A PHYTOCHEMICAL STUDY OF *SCHEFFLERA UMBELLIFERA* AND *ELEPHANTORRHIZA ELEPHANTINA*

by

XOLANI SABELO MTHEMBU

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

Pietermaritzburg
2007

PREFACE

The experimental work described in this dissertation was carried out in the School of Chemistry, University of KwaZulu-Natal, Pietermaritzburg, from January 2005 to January 2007, under the supervision of Professor Fanie van Heerden.

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

Signed.....

Xolani Sabelo Mthembu

(Candidate)

I hereby certify that this statement is correct.

Signed.....

Professor F. van Heerden

(Supervisor).

Signed.....

Doctor G. Fouche

(Co-supervisor)

Acknowledgements

First and foremost, I wish to thank my supervisor, Prof Fanie van Heerden and co-supervisor, Dr Gerda Fouche' for their enormous amount of support and teaching throughout my thesis.

I am also greatly indebted to Dr Louis Ackerman who, when I had only the vaguest idea of what research was, was curious to teach me more and offer encouragement.

Many thanks to the Biosciences, Bioprospecting platform staff for being there for me during some testing times and a special thanks to my research group leader, Dr Vinesh Maharaj, for his support and the opportunity granted during the writing of this thesis. Flip Kotze, the computer skills you taught me will always be appreciated. Dr Paul Steenkamp, the good analytical data you provided-you are in a class of your own.

This thesis is dedicated to my grateful parents, my late mother, Monica Mjadu Mthembu, I forever miss you. To you my grandmothers, your prayers and encouragements keep me alive and healthy.

Special thanks to my laboratory mates and friends, Jerry Senabe, Eric Khorombi and Rudzani Nthambeleni, "together we stand and divided we fall". If your name is not listed, rest assured that much gratitude is not less than for those listed above.

I would also like to thank the National Research Foundation and the University of KwaZulu-Natal, Pietermaritzburg for their financial support.

ABSTRACT

In this study, two plant species, *Schefflera umbellifera* (Araliaceae) and *Elephantorrhiza elephantina* (Fabaceae), were investigated for their biological activity. The crude organic extract of the leaves of *S. umbellifera* exhibits promising antimalarial activity. Bioassay-guided fractionation of the dichloromethane extract of *S. umbellifera* yielded an active compound, betulin, which exhibited good antiplasmodial activity (IC_{50} 3.2 µg/mL), when tested against a chloroquine-susceptible malarial strain (D10). The reference compound (chloroquine) gave an IC_{50} of 27.2 ng/mL. Two other known compounds were also isolated from the organic extract, namely 7-hydroxy-6-methoxycoumarin and *ent*-kaur-16-en-19-oic. These two compounds did not exhibit any significant antiplasmodial activity. An HPLC comparative study was conducted on four extracts of *S. umbellifera* collected from different geographical areas and similar chemical profiles were obtained, with common major compounds betulin, oleonolic acid and *ent*-kaur-16-en-19-oic acid as well as a minor compound, 7-hydroxy-6-methoxycoumarin.

A phytochemical study of *Elephantorrhiza elephantina* afforded β-sitosterol and seven phenolic compounds, namely gallic acid, methyl gallate, quercetin 3-*O*-β-D glucoside, 3-*O*-galloyl-3,3',5',5,7-pentahydroxyflavone, taxifolin 3´-*O*-β-D-glucoside, catechin and epicatechin. A mixture of the two flavonoid glycosides, one flavone as well as catechin and epicatechin inhibited the steroid 5α-reductase enzyme (80% inhibition) when tested at 100 µg/mL. These compounds did not exhibit activity when tested individually at 1 µM. Of these compounds isolated, 3-*O*-galloyl-3,3',5',5,7-pentahydroxyflavone showed good antimalarial activity (1.8 µg/mL) when tested against a chloroquine-susceptible strain (D10) of *Plasmodium falciparum* with chloroquine as reference compound (IC_{50} of 34.4 ng/mL). Of all the compounds isolated, only gallic acid showed good anticancer activity with a TGI of 1µg/mL. *E. elephantina* aqueous extract of the rhizomes and 3-*O*-galloyl-3,3',5,5',7-pentahydroxyflavone both showed good antioxidant activity (83% and 64% radical scavenging capacity, respectively) when tested at 6.25 ppm concentration compared to the antioxidant activity of green tea (40%) tested at the same concentration.

ABBREVIATIONS

br	Broad resonance
brs	Broad singlet
calc.	Calculated
δ	Chemical shift in ppm
cm	Centimeter
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance Spectroscopy
COSY	Correlated Spectroscopy
CSIR	Council for Scientific and Industrial Research
^{o}C	Degrees Celcius
d	Doublet
dd	Doublet of doublets
DEPT	Distortionless Enhancement by Polarisation Transfer
dt	Doublet of triplets
EIMS	Electron Impact Mass Spectrometry
GCMS	Gas Chromatography Mass Spectrometry
HMBC	Heteronuclear Multiple Bond Correlation
^{1}H NMR	Proton (^{1}H) Nuclear Magnetic Resonance spectroscopy
HRMS	High Resolution Mass Spectrometry
HPLC	High Performance Liquid Chromatography
HSQC	Heteronuclear Single Quantum Coherence
Hz	Hertz
J	Spin-spin coupling constant in

	Hertz
lit	Literature
m	Multiplet
µg/mL	Microgram per milliliter
min	Minute
m.p.	Melting point
MRC	Medical Research Council
NCI	National Cancer Institute
nm	Nanometer
NOESY	Nuclear Overhauser Effect Spectroscopy
ppm	Parts per million
q	Quartet
ref	Reference
rpm	Revolutions per minute
s	Singlet
SANBI	South African National Biodiversity Institute
[α]	Specific optical rotation
SRB	Sulforhodamine B
t	Triplet
TLC	Thin Layer Chromatography
UCT	University of Cape Town
UP	University of Pretoria
UV	Ultra Violet Spectroscopy
WHO	World Health Organization

Table of Contents

Chapter 1

Introduction

Since ancient times, throughout the world, humans have used plants for food as well as medicine[1]. Plants and herbals are part of many traditional healing practices including Chinese medicine, Ayurveda (a holistic system originated in the Vedic civilization of India), Curanderismo (a Mexican American healing tradition) and Western herbalism. The World Health Organization estimates that in Africa up to 80% of the population still depends on herbal medicines[2]. The renewed interest in medicinal plants in recent years has led to ongoing research in the identification of lead compounds for drug development, especially drugs that either control or eradicate tropical diseases such as malaria, tuberculosis, etc[3].

Africa as a continent is facing major health challenges posed by tropical diseases such as malaria, tuberculosis, onchocerciasis, schistosomiasis, HIV/AIDS, etc. The severity of these diseases is due to a number of factors including climate variations, global warming, widespread poverty, poor sanitation and drug resistance[4]. Consequently, there is an urgent and ever-present need to control these diseases. To help combat these epidemics in Africa, Western medicines have been utilized but have limitations such as a lack of efficacy, safety and affordability. As a result, there has been a major paradigm shift towards the use of traditional herbal medicine to treat these diseases.

Malaria is a major impediment to health and development in sub-Saharan Africa. More than one million people die from the disease every year and about half a

---

1. B. Patwardhan, D. Warude, P. Pushpagandan, N. Bhatt, *Evid. Based Complemen. Alternat. Med.* 2005, **2**, 465-473.
2. World Health Organization Website: http://www.who.int/mediacentre/factsheets/fs134/en Accessed on 16 December 2006.
3. S. Taylor, V. Berridge, *Trans. Roy. Soc. Trop. Med. Hyg.*, 2006, **100**, 707-714.
4. B.M. Greenwood, K. Bojang, C.J.M. Whitty, G.A.T. Targett, *Lancet*, 2005, **365**, 1487-1498.

billion are afflicted in some or other way[3]. There have been numerous approaches to help eradicate this epidemic including the use of plant-derived antimalarial drugs such as quinine and artemisinin. The presence of multidrug-resistant strains of the malaria parasite has exacerbated the danger of malaria and has a direct negative impact on the economic development of Africa.

The subcontinent southern Africa has a rich floral diversity, compromising approximately 24 000 species. Of these, about 4 000 species are used in traditional medicine as remedies for different diseases. These traditional medicines have not been systematically investigated for the treatment of malaria and other diseases and South Africa could benefit from the scientific evaluation of this indigenous knowledge base.

To formulate an integrated approach, a multi-disciplinary scientific consortium was formed by the Council for Scientific and Industrial Research (CSIR), South African National Biodiversity Institute (SANBI), University of Cape Town (UCT), Medical Research Council (MRC) and University of Pretoria (UP), aiming to develop new medicines for malaria, based on the knowledge of indigenous plants. Each member of the consortium was tasked with a certain aspect that would systematically contribute to this research. SANBI submitted their database containing 2300 records of 700 plants claimed to be used in the treatment or prevention of malaria and fevers. The Bioprospecting Platform of Biosciences, CSIR, conducted research on drug discovery by employing bioassay-guided fractionation of crude plant extracts to give pure compounds. The Department of Pharmacology, UCT, was responsible for screening these hits against the *Plasmodium falciparum* strains.

One hundred and thirty-four plant taxa native to or naturalised in South Africa, representing 54 families, were selected. Of the 134 species, 49% showed promising antiplasmodial activity and were regarded as hits. The consortium prioritized the plants based on their anecdotal and literature information as well as primary activity against an *in vitro* susceptible strain of *P. falciparum* using a parasite lactate dehydrogenase activity (pLDH) assay. Plants showing good primary activity were given priority for further research and development.

Schefflera umbellifera (Sond.) Baill. (Araliaceae) is one of the plants that exhibited good primary activity. It has been reported that the bark of this plant is used traditionally for malaria in Zimbabwe[5]. Literature searches revealed limited published information on chemical compounds isolated from this plant species.

The CSIR has also an ongoing collaboration with the Traditional Healers Committee (THC) on projects involving traditional medicines. A secrecy agreement has been signed by both parties to help protect knowledge dissemination. Traditional healers provide anecdotal information on the use of plants and the CSIR scientifically validate it. This interaction helps to identify new plant candidates for further research. Traditional healers brought in a plant which was identified by SANBI as *Elephantorrhiza elephantine* (Burch.) Skeels (Fabaceae) for the treatment of a condition called benign prostatic hyperplasia.

The aim of this study was:

- To employ bioassay-guided fractionation of extracts from *Schefflera umbellifera* to isolate, purify and identify compounds
- To screen the isolated compounds for antimalarial activity
- To compare extracts of plants of this species from different geographical areas using high-performance liquid chromatography
- To employ fractionation of extracts of *Elephantorrhiza elephantina* to isolate and identify pure compounds showing anti-benign prostatic hyperplasia activity and evaluate their cytotoxicity against Chinese Hamster Ovarian cell lines.

5. A. Hutchings, A.H. Scott, G. Lewis, A. Cunningham, Zulu medicinal plants. An inventory. University of Natal Press, Pietermarizburg, 1996, p. 221.

