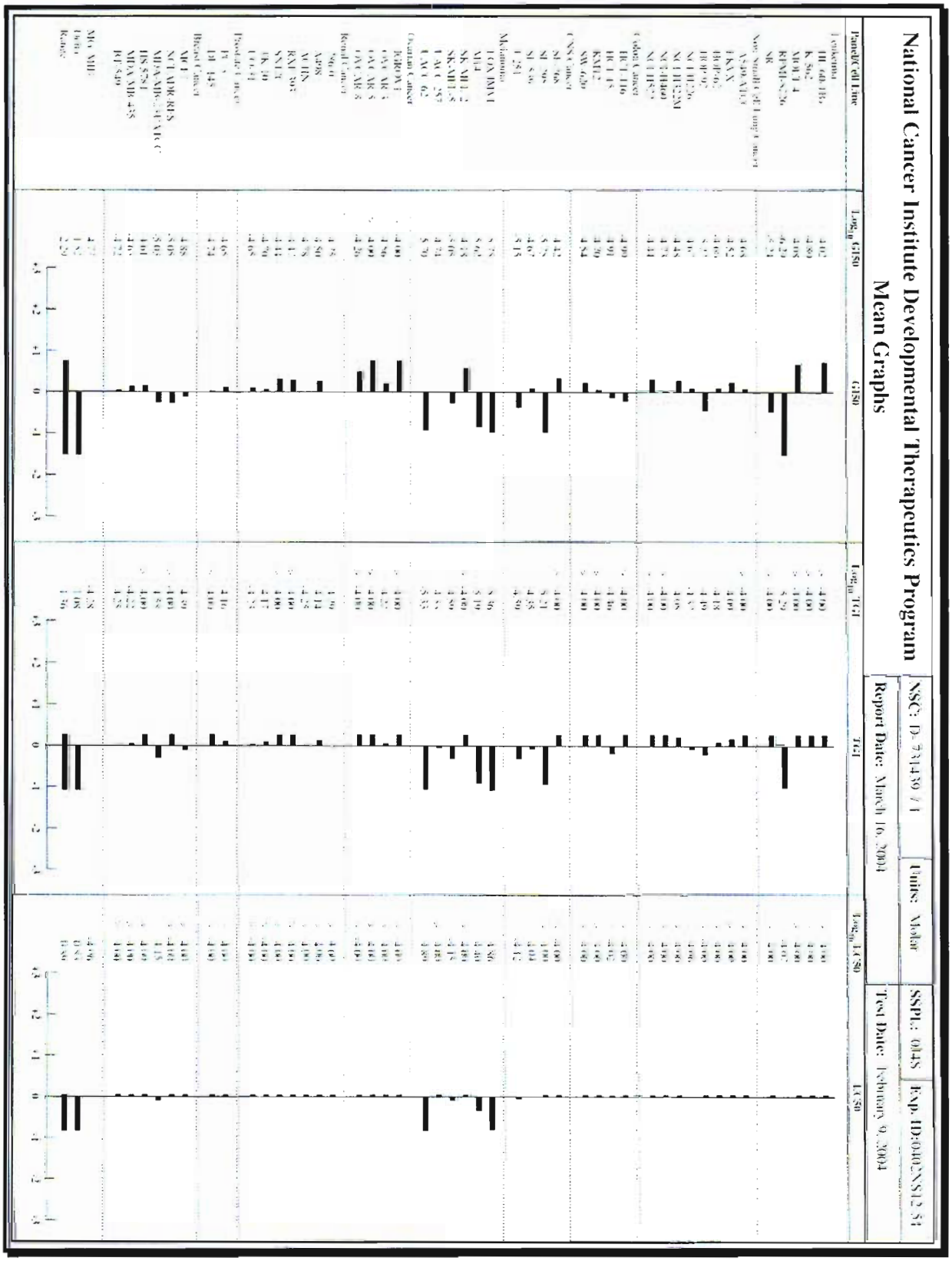


Figure 6.10: Mean graphs representation of the NCI antitumor screening results for compound **6.4**

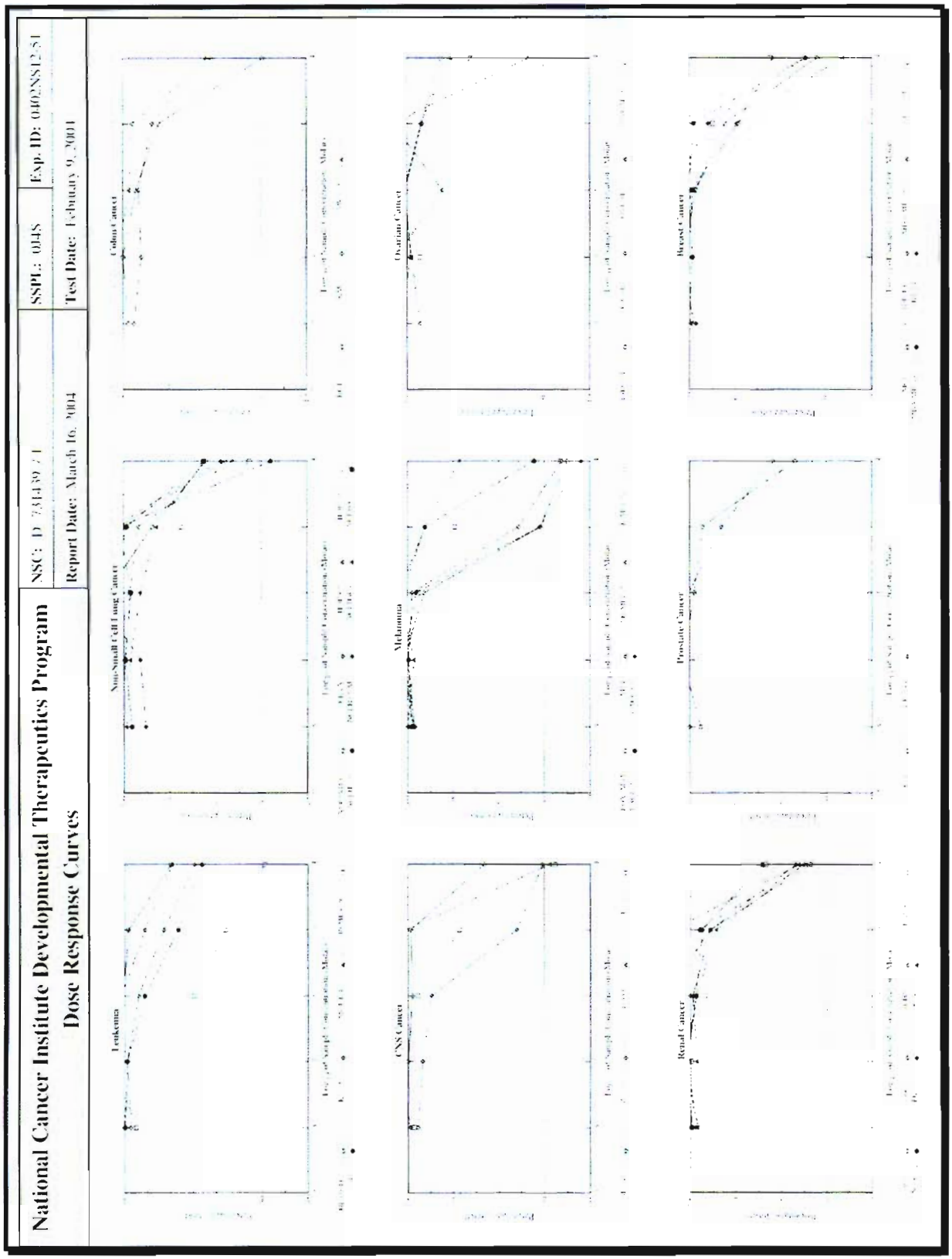


Figure 6.11: Dose responds curves of the anticancer screening results of compound **6.4** from NCI 60 cell line screen.

6.5.7. Antibacterial activity

Sample B was the highest contributor to the PDE 5 activity, thus this extract was chosen for the evaluation of the antibacterial properties of the plant. The crude organic extract was resuspended in a small amount of DMSO and made up to a concentration of 20 mg/ml concentration with water.