Chapter 2

Literature overview

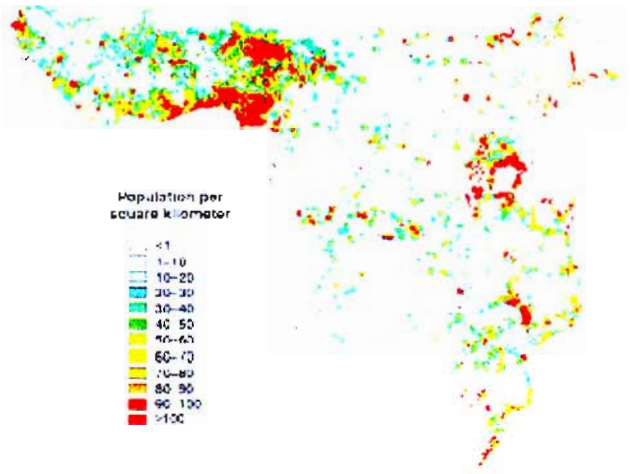
This chapter entails a summary of literature on two therapeutic areas, namely malaria and benign prostatic hyperplasia. It includes a background of malaria - distribution, cause and prevention, drug resistance, combination therapy, plants as a source of antimalarials, and commercial antimalarial drugs. For benign prostatic hyperplasia, the background of the disease, causes, different methods of treatment and the models used for studying it, as well as the structures of compounds used against it, are provided.

2.1 Malaria

2.1.1 Background to malaria

Malaria still remains among the three or four most devastating diseases occurring in the world today and it is estimated that around 100 million clinical cases may occur every year in tropical Africa alone, where changes in the epidemiological situation have resulted in the last few years in an increased frequency of the disease, often of epidemic proportions in countries such as Ethiopia, Madagascar, Rwanda and northern Sudan[1]. The damage done by malaria has resulted in the decline of productivity at the work place hence affecting the economy. In South Africa, malaria prevalence occurs mostly in the north of KwaZulu-Natal Province close to the Mozambique border. The distribution of malaria in Africa is shown in Fig. 2.1.

1. G.A.T. Targett, *Nature*, 1991, **349**, 199.



Source: R.W Snow et al, 1999

Figure 2.1: Distribution map for malaria infection in Africa

Malaria is a tropical infectious disease caused in humans by four species of the *Plasmodium* genus of protozoan (*P. falciparum, P. vivax, P. ovale* and *P. malariae*) that are transmitted by many species of *Anopheline* mosquitoes[2]. *P. falciparum* is the most widespread and also the most serious form. In the most fatal form of *P. falciparum,* severe cerebral complications occur and the only remedy is to treat it while it is still in the early stages, thus preventing progression to the severe form.

The most common symptoms of malaria are a flu-like fever, headaches, shaking chills etc. This is usually observed seven to nine days after being bitten by a female mosquito. The mechanism by which malaria manifests itself is very complicated and is illustrated schematically in Fig. 2.2.

2. A. Prakash, D.R. Bhattacharyyn, P.K. Mohapatra, J. Mahanta, *Ann. Trop. Med. Parasitol.,* 2004, **98**, 559-568.

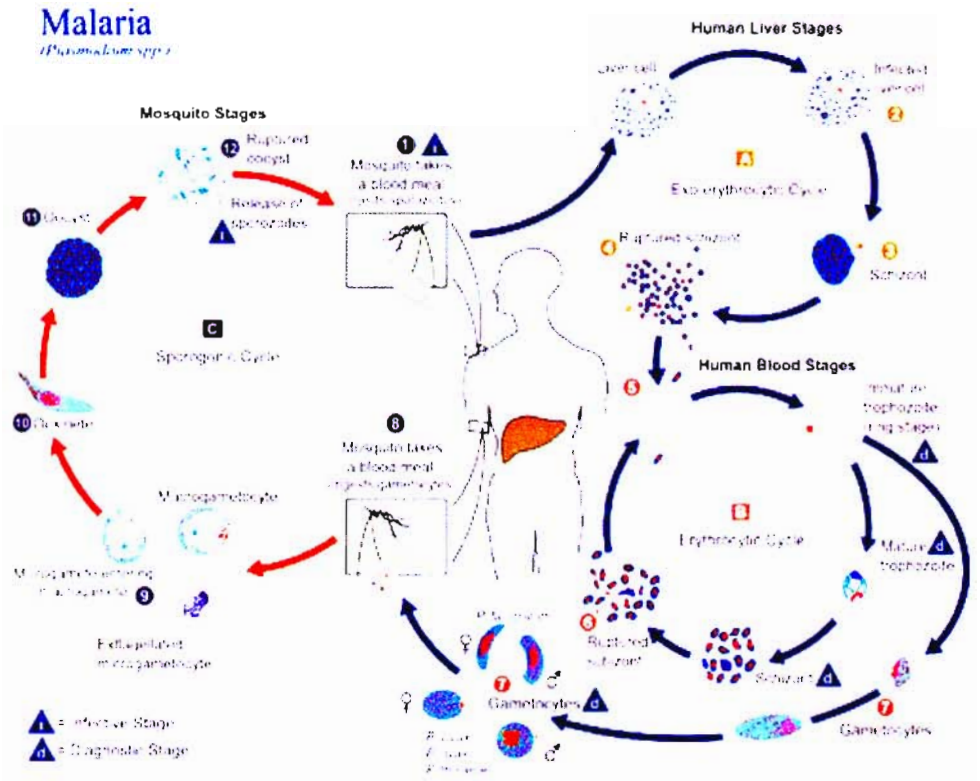


Figure 2.2: Malaria life cycle

The figure above illustrates how malaria is transferred from a female mosquito to a human being. This life cycle involves both intracellular and extra-cellular stages in both human host and mosquito vector[3]. This cycle is two fold, i.e. a sexual cycle that occurs in the mosquito and an asexual cycle that occurs in humans. The parasite enters the host's blood as the mosquito bites a human skin. Half an hour later sporozoites enter the bloodstream, invade the human liver cells and start multiplying. The sporozoites then enter red blood cells and form trophozoites. During cell division, these affected cells multiply giving rise to the formation of more merozoites and some of these malarial cells relapse weeks later. Inside the mosquito itself, female and male sporozoites come together to form a zygote-oocyte and cell division and multiplication starts happening at a phenomenal rate. These zygote-oocytes migrate to the salivary gland and wait for a re-infection to take place. Fever strikes when the infected red blood cells rupture and release merozoites.

3. J.B. Sacci, U. Alam, D. Douglas, J. Lewis, D.L.J. Tyrrell, A.F. Azad, N.M. Kneteman, *Intern. J. Parasitol.*, 2006, **36**, 353-360.

2.1.2 Malaria prevention

Early attempts to control this disease led to the discovery of the first potent antimalarial drug, quinine, isolated from the *Cinchona* tree from Peru and named in 1820 by French researchers, though it was first used in an un-extracted form in the early 1600s[4]. Quinine is the oldest and most important drug still in use today against malaria[4]. Limited access to *Cinchona* plant material during the Second World War led to the development of the synthetic drug chloroquine. Since then chloroquine has been used as a monotherapy for people affected with malaria. Chloroquine is structurally related to quinine and for years it has shown the qualities of being a prototype drug: high efficacy, cheap with tolerable adverse effects (affordable to poor people), smooth diffusion between the body cells and low toxicity.

The mechanism of chloroquine resistance is still somewhat unclear although it appears to be caused by mutations in a *Plasmodium* transmembrane protein, PfCRT (*Plasmodium falciparum* chloroquine resistance transporter). The mutant forms seem to lower the accumulation of chloroquine in the parasitized cell. Thus, new drugs have every prospect of being active even in CQ-resistant parasites, providing they circumvent the effects of PfCRT. CQ seems to target either haematin or haemozoin which are the catabolic by-products of haemoglobin and remain highly attractive as a target for new drugs. Haematin has always been a target of the most effective antimalarials because its biochemistry in *Plasmodium* is completely different to that of the host.

Generally, during the blood stages, the malaria parasite lives inside a red blood cell of the human host and since the major constituent of the red blood cells is haemoglobin, the parasite uses it as a primary source and degrades it. This process occurs in a food vacuole. A portion of the haem is oxidized and incorporated into haemozoin (malaria pigment). However, the structure of haemozoin has been identified as a cyclic dimer of ferriprotoporphyrin and is similar to a synthetic haematin product known as β-haematin. Haematin is

4. S. Saxena, N. Pant, D.C. Jain, R.S. Bhakuni, *Curr. Sci.*, 2003, **85**, 1314-1329.

apparently the target for chloroquine and other antimalarial drugs and these also inhibit synthetic haemozoin formation. This hypothesis is still not yet confirmed as to whether the inhibition process occurs in the parasite or not[5].

There has been many antimalarial agents synthesized after chloroquine and have proved to be effective and they all have different half lives namely, mefloquine, halofantrine, primaquine and some other antibiotics. Most of these synthetic drugs or prophylactic agents have some side effects[6].

2.1.3 Drug resistance and combination therapy

In sub-Saharan Africa, *P. falciparum* with its complex life cycle has in recent times showed resistance against chloroquine, resulting in a paradigm shift in terms of the search for new, effective drugs for malaria. Drug resistance in general can be defined as an infection that survives after drug administration[7]. This resistance was noticed late in the 1950's and early 1960's and presently it is experienced world wide[8].

Genetically, drug resistance occurs as a result of mutations that alter protein structure to reduce drug binding capacity or that alter gene expression. This physiological change is also as a result of the change in parasite metabolism which determines the level of resistance. Since the enzyme undergoes so many alterations, this has a direct impact on the cost of developing drugs as more complex enzyme dynamics and pathways have to be investigated[9]. This multi-drug resistance is a present challenge in Africa and south-east Asia as no single drug is currently effective against it. While most of the antimalarial agents have been utilized as monotherapies, there have recently been many developments by of combining two drugs to provide a powerful potential therapeutic agent, e.g. atovaquone-proguanil (AP), sulfadoxine-pyrimethamine (SP), chloro-proguanil plus

5. T.J. Egan, *Drug Disc. Today: Targets*, 2003, **2**, 115-124.
6. W.K. Amery, *J. Head face Pain*, 1983, **23**, 70.
7. I.M. Hastings, W.M. Watkins, *Trends Parasitol.*, 2006, **22**, 71-77.
8. U. Farooq, R.C. Mahajan, *J. Vect. Bor. Diseas.*, 2004,**41**, 45-53.
9. I.M. Hastings, M.J. Donnelly, *Drug Resist. Upd.*, 2005, **8**, 43-50.

artesunate. This type of approach is very useful for the treatment of non-severe malaria[10].

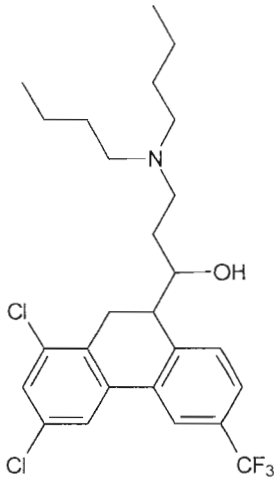
The combination of antimalarial drugs helps reduce treatment failure, recrudescence and gametocyte carriage[11], thereby affecting the rapid clearance time and thus reducing the incidence of gametocytaemia during follow-ups[12] and also offer improved efficacy[13]. However, when the two combined drugs have different half lives and pharmacokinetics, it lead to side effects[10]. Resistance is associated with drug half life. The longer the half-life of a drug, the better is the chances of drug resistance development[14]. There are many hypotheses supporting the origins and dynamics of chloroquine resistance and the quest is still continuing. People with access to medication also experience resistance as a result of incomplete healing or overdose of drug administration[11]. It also affects pregnant women who take medication and infects their foetus indirectly.

Another potential problem associated with *P. falciparum* other than resistance is recrudescence. This hampers the success of the treatment after a single or combination of drugs has been administered successfully as the parasite start reappearing in the long-term[15]. An ideal antimalarial drug is the one that will not only kill the parasite but also kill the gametocytes to block propagation stages of the parasite lifecycle[16].