Bioautography was performed as a supplementary qualitative analysis of the presence of antibacterial compounds. In this technique, the samples were loaded and separated on a thin layer chromatography (TLC) plate, and after removal of eluant, sprayed with a suspension of *Staphylococcus aureus*. After overnight incubation, the plate was sprayed with a tetrazolium salt to identify the presence of antibacterial compounds in the samples. These active compounds appeared as white spots (no bacterial growth) on a pink background of dividing bacteria. The organic extract, sample B, displayed noticeable antibacterial activity in this assay. Bioautography was done on TLC plates using polar (ethyl acetate: methanol: water, 45:5:4.4 v/v/v) or non-polar (hexane: ethyl acetate, 2:1 v/v) solvent combination and thereafter sprayed with a suspension of *Staphylococcus aureus*.

The non-polar solvent system did not move the active compounds from the origin. There appeared to be only one major antibacterial compound present in the extract when using the polar solvent system or at least the TLC separation method did not separate the components.

Table 6.15: Minimal Inhibitory Concentrations (MIC) of samples against test bacteria

Sample	**MIC** ($mg.ml^{-1}$)			
	E. coli	***E. faecalis***	***P. aeruginosa***	***S. aureus***
Sample B	>5	1.25	>5	0.032

A microplate serial dilution method by Eloff[21] was used to quantify the antibacterial activity of extracts. The extract was screened against the bacteria, *Escherichia coli, Enterococcus faecalis, Pseudomonas aeruginosa* and *Staphylococcus aureus,* and displayed a noticeable antibacterial activity in this assay (Table 6.15). The extract had a

high activity against *S. aureus* and this result was corroborated by the bioautography data using only this pathogen. Because only *S. aureus* was sensitive to the extract tested, bioautography was not carried out with other pathogens.

6.6. CONCLUSION

The plant *Monsonia angustifolia* is traditionally used for the treatment of erectile dysfunction problems and also to enhance male and female libido. The extracts of *M. angustifolia* were scientifically validated for the reported claim in *in vivo* and *in vitro* models. Activity was shown for the methanol-dichloromethane extract (1:1, v/v) as compared to the aqueous extract which is the preferred preparation used by the traditional healers for the treatment of erectile dysfunction problems. The extracts of *M. angustifolia* also showed anticancer and antibacterial properties.

For the treatment specific to the enhancement of libido, the extract mixture (organic and aqueous) was tested in the *in vivo* sexual activity, mating frequency/pregnancy model (MFM) which is a measure of male libido. The extract mixture exhibited a significant increase in mating frequency (243 mounts) as compared to the vehicle control (82 mounts). Together with the number of pregnant rats (14) as compared to the vehicle control (8), the extract has clearly demonstrated increased male libido. This result was considered significant in the treatment aimed towards enhancing the sexual drive in rats and henceforth it is envisaged that it can be useful to humans suffering from the same condition.

The extract were tested *in vitro* to establish the possible mode of action and the methanol-dichloromethane extract was found to inhibit the phosphodiesterase 5 (PDE 5) enzyme (68-96%) and also effectively relaxed the pre-contracted smooth muscle (46-50%). It is believed that one of the pathways essential to the treatment of erectile dysfunction is via the inhibition of the PDE 5 enzyme which then brings about the relaxation of the smooth muscle. It was also noted that the commercial synthetic drug Viagra operates via the same mode of action.

Phytochemical investigation of the extracts in order to identify the active components was done specifically for the erectile dysfunction therapeutic area. The resulting five lignans

showed varying degrees of the inhibition of the PDE 5 enzyme ranging between 11 and 53%. Four compounds, 5-methoxyjusticidin A (**6.4**, 62%), chinesinaphthol (**6.6**, 75%), suchilactone (**6.7**, 71%) and retrochinesinaphthol methyl ether (**6.8**, 57%) relaxed the rabbit corpus cavernosum smooth muscle significantly. However, the effect on PDE 5 and rabbit corpus cavernosum smooth muscle was much lower than that of sildenafil (Viagra). Furthermore, the water extract also show activity. As yet, we have not characterised the water extract fully.

The compounds were also tested for their cytotoxic effect against a panel of three cell line at the CSIR and only compounds, 5-methoxyjusticidin A (**6.4**) and justicidin A (**6.5**), exhibited significant anticancer activity and specific for the melanoma cell line. 5-Methoxyjusticidin A was selected by the National Cancer Institute, USA, for testing in their 60 cell line screen, but research on this compound was not continued because of lack of sufficient potency.