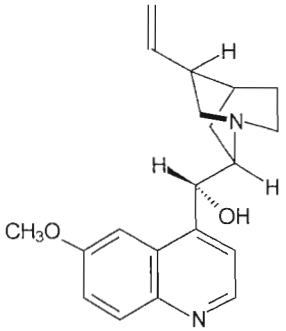
---

10. E.W. Nduati, E.M. Kamau, *Acta Trop.*, 2006, **97**, 357-363.
11 P. Garner, *Lancet,* 2004, **363**, 9-17.
12 F. Abacassamo, S. Enosse, J.J. Apunte, F.X. Magnussin, A.M. Ronn, R. Thompson, P.L. Alonso, *Trop. Med. Int. Heal.,* 2004, **9**, 200-208.
13. B.M. Greenwood, G.AT. Targett, *Lancet,* 2005, **365**, 1487-1498.
14. J. May, C.G. Meyer, *Trends Parasitol.*, 2003, **19**, 423-435.
15. M.M. Thapar, J.P. Gil, A. Bjorkman, *Trans. Roy. Soc. Trop. Med. Hyg.*, 2005, **99**, 62-70.
16. I. Bathurst, C. Hentschel, *Trends Parasitol.*, 2006, **22**, 301-307.

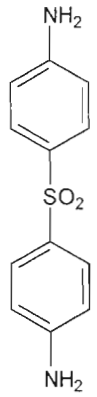
2.1.4 Chemical structures of some commercial antimalarials



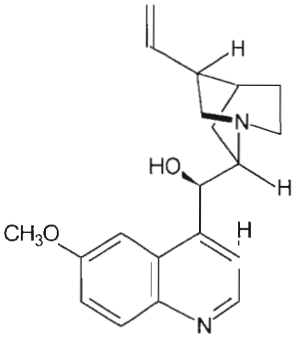
Halofantrine



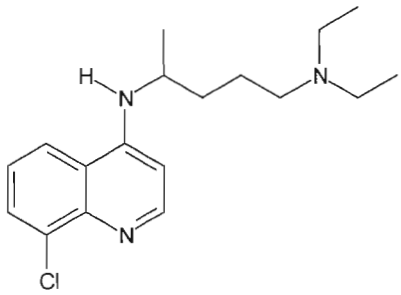
Quinine



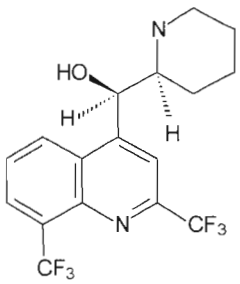
Drapzon



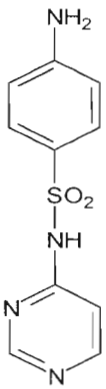
Quinidine



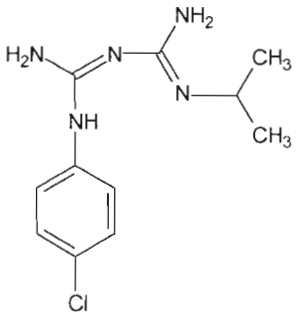
Chloroquine



Mefloquine



Sulfadiazine



Proguanil

Figure 2.3: Structures of antimalarial compounds

2.1.5 Plants as sources of antimalarials

Resistance developed by the malaria parasite against synthetic drugs has led to a renewed interest in evaluating plant metabolites as possible antimalarials. Quinine was isolated from the bark of the *Cinchona* (Rubiaceae) tree in the early 1600s and proved to be safe, affordable and very effective against malaria throughout the world. Quite a number of antimalarial compounds have been isolated from plants and these classes of compounds include alkaloids, sesquiterpenes, triterpenes, diterpenes, flavonoids, sterols etc.

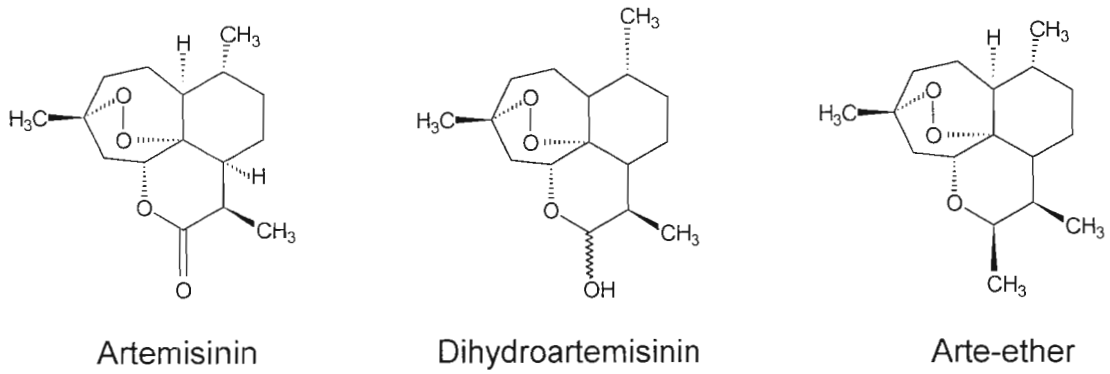
2.1.6 Discovery of artemisinin

Thirty years ago Chinese scientists investigated a plant species, *Artemisia annua*, and managed to isolate a sesquiterpene lactone endoperoxide called qinghaosu or artemisinin[17]. The plant itself has been used for more than 50 years in China as an herbal tea to treat malaria and haemorrhoids. However, there has been much debate as to the identification of the correct plant which contains major quantities of artemisinin as there were two sources of plant material i.e. *A. apiacea* and *A. annua*. The only distinguishing factor between the two plant species is their natural habitat, i.e. *qing hao* (*A. annua*) known as "blue-green" and grows in wastelands whereas *chou hao* (*A. apiacea)* known as "stinking hao" grows in ruderals[18].

Despite these uncertainties about identification of the relevant plant, the same method of preparation was used in which the entire green plant was soaked directly in water thus extracting the juice from the plant[17]. Much research has been done to validate this drug in terms of mechanism of action, pharmacokinetics, toxicity and combination therapy. Artemisinin is insoluble in water and oil and after administration it is converted to an active form, dihydroartemisinin. Scientists have synthesized artemisinin derivatives in order to improve efficacy and solubility and these include artemether, arte-ether and artelinate - all sesquiterpene

17. J.M. Liu, M.Y. Ni, J.F. Fan, Y.Y. Tu, Z.H. Wu, Y.L. Wu, W.S. Chou, *Act. Chim. Sin.*, 1979, **37**, 129-143.
18. E. Hsu, *Trans. Roy. Soc. Trop. Med. Hyg.*, 2006, **100**, 505-508.

endoperoxides[17]. So far, these compounds have all been effective against drug-resistant strains of *P. falciparum*. There has been many arguments regarding the effectiveness of these artemisinin drugs and it has been suggested that the endoperoxide moiety is essential for activity[19].



Artemisinin Dihydroartemisinin Arte-ether

Figure 2.4: Structures of artemisinin and its derivatives

Mostly, artemisinin's effectiveness as a monotherapy drug has made it the first line treatment method but this has been improved yet further by combining it with other drugs to avoid the risk of resistance formation by parasites. This was initiated so as to secure a constant supply of the drug without causing extinction of the plant source and to circumvent multi-drug resistance. Although this approach has had success in terms of efficacy, its safety prospects remains largely challenged.

These types of drugs cause dramatic side effects, i.e. neurotoxicity and neuronal degeneration in animals and contra-indication to pregnant species[13]. More potential drugs needs to be researched further to meet all the requirements of a good drug lead for a specific country as no drug does fit the needs of every country. In future, still more emphasis should still be put on strategies to finance viable research and development to reduce the hardships imposed by malaria, to strengthen public sector health systems, educate the public more about the disease (prevention strategies) and to deliver affordable treatment to needy people, especially in Africa[20]. Scientific research should focus more strongly on the pharmacodynamics and pharmacokinetics studies of the existing and new

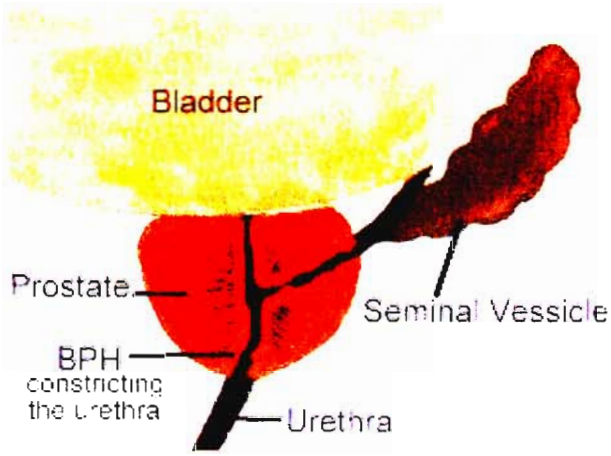
19. S. Tonmunphean, V. Parasuk, S. Kokpol, *Bioorg. Med. Chem.*, 2006, **14**, 2082- 2088.
20. P. Olumese, *Acta Trop.*, 2005, **95**, 265-269.

emerging antimalarials so as to formulate methods to help combat the malaria epidemic.

2.2 Benign Prostatic Hyperplasia

2.2.1 Background to benign prostatic hyperplasia

Benign prostatic hyperplasia (BPH) is a common, significant problem that affects mainly males over the age of 50 although the disease itself might have manifested itself earlier[21]. It affects about 80% of men 70 to 80 years old and is a major public health problem[22]. The main symptoms are the physical enlargement of the prostate gland that then results in urinary frequency, urgency, nocturia and dribbling or a slow stream. If this condition is not treated, it can lead to urinary tract infections, urinary retention and in rare cases, kidney disease[23].



Source: http://www.leaddiscovery.co.uk.

Figure 2.5: Location of the prostate, seminal vesicle, urethra and bladder

The cells of the prostate undergo changes, the microscopic foci grow to form macroscopic nodules and these then displace normal prostatic tissue that result in urethra compression. The compression itself results in either physical enlargement

21. P.J. Hieble, *Therap. Strat.*, 2004, **1**, 243-248.
22. F. Bravi, C. Bosetti, L.D. Maso, R. Talamini, M. Montella, E. Negri, V. Ramazzotti, S. Franceschi, C.L. Vecchia, *Ad. Urol.*, 2006, **67**, 1205-1211.
23. M.K. Brawer, *Urol. Tim.*, 1999, **27**, 13-18.

of the prostate or contraction of the prostate and urethral smooth muscles in response to nerve stimulation[20]. BPH prevalence has a serious impact on the quality of life of older males as it leads to isolation and death. However, vegetarian men have a lower incidence of prostate cancer than omnivorous men which is ascribed to flavonoids in vegetables which play a preventive role against this disease[24]. Invariably, the symptoms of BPH develop long before diagnosis hence the treatment only provides short-term relief and the treatment depends on the severity of the symptoms.

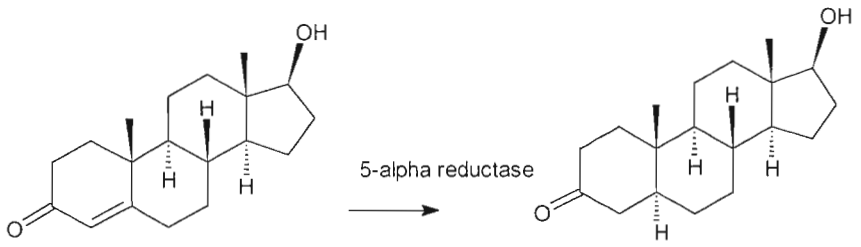
Over the years, scientific research has been undertaken in an attempt to study the mechanism of action and pathogenesis of BPH to help treat sufferers. The origin of the causes of this disease remains unresolved as there are many different hypotheses reported to date[25]. However, hormonal and histological changes within men over 50 years are considered to be the major contributing factors[26].

2.2.2 The causes of BPH

In the human prostate, androgens are responsible for the normal growth, cell differentiation and maintaining the maturing of the gland. As men grow older, the function of androgen continues and this could lead to risk factors and susceptibility against diseases such as prostate cancer[27]. The conversion of the androgenic hormone, testosterone to dihydrotestosterone (DHT) that is regulated by the steroid 5α- reductase enzyme seems to be one of the causative factors for benign prostatic hyperplasia[28]. It is believed that the higher conversion of testosterone to dihydrotestosterone contribute to the pathogenesis of BPH[25]. Steroid 5α-reductase has two different iso-forms, i.e. type 1 (5α-R1) and type 2 (5α-R2)[29]. These two types are characterized in humans, monkeys, rats and mice and are expressed by different genes. Type 1 is mainly responsible for androgen metabolism, whereas

24. L.J. Denis, M.S. Morton, K. Griffiths, *Europ. Urol.*, 1999, **35**, 5-6.
25. M.A. Cabelin, A.E.Te, S.A. Kaplan, *Curr. Opin.Urol.*, 2000, **10**, 301-306.
26. V. Mirone, F. Fusco., P. Verze, C. Schulman, F. Debruyne, C. Imbimbo, *Europ. Urol. Suppl.*, 2006, **5,** 410-417.
27. R. Ross, L. Bernstein, H. Judd, R. Hanisch, M. Pike, B. Henderson, *J. Natl. Cancer Inst.*, 1986, **76**, 45-48.
28. G.F. Verlag, *Phytomedicine*, 1996, **3**, 121-128.
29. Y. Jin, T.M. Penning, *Best Pract. Res. Clin. Endocrinol Metab.*, 2001, **15,** 79-94.

type 2 plays a role in prostate cancer[26]. It is also believed that the same enzyme is responsible for male baldness, acne, hirsutism and BPH.



Testosterone Dihydrotestosterone(DHT)

Figure 2.6: Irreversible enzymatic conversion of testosterone to dihydrotestosterone(DHT)[30]

This reaction occurs within the prostate, after DHT is formed, it is further metabolized into 3α- and 5α–diols which are water soluble and inactive as androgens and cannot re-form DHT.

2.2.3 Treatment of BPH

2.2.3.1 Surgical approach

Traditionally, surgical therapy has always been proved to be the effective method for the treatment of BPH. It provides immediate urinary flow rate improvement although the risks associated with surgical procedures are immense[31]. These risks include infections, bleeding, sexual problems and even death. Surgery was introduced due to the lack of relevant medical treatment for the disease. The older types of surgery include trans-urethral resection of the prostate, trans-urethral incision of the prostate, open prostatectomy etc. The newly improved techniques are high-intensity focused ultrasound, balloon dilation and urethral stents. All of these techniques are dangerous as they can cause permanent damage to the prostate and offer only short-term relief. The decline in surgical therapy has

30. A.W. Partin, D.S. Coffey, *Recent Prog. Horm. Res.*, 1994, **49**, 293-331.
31. W. Mahapokai, F.J. van Sluijs, J.A. Schalken, *Prost. Can. Prost. Diseas.*, 2000, **3,** 28-33.

resulted in patients opting for prostatectomy which is a complete removal of the prostate and is less invasive[32].

2.2.3.2 Prescription medicine

The prevalence of BPH has led to the discovery of the prescribed chemopreventive drugs on the market. The introduction of these drugs led to the decline in usage of the surgical methods. The two types of prescribed drugs are α-blockers and 5α-reductase inhibitors. α-Blockers help to relax the smooth muscle in the prostate that constricts the urethra and bladder neck. They tend to be non-specific and these include doxazosin (Cardura®), prozosin (Minipress®), tamsulosin (Flomax®) and terazosin (Hytrin®)[20]. These types of drugs are used worldwide as they offer quick relief, no adverse effect on erectile or urinary function, can be used with any size of the prostate and are less expensive. Although these drugs are used, they have shown considerable side effects such as hypotension, dizziness, upper respiratory symptoms, headache, fatigue and sexual disturbances[33].

The enzyme inhibitors (5α-reductase inhibitors) offer much more relevant effects as they block the conversion of testosterone to dihydrotestosterone which then results in reduction in prostate sizes by approximately 25%. The only enzyme inhibitor available so far is a Merck product, finasteride (Proscar®). Its main advantages are its ability to lower the DHT level by 70% to 80%, improving urinary flow rate (15% to 20%), while causing no cardiovascular side effects and hence reducing the need for prostate surgery. This drug is not very effective in men with smaller prostates as it may cause reduction in erection rate by up to 8%, sex drive by 6% and semen volume reduction by 4%.

32. P. Cathcart , J. Armitage, J. van der Meulen, J.M. Emberton, *Eur. Urol. Suppl.*, 2006, **5**, 158.
33. Y.T. Logan, M.T. Belgeri, *Amer. J. Geriat. Pharm.*, 2005, **3**, 103-113.

2.2.3.3 Herbal treatments / Phytotherapies

It is not well established whether herbal or nutritional treatments have a direct impact on BPH. Research has been conducted to investigate and scientifically validate claims of the beneficial properties of herbal treatments. There are several herbs which have been evaluated and shown to inhibit the action of steroid 5α-reductase enzyme, although little is known about their specificity on BPH and their pharmacokinetics and pharmacodynamics.

The following herbs have been investigated and reported to be effective against steroid 5α-reductase enzyme activity: saw palmetto (*Serenoa repens,* previously *Sabal serrulata), Prunus africana* (previously known as *Pygmeum africanum*) and *Urtica dioca* (stinging nettles*)*. Saw palmetto has been popular in America for the treatment of urino-genital disorders and for the treatment of prostate problems since the early 1890's[34,35].

In the 1990's, saw palmetto gained recognition as one of the leading herbs for the treatment of BPH as its fruits are rich in sterols, which in turn appear to be the active components, although the mechanism is still unknown[36]. Many studies have been conducted and proved that saw palmetto extract provides mild to moderate improvement to urinary symptoms compared to the placebo and this has led to the Germans approving it as a tonic for the treatment of symptomatic BPH[37]. There has been no scientific evidence that saw palmetto could treat BPH. β-Sitosterol has been reported to be the major active ingredient in saw palmetto and it is not clear whether this compound is responsible for reducing the prostate size[38]. β-Sitosterol is believed to inhibit some growth factors, hormones and enzymes.

P. africana has been quite effective against BPH compared to finasteride and some α-blockers (Tamsulosin). Significant improvements were seen in 43% of

34. A.W. Smith, *Trans. Amer. Mater. Med. Assoc.*, 1914, **1**, 1-158.
35. J. Eng, D. Ramsum, M.Verhoef, E. Guns, J. Davison, R. Gallagher, *Interg. Can. Therap.*, 2003, **29**, 212-216.
36. K. Bone, *Europ. J. Herb. Med.* 1998, **4**, 15-24.
37. S. Bent, C. Kane, K. Shinohara, J. Neuhaus, E.S. Hudes, H. Goldberg, A.L. Avins, *N. Engl. J. Med.*, 2006, **354**, 557-566.
38. R. Sahelian, *Amer. J. Nat. Med.*, 1997, **4**, 1-3.

patients on phytotherapy with *S. repens* or *P. africana* compared to 57% of those on finasteride and 68% on α-blockers[39].

U. dioica is another herb that has widely been used by the Germans for treating BPH as the extract has been shown to suppress cell growth and hence block the hormone receptors. Phytotherapy treatment of BPH has showed great potential benefits with few or no side effects. A comparative study[40] of these herbs against each other and prescribed drugs and in combination was also performed to investigate the possibility of discovering new agents with higher efficacies, less side effects and increased affordability. During the study, *U. dioca* extract had an influence on the steroid 5α-reductase at high concentrations and showed a synergistic effect when combined with *P. africana* extract[40].

2.2.3.4 Combination therapy

Most drugs or therapeutic agents have failed or found not to be effective enough when utilized as monotherapies. Although short-term studies have been done on combination therapy and positive results have been obtained, more research need to be done to combine two or more agents to see whether new powerful medicines could be discovered.

An *in vitro* study has been undertaken whereby *P. africanum* and *U. dioca* extracts were combined at a specified concentration and tested against BPH and the mixture showed an improved activity due to a synergistic effect[40]. This extract is known to contain sterols, aliphatic alcohols, acidic phenols, terpenic acids and triterpenes and has several sites of action potentially relevant to the treatment of patients with BPH[41]. Another comparative study was undertaken when saw palmetto (herbal remedy) was evaluated against finasteride (synthetic drug) for the treatment of BPH in terms of safety and efficacy. Saw palmetto outclassed finasteride[42]. Patients have since stopped using finasteride as it has side effects

39. A. Hutchison, R. Farmer, K. Verhamme, R. Berges, R.V. Navarrete, *Europ. Assoc. Urol.*, 2007, **51,** 207-216.
40 R.W. Hartman, M. Mark, F. Soldat, *Phytomedicine*, 1996, **3,** 121-128.
41 M-C. Andro, J-P. Riffaud, *Curr. Therap. Res.*, 1995, **56**, 796-805.
42. D.J. Brown, *J. Amer. Bot. Counc.*, 2003, **58**, 20-21.

and is very expensive compared to *S. repens,* which is cheap, safe and accessible to needy people. The problem in using saw palmetto is that it has not shown any combination effect with other drugs and it's mechanism of action remains obscure[43,44] .

2.2.3.5 Effect of nutrition and diet on BHP prevalence

Food plays a major role in preventing and fighting diseases. Little is known about geographic variations, health resources and socio-demographic factors that might be the contributing factors to the prevalence of the BPH condition[45].

Most of the traditional Western diseases i.e. breast and prostate cancer, have been associated with diet. In general, a Western diet is rich in fat and low in fibre while Asian diet is low in fat and high in vegetables which is a rich source of plant oestrogens, lignans, flavonoids and isoflavonoids that help protect against cancer[46]. Polyphenolic compounds are antioxidants and radical scavengers and are commonly present in most plant species[40]. Ingestion of plants rich in these compounds could thus provide protection against many diseases including BPH. It has been reported that an excess calorie intake (obesity) does have an influence on BPH and prostate cancer[47]. A change in the type of diet, especially from fat to vegetable, could thus be beneficial when it comes to the prevention of BPH or reduction of prostate size[48]. Food rich in fibre, soya proteins, fruit, wine, vegetables and tea is recommended as these are rich in polyphenols. Consumption of these components can be chemo-preventative as opposed to drug administration.

---

43. G.M. Allen, M.D. Bond, M.B. Main, *Circular*, 1999, **1439**, 1-22.
44. S. Bent, C. Kane, K.Shinohara, J. Neuhaus , E.S. Hudes, H. Goldberg, A.L. Avins, *N. Engl. J. Med,* 2006, **354**, 557-566.
45. J.C. Sung, L.H. Curtis, K.A. Schulman, D.M. Albala, *J. Urol.*, 2006, **175,** 1023-1027.
46. M.S. Morton., A.Turkes , L. Denis, K. Griffiths, *BJU International,* 1999, **84**, 549-554.
47. T. Bocchicchio, *Amer. Urol. Assoc.,* 1999, **18,** 298-303.
48 A.B. Miller, F. Berrino, M. Hill, P. Pietinen, E. Riboli, J. Wahrendorf, *Eur. J. Cancer,* 1994, **30A**, 207-220.