The lignans of the same structure framework as these isolated are amongst the compounds researchers focus on. Podophyllotoxin and derivatives generally show cytotoxic effects but are found in most fruits and foods as harmless derivatives in low concentrations. They posses a wide range of biological activities such as anti-HIV, antimalaria, anti-inflammatory and anticancer activity. Etoposide is a lignan glycoside structurally related to the isolated compounds and is one of the clinically tested agents for the treatment of cancer of the skin. 5-Methoxyjusticidin A (**6.4**), justicidin A (**6.5**), chinesinaphthol (**6.6**), suchilactone (**6.7**) and retrochinesinaphthol methyl ether (**6.8**) also showed an interesting pattern of cytotoxic activity and future work may focus on the synthetical modification of their functional groups in order to provide derivatives with improved anticancer activity but with lower toxicity.

6.7. EXPERIMENTAL

HPLC-MS

The flow from the pump was split after the column with 90% going to the variable UV, Waters 996 photodiode array detector (PDA) and 10% to the mass spectrometer. The samples concentrations of 5000 ppm for the extracts and 2500 ppm for the fractions were prepared by dissolving 5-10 mg of the sample in methanol-acetonitrile (1:1) and injecting 25 µl volumes on the column.

Plant material

Plant material was collected in the Free State by a traditional healer. The plant was visually classified as a shrub with purple flowers and a specimen was deposited at SANBI in Pretoria for identifications purposes. The plant was identified as *Monsonia angustifolia* E.Mey. Ex A.Rich (Geraniaceae) (Genspec no. 39250002).

Extract preparations and isolation of compounds

The extracts were prepared from dried plant material (408 g) of *Monsonia angustifolia.* The plant parts supplied comprised of the roots, stems, leaves and purple flowers. The dry plants were ground in a hammer mill to 1-2 mm pieces (352 g). The plant material was extracted and partitioned as illustrated in Fig. 6.2.

The aqueous extract was prepared by a method similar to making a tea infusion. One liter of de-ionized water was added to 86 g of the ground material and boiled for an hour. The water solution was left to cool to room temperature, filtered and freeze-dried for 24 hours to yield 22.16 g of a brownish fluffy powder (**sample A**).

The organic extract was prepared from 266 g of ground plant material that was extracted with 6 L of methanol-dichloromethane (1:1 v/v) for an hour with occasional stirring. The solution was filtered and the residual plant material was extracted again with 3 L of methanol-dichloromethane (1:1 v/v). The filtered solvents were combined and evaporated under reduced pressure to give 27.32 g of a dark-green sticky extract (**sample B**).

The organic extract (**sample B**, 1.5g) was partitioned between 1L of methanol-water (90% v/v) and 500 ml of hexane. The hexane layer was separated and evaporated to yield 395.8 mg of a sticky greenish fraction (**sample D**). The methanol-water layer was evaporated under reduced pressure. Water was added to this layer (total volume of 500 ml) and the aqueous solution extracted with dichloromethane (3 x 500 ml). The combined dichloromethane layers were evaporated under reduced pressure to yield 543 mg of a brownish extract (**sample C**). The residual water was freeze-dried to give a brownish powder (**sample E**, 474 mg).

The aqueous extract, sample A (200 mg) was dissolved in 2 ml of water and fractionated on a C18 reverse phase silica gel flash cartridge using 100 ml of water, followed by 100 ml of methanol-water (1:1 v/v) and 100 ml of methanol. A disaccharide was isolated from the methanol fraction and it was identified as sucrose (**6.10**, 21 mg). The NMR data of sucrose is collated in Table 6.7.

Fractionation of the organic extract (sample B, 6.0 g) was achieved on flash chromatography (silica gel 60, Merck, 230-400mesh) with ethyl acetate-hexane mixtures (1.5:8.5, v/v) as the eluent. β-Sitosterol (**6.9**, 164 mg) and five lignans, **6.4** (35 mg), **6.5** (19 mg), **6.6** (27 mg), **6.7** (36 mg), **6.8** (16 mg) were isolated.