2.2.4 Models for studying BPH

Many attempts have been made in the last decade in trying to obtain a thorough understanding of the what, why and how of BPH. In spite of this, the aetiology and pathophysiology of this disease remain obscure[49]. Firstly, non-surgical modes of treatment have been studied intensively to find satisfactory and cost-effective therapeutic options for patients in whom surgical intervention carries a high risk. The obstacles encountered are due to BPH being rare in species other than man and has only recently been described in dogs and also in chimpanzees.

In vitro models include the culturing of prostate epithelial cells, but most of the available human prostate cell lines are derived from malignancies and hence not suitable for studying normal prostate physiology or initiation and development of tumours. To date, very few prostate cell lines are known to be non-tumourigenic. *In vivo* models include Xenograft models of human BPH and these have shortcomings when it comes to tissue implants. Transgenic mice have also been used but the fibromuscular stromal hyperplasia commonly seen in humans is absent in rats, so that the differences in cellular and structural components poses a challenge. Only dogs can be used with success as they have many common features of human BPH. Unfortunately, there is a lack of commercially available ageing male dogs and ethical as well as financial matters need to be considered.

2.2.5 Chemical compounds showing biological activity against BPH

Polyphenolic compounds have been reported to play a critical role in the inhibition of the steroid 5α-reductase enzyme; these include myricetin, quercetin, baicalein, fisetin, genistein, kaempferol and daidzein[50]. It has been reported that green tea is a main source of certain classes of compounds called catechins and these have proven to contribute immensely towards the inhibition of 5α-reductase activity[51].

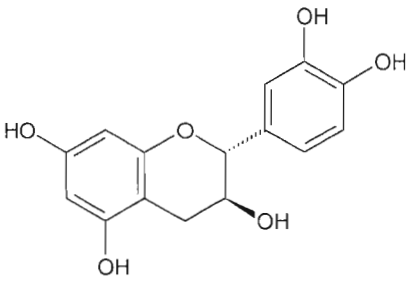
---

49. W. Mahapokai, J.A. Schalken, *Prost. Can. Prost. Disease,* 2000, **3,** 28-33.
50. R.A. Hiipakka, H.-Z. Zhang, W. Dai, Q. Dai, S. Liao, *Biochem. Pharm.*, 2002, **63**, 1165-1176.
51. S. Liao, T. Liang, *United States Patent,* 1997, **5,605,929.**

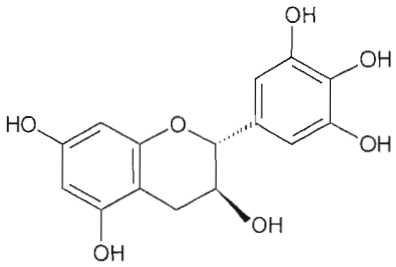
Many compounds including fatty acids have been evaluated for steroid 5α-reductase activity and it has been shown that catechin and its derivatives (especially gallates) have a significant effect on activity as they have an ability to reduce body, organ and tumour growth in animals[52]. Catechins have unique structural features i.e. a C_6-C_3-C_6 system with hydroxyl groups attached at different positions to define and differentiate other structural analogues. It has been established that the more hydroxyl groups attached to each molecule, the more active it becomes against BPH. Catechin derivatives include epicatechin gallate (ECG), epigallocatechin gallate (EGCG), catechin gallate (CG) and other conjugated substances such as theaflavins etc.

Theaflavins are isolated from black tea and have also shown good anti-carcinogenic properties[53]. However, the activity of catechin and its derivatives have appeared to be synergistic because when these compounds are tested as individuals, they lose activity. During this study, compounds for example, EGCG and ECG, were found to be more effective and this might be due to the higher number of hydroxyl groups attached to them. It has been demonstrated that these compounds have produced no allergic reaction to humans. The structures of these compounds are shown in Figure 2.7.

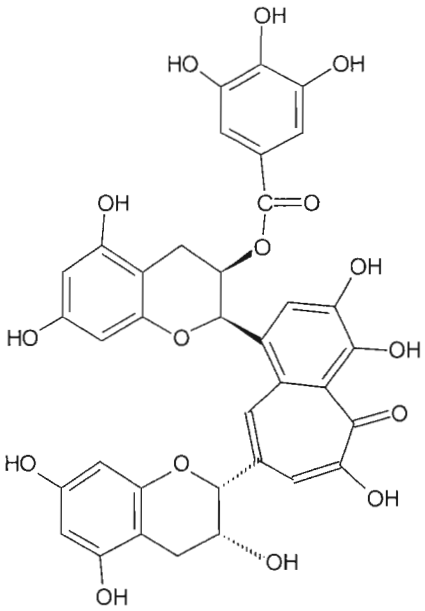
52. S. Liao, R.A. Hiipakka, *Biochem. Biophys. Res. Commun.*, 1995, **214**, 833-838.
53. H. Lee, C. Ho, J. Lin, *Carcinogen.*, 2004, **25,** 1109-1118.



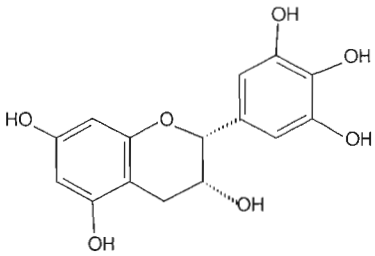
(+)-Catechin



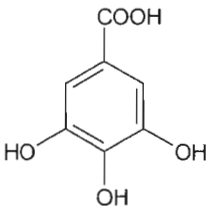
(+)-Gallocatechin



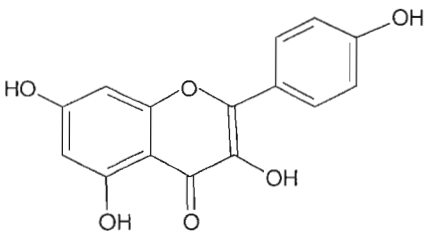
Theaflavin gallate



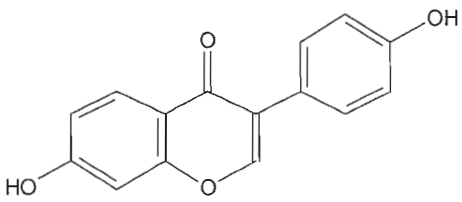
(-)-Epigallocatechin



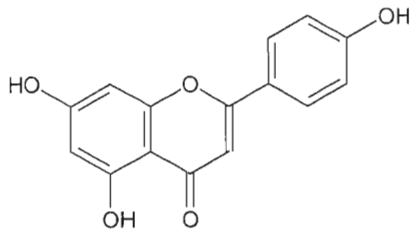
Gallic acid



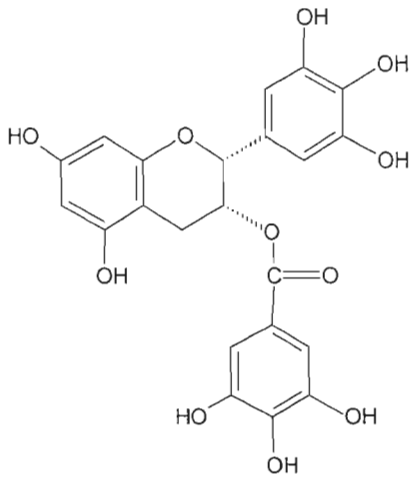
Kaempferol



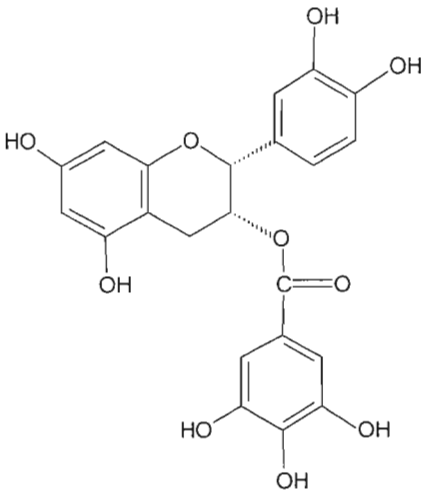
Daidzein



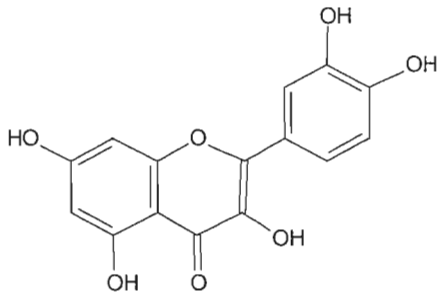
Genistein



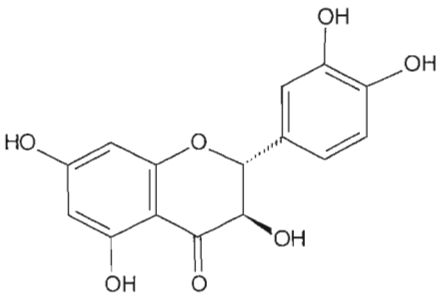
(-)-Epigallocatechin gallate



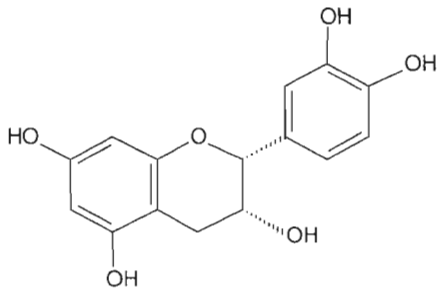
(-)-Epicatechin gallate



Quercetin



(-)-Taxifolin



(-)-Epicatechin

Figure 2.7: Structures of green and black tea ingredients that inhibit steroid 5α-reductase enzyme[26, 48, 51, 52]

2.3 Conclusion

The quest to find new, safe, and efficacious antimalarial agents and drugs to treat benign prostatic hyperplasia continues. Scientific evidence needs to be provided on use of natural products as herbal remedies against diseases affecting man.

Chapter 3

Materials and Methods

3.1 Plant collection

Plants were collected by the South African National Biodiversity Institute (SANBI) and by an independent botanist (Ralph Peckover). Plant specimens were pressed, dried and stored in a safe environment. Voucher specimens were generated after the plant had been identified by an experienced botanist. The collection sites were chosen based on endemicity and the popularity of the plants in that particular area. Specimens of a particular species from different geographical areas were collected, identified and their voucher specimens generated. The content of the voucher specimens included: collection site, plant name, province, habitat, locality, geographical point system coordinates, collectors name, collection number and the date of collection.

3.2 Extraction of plant material

Preparation of plant extracts depended on the anecdotal information and on the application. The plant material was first dried in an oven at 60 °C and ground to a fine powder. Two extraction methods were followed. In method A, the plant material was separated into two portions and one portion was immersed in distilled water and boiled to produce a tea. The infusion was filtered through a cheese cloth and then through Whatman filter papers after which the filtrate was freeze-dried. The second portion was extracted for 24 h with enough methanol/dichloromethane (1:1) to cover the plant material. The suspension was filtered and the solvent evaporated using a rotary vacuum evaporator set at 60 °C. The semi-dried extract was dried further in a desiccator overnight and the recovered extract yields were recorded thereafter. In method B, a sequential extraction was performed where a methanol/dichloromethane (1:1) extract was first prepared, filtered and dried. The

resultant plant material was then dried overnight in an oven at 60 °C and then immersed into cold distilled water for half a day and then extracted. The water extract was filtered and the filtrate was freeze-dried.

3.3 Chromatography

The purification of the crude extracts was performed using the following chromatographic techniques: column (CC), flash (FC) and thin-layer chromatography (TLC). High-performance liquid chromatography (HPLC) was utilized to analyze, identify and quantify the extracts and compounds isolated. An HPLC-UV-MS Waters Alliance 2690 using a Waters 996 photodiode array detector as well as Masslynx software coupled to a Quattro micromass spectrometer was utilized. The ODS Hypersil C-18 column dimensions were 250 x 4.6 mm, 5 µm particle size and 100 Å pore size. The column temperature was maintained at 40 °C for all experiments. To prevent blockage of the column and guard column, all samples were filtered using a 0.45 µm membrane.

Solutions of the extracts were prepared to a concentration of 5000 ppm and 20 µL of the solutions was injected onto the column. For pure compounds, 100 ppm solutions were prepared and 20 µL was also injected onto the column. HPLC grade solvents such as acetonitrile, methanol and distilled water were used either in a gradient or isocratic form in all experimental runs. An Agilent HP 6890/5973 gas chromatograph with a mass selective detector equipped with a narrow-bore fused silica capillary column (0.25 µm film thickness of 5-phenylmethylsilicone) coupled to an HP computer with Wiley 138.1 software for library matching of compounds was utilized.

Column chromatography employed different column sizes based on the complexity of the fraction or the crude extract, usually 20-90 cm long and 4 cm wide. Silica gel 60 purchased from Merck was used as the stationary phase with particle size ranging from 0.063-0.2 mm. Flash silica gel was also used to separate mixtures of compounds and its particle sizes ranged from 35-75 micron. Thin-layer chromatography was employed to monitor the progress of the column chromatography technique. Plates used were 0.2 mm thick pre-coated silica gel

glass plates (F_{254} which is UV active). These plates were purchased from Merck or Sigma Aldrich.

Liquid-liquid partitioning of plant material was also employed to separate extracts based on their polarities. An extract was dissolved in a few millilitres of methanol and partitioned between hexane, dichloromethane and water using a separating funnel sequentially. Hexane and dichloromethane layers were evaporated under reduced pressure and stored overnight in a desiccator, while water extracts were freeze-dried.

3.4 Spectroscopy

3.4.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

NMR analyses were carried out at the CSIR, Modderfontein using a Varian 400 MHz Unity spectrometer. For both one-dimensional and two-dimensional NMR analysis, all the compounds were dissolved, using deuterated solvents, namely chloroform, acetone, methanol and water. Tetramethylsilane (TMS) was used as an internal reference standard.

3.4.2 Mass spectrometry

The mass spectral data was obtained from a coupled HPLC-UV/MS instrument with a triple pole Quattro LC Micro mass spectrometer which was set to operate both in the ESI^- and ESI^+ modes. This work was carried out at the CSIR, Biosciences, Bioprospecting Platform. High-resolution mass spectra (HREIMS) of compounds were obtained from the University of Witwatersrand using a VG 70SEQ HRMS instrument, operating mainly on EI^+ mode using 8 kV as a standard ionization energy.

3.5 Polarimetry

A Perkin Elmer 241 polarimeter at 589 nm using a sodium lamp as a light source and a cell with path length of 1 dm was used in all experiments. These

experiments were done using either methanol or chloroform as solvent depending on the solubility of the compound.

3.6 Melting point determination

Melting points were determined using a Reichert Koffler hotstage apparatus and are uncorrected.

3.7 Description of Biological assays

3.7.1 *In vitro* antimalarial screen

This work was carried out at the University of Cape Town, Pharmacology Department.

In this *in vitro* evaluation method, compounds or crude extracts were assayed against two *Plasmodium falciparum* strains, namely chloroquine-sensitive (CQS) (D10) and chloroquine-resistant (CQR) strains (K1). Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a method of Trager and Jensen[1]. The parasites were maintained at a 5% haematocrit with RPMI 1640 (Biowhittaker) medium supplemented with Albumax II (lipid rich bovine albumin) (GibcoBRL) (25 g/l), hypoxanthine (44 mg/l), HEPES [*N*-(2-Hydroethyl)-piperazine-*N*'-(2-ethansulfonic acid)] (Sigma Aldrich) (50 mg/l). The cultures were incubated at 37 °C in an atmosphere of 93% N_2, 4% CO_2 and 3% O_2.

Quantitative assessment of *in vitro* antiplasmodial activity was determined via a parasite lactate dehydrogenase (pLDH) assay[2]. This enzyme assay differentiates between pLDH and host LDH activity by using 3-acetylpyridine adenine dinucleotide (APAD). The pLDH used APAD as a coenzyme in the conversion of pyruvate to lactate and reduces it to APADH. The formation of APADH can be measured by the subsequent reduction of a yellow nitroblue tetrazolium (NBT) salt

1. W. Trager, J.B. Jensen, *Science,* 1976, **193**, 673-675.
2. M.T. Makler, J.M. Ries, J.A. Williams, J.E. Bancroft, R.C. Piper, B.L. Gibbins, D.J. Hinrichs, *Am. J.Trop. Med. Hyg.*, 1993, **48**, 739-741.

to a blue formazan product, the absorbance of which can be monitored on a microplate reader.

The compounds or extracts were prepared as stock solutions in 10% methanol or 10% DMSO. Chloroquine (CQ) was used as a reference drug in all experiments. All samples were store at 20 °C until use. A full dose-response experiment was performed with a starting concentration of 100 µg/ml, which was serially diluted 2-fold in complete medium to give 10 concentrations with the lowest concentration being 0.195 µg/ml. All tests were performed in duplicate. The highest concentration of solvent to which the parasites were exposed to had no measurable effect on the parasite viability. The 50% inhibitory concentration (IC_{50}) values were obtained using a non-linear dose-response curve fitting analyses via Graph Pad Prism v.4.0 software.

3.7.2 *In vitro* anticancer screen

This is an in-house CSIR screen for extracts or pure compounds against three cancer cell lines, PC-3 (prostate), UACC62 (melanoma) and TK10 (renal). The method employed was obtained from the National Cancer Institute, USA. This work was carried out at the CSIR, Biosciences, Bioprospecting Platform.

The cell lines were grown in RPMI 1640 medium containing 5% fetal bovine serum and 2 mM L-glutamine. Cells were inoculated into 96-well flat bottom, polystyrene plates at plating densities ranging from 5000 to 40,000 cells/well depending on the doubling time of individual cell lines. Each cell line was plated on duplicate plates. After cell inoculation, the microtiter plates were incubated at 35 °C, 5% CO_2, 95% air and 100% relative humidity for 24 hours prior to addition of experimental drugs. After 24 hours, the plates of each cell line were fixed *in situ* with the time course assay (TCA) to represent a measurement of the cell population for each cell line at the time of drug addition. Experimental drugs were solubilized in DMSO at 400-fold the desired final maximum test concentration and stored frozen prior to use. Extracts or pure compounds were diluted in complete media with 0.1% gentamycin sulfate and dispensed into wells in a volume of 20 µL to yield a test concentration

of 100 µg/mL. NSC (adriamycin) was used as standard and was included into each plate.

Cells were fixed *in situ* by the addition of cold TCA (final concentration 10% TCA) and incubated for 60 minutes at 4 °C. The supernatant was discarded, plates washed five times with tap water and air-dried. SRB at a 0,4% (v/v) in 1% acetic acid was added to each well and the plates incubated for 10 minutes at room temperature. Unbound dye was removed by washing six times with 1% acetic acid and the plates were air-dried. Bound sulforhodamine B (SRB) was solubilized with 10 mM trizma base and the absorbance was measured at a wavelength of 515 nm.

Crude extracts were screened at a single dose concentration to evaluate their anticancer properties i.e. 100 µg/ml in all three cell lines. The extract showing a growth inhibition above 75% against two or all three cell lines were then screened at five dose concentrations i.e. 100, 50, 25, 12.5 and 6.25 ppm. The results obtained were presented as dose-response curves for each cell line. The dose-response curve describes a percentage growth inhibition (GI) of the cancerous cells against the concentration of the extract in parts per million concentration. Total growth inhibition (TGI) is represented by the concentration value where the line crosses zero.

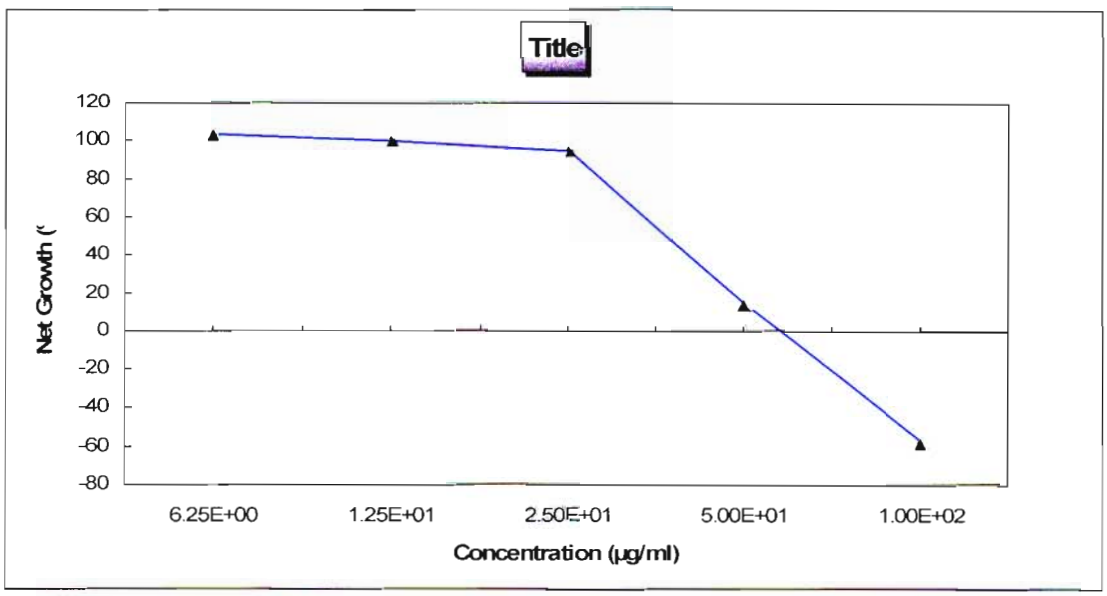


Figure 3.1: Example of an anticancer graph

Criteria for anticancer activity:

Inactive	TGI > 50 ppm
Weak	TGI between 15 and 50 ppm for two or three cell lines.
Moderate	TGI between 6.25 and 15 ppm for two or three cell lines.
Potent	TGI < 6.25 ppm for two or three cell lines.

3.7.3 *In vitro* cytotoxicity

This work was carried out at the University of Cape Town, Pharmacology Department.

This assay was used to measure the *in vitro* toxicity of a pure compound or extract to normal cells. The cell line chosen for this evaluation was a mammalian cell line, Chinese Hamster Ovarian (CHO), using the 3-(4,5-dimethylthiazol-2,5-diphenyltetrazoliumbromide (MTT) assay[3]. Toxicity was reported as LC_{50}, the concentration of compound/extract that is lethal to 50% of the cells.

For this test, all samples were done in triplicate on two separate occasions. The MTT assay was used as a colorimetric assay for cellular growth and survival and compares well with other available assays. This colorimetric assay is based on the ability of viable cells to metabolise a yellow water-soluble tetrazolium salt into a water-insoluble purple formazan product. The amount of formazan produced can be measured spectrophotometrically and is proportional to the metabolic activity and the number of cells in the test plate.

The CHO cells were cultured in Dulbecos Modified Eagles Medium (DMEM): Hams F-12 medium (1:1) supplemented with 10% heat inactivated fetal calf serum (FCS) and gentamycin (0.04 µg/mL). The medium reagents were obtained from Highveld Biological, South Africa.