6.8. PHYSICAL DATA

6.8.1. COMPOUND 6.4

Systematic name: 9-(1',3'-Benzodioxol-5'-yl)-4,5,6,7-tetramethoxynaphtho[2,3-*c*]furan-1(3*H*)-one

Alternative name: 5-Methoxyjusticidin A

Yield: 35 mg (0.58%) relative to the extract

Physical description: White amorphous solid

UV data: λ_{max} 365, 263 and 237 nm.

Mass spectrum: *m/z* 424, $C_{23}H_{20}O_8$

^{1}H NMR data: See Table 6.1 ($CDCl_3$)

^{13}C NMR data: See Table 6.1 ($CDCl_3$

6.8.2. COMPOUND 6.5

Systematic name: 9-(1',3'-Benzodioxol-5'-yl)-4,6,7-trimethoxynaphtho[2,3-*c*]furan-1(3*H*)-one

Alternative name: Justicidin A

Yield: 19 mg (0.31%)

Physical description: White amorphous flakes

UV data: λ_{max} 261 nm.

Mass spectrum: *m/z* 395, $C_{22}H_{18}O_7$

^{1}H NMR data: See Table 6.2 ($CDCl_3$)

^{13}C NMR data: See Table 6.2 ($CDCl_3$)

6.8.3. COMPOUND 6.6

Systematic name: 9-(3',4'-Dimethoxyphenyl)-4-hydroxy-6,7-methylenedioxynaphtho[2,3-*c*]furan-1(3*H*)-one

Alternative name: Chinensinaphthol

Yield: 27 mg (0.45%)

Physical description: White amorphous solid

UV data: λ_{max} 322, 266 and 228 nm

Mass spectrum: (*m/z*) of 381, $C_{21}H_{16}O_7$

^{1}H NMR data: See Table 6.3 ($CDCl_3$/CD_3OD)

^{13}C NMR data: See Table 6.3 ($CDCl_3$/CD_3OD)

6.8.4. COMPOUND 6.7

Systematic name:	3-(1',3'-Benzodioxol-5'-ylmethylene)-4-(3",4"-dimethoxybenzyl)dihydrofuran-2(5*H*)-one
Alternative name:	Suchilactone
Yield:	36 mg (0.60%)
Physical description:	yellowish and crystalline
UV data:	λ_{max} 331, 257 nm
Mass spectrum:	*m/z* 368, $C_{21}H_{20}O_6$
^{1}H NMR data:	See Table 6.4 ($CDCl_3$)
^{13}C NMR data:	See Table 6.4 ($CDCl_3$)

6.8.5. COMPOUND 6.8

Systematic name:	4-(3',4'-Dimethoxyphenyl)-9-methoxy-6,7-methylenedioxynaphtho[2,3-*c*]furan-1(3*H*)-one
Alternative name:	Retrochinensinaphthol methyl ether
Yield:	16 mg (0.26%)
Physical description:	White amorphous solid
UV data:	λ_{max} 316, 262 nm
Mass spectrum:	*m/z* 395, $C_{22}H_{18}O_7$
^{1}H NMR data:	See Table 6.5 ($CDCl_3$)
^{13}C NMR data:	See Table 6.5 ($CDCl_3$)

6.8.6. COMPOUND 6.9

Systematic name:	17-(4-Ethyl-1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-1*H*-cyclopenta[*a*]phenanthren-3-ol
Alternative name:	β-Sitosterol or (3β)-Stigmast-5-en-3-ol
Yield:	164 mg (2.70%)
Physical description:	White amorphous solid
^{1}H NMR data:	See Table 6.9 ($CDCl_3$)
^{13}C NMR data:	See Table 6.9 ($CDCl_3$)
Other data:	See section 5.3.2.

6.8.7. COMPOUND 6.10

Systematic name:	2-(3,4-Dihydroxy-2,5-bis-hydroxymethyl-tetrahydro-furan-2-yloxy)-6-hydroxymethyl-tetrahydro-pyran-3,4,5-triol **or** 13-O-Fructofuranosyl α-D-glucopyranoside
Alternative name:	Sucrose
Yield:	21 mg (10.5%)
Physical description:	White crystals
^{1}H NMR data:	See Table 6.7 (DMSO)
^{13}C NMR data:	See Table 6.7 (DMSO)
Other data:	See section 5.3.1.

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