3. T. Mosmann, *J. Immunol. Meth.*, 1983, **65**, 55-63.

Samples were dissolved in 10% methanol or 10% DMSO. The initial concentration of stock solutions was 2 mg/mL for all samples. Samples not properly/completely dissolved were tested as suspensions and stored at -20 °C until use. The highest concentrations of solvent to which the cells were exposed to had no measurable effect on the cell viability. Emetine was used as a reference drug 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 test samples with an initial concentration of 100 µg/mL to give 5 concentrations, with the lowest being 0.01 µg/mL.

The 50% inhibitory concentration (IC_{50}) values for all samples were obtained from dose-response curves, using a non-linear dose-response curve fitting analysis via Graph Pad v.4 software. The above work was done by Dr Clarkson, UCT, Pharmacology Department.

3.7.4 *In vitro* steroid 5α-reductase assay

This work was performed at the MDS Pharma Services, Pharmacology Laboratories, Taiwan.

This assay is an *in vitro* evaluation of an extract or pure compound's ability to inhibit the steroid 5α-reductase enzyme from metabolizing testosterone to dihydrotestosterone. This is an enzyme-immunoassay (EIA) for quantitative determination of testosterone in human serum or plasma. The significance of this type of inhibition is that it could lead to eradication of a condition called benign prostatic hyperplasia (BPH). Two distinct isozymes are found in mice, rats, monkeys and humans: type 1 and II. Each of these isozymes is differentially expressed in tissues and developmental stages. In human, type 1 steroid 5α-reductase is predominant in the sebaceous glands of most regions of skin, including scalp and liver and is responsible for approximately one third of circulating DHT. Inhibitors of steroid 5α-reductase may be of benefit in the treatment of androgenetic alopecia. This is a specific binding assay whereby the

biochemical assay results were expressed as percentage inhibition. Finasteride was used as a reference compound in all experiments.

The method employed in this study was adapted from the scientific literature to maximize reliability and reproducibility[4]. The steroid 5α-reductase enzyme was isolated from the liver of Wistar rats. The test compound is incubated with 20 µg/mL of steroid 5α-reductase preparation which contains 1 µM testosterone and 50 µM NADPH in DTT buffer, pH 6.5 for 30 minutes at 37 °C. The reaction is stopped by addition of 1N HCl and neutralized by 1N NaOH and testosterone is quantified using a testosterone EIA Kit. Compounds are screened at 10 µM.

Reference compound data:

Compound	IC_{50} (µM)
* Finasteride	0.025
γ-Linolenic acid	14

* refers to the reference compound used in this study.

3.7.5 Antioxidant screen

This evaluation was carried out at the CSIR, Biosciences, Bioprospecting Platform. The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was used to investigate the scavenging properties of extracts and pure compounds. Basically, a DPPH radical is scavenged by the potential antioxidant by donating a proton, forming a reduced DPPH and this resulted in a colour change from purple to yellow. This is quantified by the decrease of absorbance at wavelength 515 nm in the spectrophotometer.

$$DPPH^{\bullet}\ (purple) + (A\text{-}H)_n \rightarrow DPPH\text{-}H\ (yellow) + (A^{\bullet})_n$$ [5]

Since this was a colorimetric test, the appearance of the yellow colour was measured using a chromameter. The final appearance of a more intense yellow

4. T. Liang, M.A. Cascieri, A.H. Cheung, G.F. Reynolds, G.H. Rasmusson, *Endocrinol.*, 1985, **117**, 571-579.
5. C. Soler-Rivas, J.C. Espin, H.J. Wichers, *Phytochem. Anal.*, 2000, **11**, 330-338.

colour was used as a good indication of the radical scavenging ability of the extract or compound and this is directly proportional to its antioxidant activity. The antioxidant activity was directly related to the total amount of phenolics and flavonoids present in that particular extract. This method is sensitive enough to indicate and monitor the presence of phenolic-type compounds.

Dilutions of crude extracts are prepared at a single dose (100 ppm) concentration and spotted on a TLC silica gel layers in a form of a dot-blot test and layers are then stained with the 2,2-diphenyl-1-picrylhydrazyl radical. Spots with good radical scavenging properties turn yellow and the intensity is measured by a chromameter[5]. The active crude extract or compound is then serially diluted at a five dose concentration, i.e. 100, 50, 25, 12.5 and 6.25 ppm to determine their percentage radical scavenging capacity (%RSC). The activity of the compound or extract is measured against the reference compound, epigallocatechin gallate.

Chapter 4

Bioassay-guided isolation of antimalarial and cytotoxic compounds from *Schefflera umbellifera* (Sond.) Baill.

4.1 Introduction

Schefflera umbellifera (Sond.) Baill. is a semi-deciduous tree of 6-20 m in height. It has a tall, straight, usually unbranched main stem ca. 0.6 m in diameter and a much-branched, rounded crown (Fig. 4.1). The stem has a rough, longitudinally fissured and a grey-brown bark[1]. This tree has digitate leaves at the end of the branches with 3-5 m oblong leaflets that are leathery, dark green above and paler beneath. The leaf stalks are up to 200 mm long. It bears flowers between January and May[1]. In 2005, this tree was selected as one of the South African 'Trees of the Year'.

This plant belongs to the Araliaceae family and was previously known as *Cussonia umbellifera* and *Neocussonia umbellifera*, while its common names are basterkiepersol, false cabbage tree and its Zulu names are umsengembuzi, umbegele, umgezisa, umsenge and umbumbu[2].

4.1.1 The species *Schefflera umbellifera*

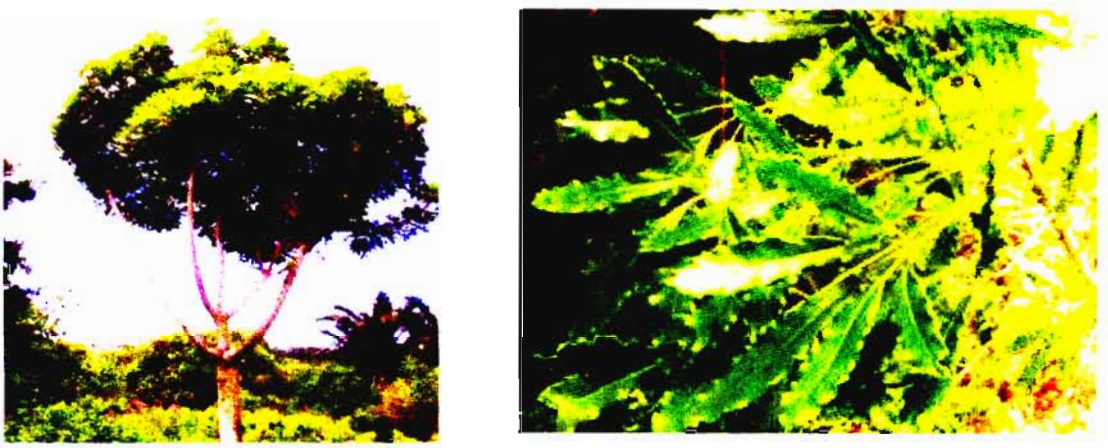
The genus *Schefflera* J.R.Forst. & G.Forst. has about 650 species and was named in 1776 by G. and J.R. Forster in honour of J.C. Scheffler of Danzig[1]. This is the only South African member of the genus which grows in warm and tropical regions and is very closely related to *Cussonia,* even the Xhosa and Zulu common names

1. www.plantzafrica.com. Accessed on the 18 December 2006.
2. A. Hutchings, G. Lewis, A.H. Scott, A.B. Cunningham. Zulu medicinal plants. An inventory, University of Natal Press, 1996, pp. 221-222.

are the same. According to Palmer and Pitman[3], the specific epithet *umbellifera* refers to the umbellate arrangement of the flowers.

4.1.2 Distribution of *S. umbellifera*

This plant is widely distributed in Malawi, Mozambique and Zimbabwe as well as in South Africa (KwaZulu-Natal, Northern and Western Cape Province and Gauteng). The plants are growing in warm places and in mountain forests[1].



Source: www.plantzafrica.com

Figure 4.1: Pictures of *S. umbellifera*

4.2 Ethnopharmacology

The leaves of this plant have been used traditionally to treat rheumatism, colic and insanity and for malaria, a bark extract is drunk[4]. The Vhavenda people use the roots of this plant as a diuretic and laxative, for bathing, for weaning infants and for malaria, venereal diseases and nausea[2]. The bark is used for stomach ulcers and magical purposes[2]. In Tanzania, leaves are used for indigestion while roots are used for fevers and venereal disease, in emetics for nausea and in cold infusions

3. E. Palmer, N. Pitman, Trees of southern Africa, covering all known indigenous species in the Republic of South Africa, South-West Africa, Botswana, Lesotho & Swaziland. 1972, A.A. Balkema, Cape Town.

4. J.M. Watt, M.G. Breyer-Brandwijk, The medicinal and poisonous plants of southern and eastern Africa. 2nd Edition, E & S Livingston Ltd, Edinburgh and London, 1962, p. 117.

for skin irritation in new-born babies[2]. Rootbark decoctions are administered for mental illness[5].

4.3 Previous phytochemical studies

The secondary metabolites characteristic of the Araliaceae family are triterpene glycosides (saponins)[6] and polyacetylenes [e.g. falcarinol[7]]. Caffeic acid derivatives[8] have also been isolated. No phytochemical studies of *S. umbellifera* have previously been reported. The compounds isolated from Araliaceae species have a wide range of biological properties including antifungal, antimalarial, anti-inflammatory, anti-brain tumour, and antibacterial activity[9].

Two known flavonol glycosides, rutin and kaempferol rutinoside, as well as *ent*-kaur-16-en-19-oic acid has been isolated from *Cussonia vantsilana and C. racemosa*[10]. Triterpene glycosides have been isolated from *S. bodinieri* roots[11]. An oleanolic acid derivative, 2,3-dihydroxy-23-oxo-12-oleanen-28-oic acid has been isolated from *S. divaricata* and a lupenoic acid derivative has been isolated from *S. bodinieri*[12].

4.3 Isolation of active compounds.

5. S.C. Chhabra, F.C. Uiso, E.N. Mshiu, *J. Ethnopharm.*, 1984, **11**, 157-179.
6. J. Gunzinger, J.D. Msonthi, K. Hostettmann, *Phytochemistry*, 1986, **25**, 2501-2503.
7. L. Hansen, P.M. Boll, *Phytochemistry,* 1986, **25**, 529-530.
8. Y. Li, P.P.H. But, V.E.C. Ooi, *Antiviral Res.*, 2005, **68**, 1-9.
9. P. Tetyana, E.A. Prozesky, A.K. Jager, J.J.M. Meyer, J. van Staden, *S. Afr. J. Bot.*, 2002**, 68**, 51-54.
10. L. Harinantenaina, R. Kasai, K. Yamasaki, *Phytochemistry,* 2002, **61**, 367-372.
11. M. Zhu, S. Yang, J.D. Phillipson, P.M. Greengrassst, N.G. Bowery, *Phytochemistry,* 1996, **43**, 1313-1318.
12. J. Buckingham, Dictionary of Natural Products, CD-ROM. 1996. CRC Press.

In a comprehensive study on the antimalarial effect of South African plants selected on the basis of ethnopharmacological information[8], *S. umbellifera* was identified as one of the plants with promising antimalarial activity. The highest activity has been observed for the dichloromethane leaf extract of *S. umbellifera*.

Plant material was collected at Mariepskop, Mpumalanga, on the road towards the top above an electrical substation. This area was a forest with a well-drained, rocky soil and humus clay. A voucher specimen deposited at the South African National Botanical Institute (SANBI) was identified as *Schefflera umbellifera* (Sond.) Baill.

A methanol/dichloromethane (1:1) extract of the dried leaves of *S. umbellifera* was partitioned between hexane, dichloromethane and water (Fig. 4.2). A series of chromatographic separations of the dichloromethane fraction led to the isolation of three known pure compounds, namely *ent*-kaur-16-en-19-oic acid (**4.1**), 7-hydroxy-6-methoxycoumarin (**4.2**) and 3-hydroxy-20(29)-lupen-28-ol (**4.3**). The structures of these compounds were elucidated by physical, chemical and spectroscopic properties (1D and 2D NMR and confirmed by HRMS and GC/MS).

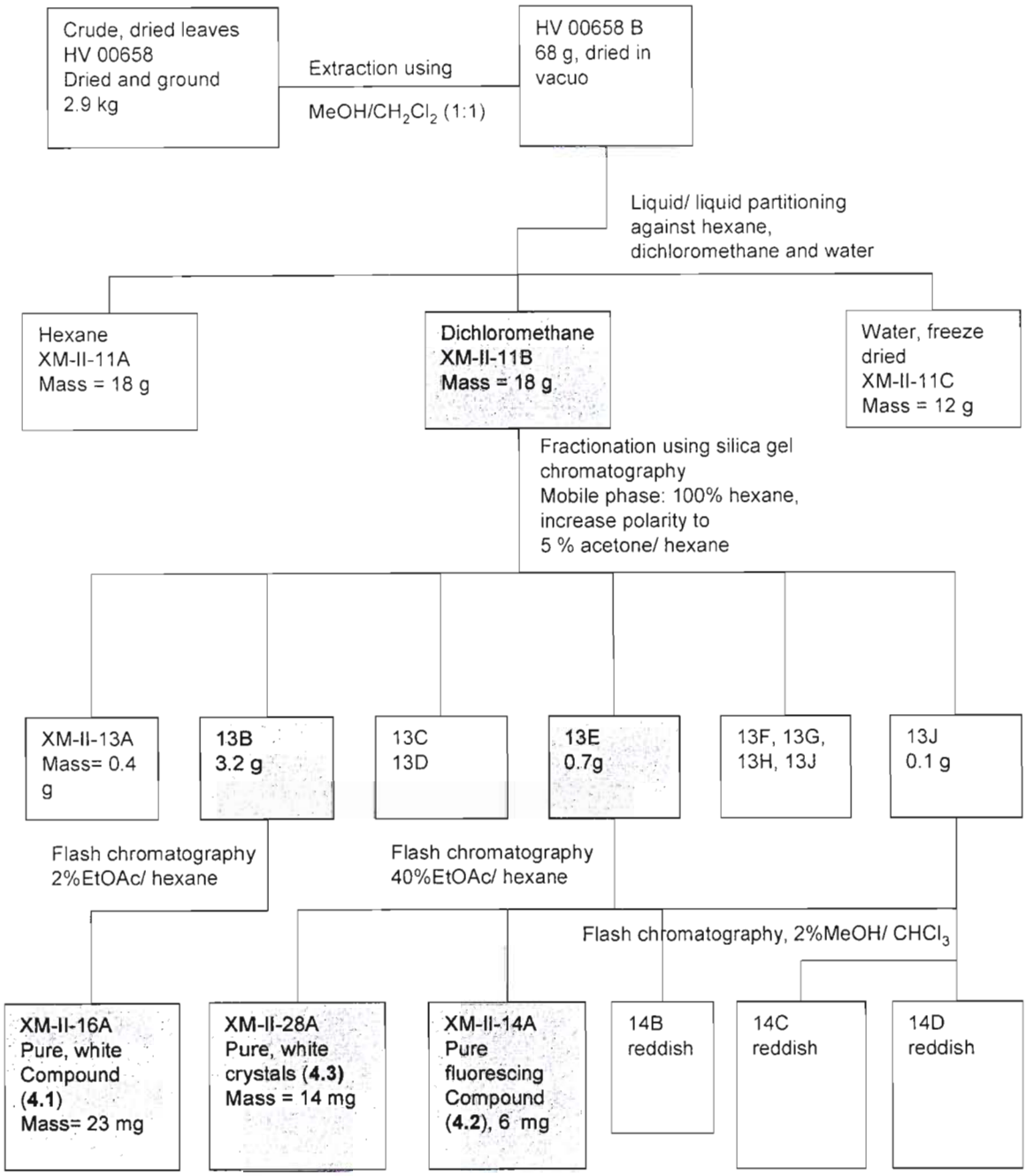
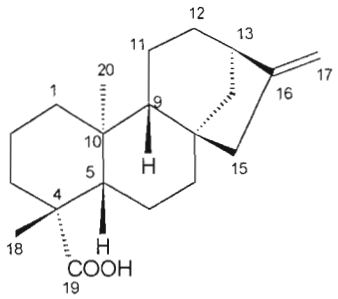


Figure 4.2: Flow diagram illustrating the fractionation of *S. umbellifera* extract

4.4 Results and discussions

4.4.1 Structure elucidation of compound 4.1



4.1

Compound **4.1** was obtained as a white crystalline solid from an acetone/hexane solvent mixture. It is not UV active but shows an intense pinkish colour after spraying with vanillin on the TLC plate. The structure of compound **4.1** was elucidated on the basis of its ^{13}C and ^{1}H NMR (400 MHz, $CDCl_3$) spectral data (Table 4.1). Inspection of ^{1}H NMR showed signals of the two methyl singlets at δ_H 1.23 and δ_H 0.93 which are typical of a kaurene-type skeleton (H-18 and H-20). A multiplet at δ_H 2.61 was assigned to H-13. Two distinct singlets signals at δ_H 4.77 and δ_H 4.71 are due to the two exocyclic methylene protons at C-17 (δ_c 103.2). No NOESY correlation was observed between the C-18 and C-20 methyl proton signals. A carboxyl group at δ_c 183.9 was observed from the ^{13}C NMR data and was assigned to C-19 (δ_H 11.2). ^{1}H-^{1}H COSY experiments showed correlations between the signals of H-11 and H-12 and H-13. Inspection of the HMBC spectrum inferred the presence of partial structures $-CH_2CH_2CH_2-$ (C-1 to C-3) and $-CHCH_2CH_2-$ (C-5 to C-7). HMBC cross peaks were observed between the signals of H-5 (δ_H 1.20, *m*) with C-1, C-3, C-6 and C-18 and this is typical of an *ent*-kaurenoid.

^{13}C NMR signals at δ_c 44.0 (s, C-4) and δ_c 55.3 (d, C-5) shifts were observed due a carboxyl function which was assigned to C-19. COSY correlations were observed between H-1 and H-3. The ^{13}C NMR spectrum showed two distinct signals at δ_c 103.2 and δ_c 156.0 which were assigned to C-17 and C-16

respectively. The stereochemistry at C-4 of this compound was deduced after NOESY spectra which showed no correlation between H-20 and H-18 hence the orientation of a carboxyl group was deduced to be axial. The DEPT spectrum exhibited 20 signals which were assigned as 3 CHs, 2 CH_3s and 10 CH_2s. The molecular formula of compound **4.1** was deduced as $C_{20}H_{30}O_2$, $[M]^+$ peak at *m/z* 302.4011 from HRMS data. GC/MS data also confirmed this molecular ion and showed fragment peaks at *m/z* 288 due to $[M\text{-}CH_2]^+$ and *m/z* 242 which corresponds to a loss of both a carboxyl and a methyl group. Compound **4.1** was identified as ***ent*-kaur-16-en-19-oic acid**, which is a well-known kaurane diterpenoid.

The spectroscopic data for compound **4.1** was in accordance with ^1H and ^{13}C NMR spectral data of other reported diterpenoids hence unequivocally proved the structure[13,14,15].

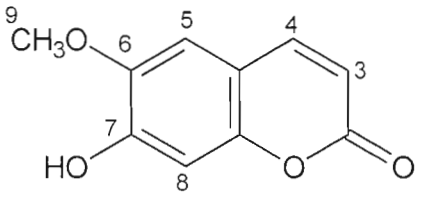
13. G.O. Lobitz, G. Tamayo-Castillo, I. Merfort, *Phytochemistry,* 1997, **46**, 161-164.
14. F.J.Q. Monte, E.M.G. Dantas, F.R. Braz, *Phytochemistry*, 1988, **27**, 3209.
15. A.G. Gonzalez, B.M Fraga, M.G. Hernandez, J.R. Hanson, *Phytochemistry,* 1973, **12**, 2721.

Table 4.1: ^{13}C NMR chemical shifts data of compound **4.1** and a reported *ent*-kaur-16-en-19-oic acid[16] (δ, ppm, $CDCl_3$, 400 MHz)

Carbon number	δ_H (*J* in Hz) Compound **4.1**	δ_C Compound **4.1**	Functional groups	δ_C *Ent*-kaur-16-en-19-oic acid
1	0.78 1.81,*td*, *J*=13.2, 4.3	40.9	$-CH_2$	41.1
2	1.49, *m*	19.3	$-CH_2$	19.8
3	0.99, *m* 2.00, *brd*, *J*=7.2	38.0	$-CH_2$	38.6
4		44.0	Quaternary C	43.8
5	1.20, *m*	55.3	-CH	55.2
6	1.84, *m*	22.0	$-CH_2$	22.5
7	1.50, *m*	41.5	$-CH_2$	41.5
8		44.4	Quaternary C	44.4
9	1.03, *m*	57.2	-CH	57.1
10		39.9	Quaternary C	39.9
11	1.50, *m*	18.6	$-CH_2$	18.6
12	1.52, *m*	33.3	$-CH_2$	33.3
13	2.61, *m*	43.9	-CH	44.2
14	1.23, *m*, 1.86, *m*	39.9	$-CH_2$	39.9
15	1.69, *m*	49.2	$-CH_2$	49.2
16		156.0	Quaternary C	155.7
17	4.77, *brs* 4.71, *brs*	103.2	$=CH_2$	103.5
18	0.93, *s*	29.5	$-CH_3$	29.3
19	11.2	183.9	-COOH	179.9
20	1.23, *s*	15.8	$-CH_3$	16.0

16. L. Rakotondraibe R. Harinantenaina, R. Kasai, K.Yamasaki, *Chem. Pharm. Bull.*, 2002, **50**, 268-271.

4.4.2 Structure elucidation of compound 4.2



4.2

Compound **4.2** is a white crystalline compound isolated using flash column chromatography and crystallized from ethyl acetate and hexane. Compound **4.2** exhibited a blue fluorescing colour under ultraviolet light at a wavelength of 366 nm. The structure of this compound was elucidated using ^{1}H, ^{13}C, HSQC and HMBC NMR data (Table 4.2). The electrospray mass spectrometer operating in a positive mode (ESI^{+}) was also used to determine the molecular formula of this compound.

The DEPT spectra showed the presence of 1 CH_3, 4 CH's and no CH_2 signals. The ^{1}H NMR data revealed a pair of doublets at δ_H 7.60 (J = 9.6 Hz) and δ_H 6.23 (J = 9.6 Hz); this is characteristic of cis-olefinic protons, namely C-3 (δ_c 113.6) and C-4 (δ_c 143.4). The two singlet protons appeared at δ_H 6.90 and δ_H 6.80 (C-5 δ_c 107.7 and C-8 δ_c 103.4). HMBC NMR spectra confirmed the following long-range correlations i.e. C-4a→H-3, C-2→H-4, C-7→H-5, C-6→H-8, C-5→H-4. A broad signal appeared at δ_H 8.81 due to a hydroxyl group on carbon-7 (δ_c 149.9). A methoxy singlet at δ_H 3.81 (3H) was observed and assigned to C-9 (δ_c 56.6). A ^{13}C signal at δ_c 161.5 denotes presence of a carbonyl group attached to an oxygen (O-C=O) in a ring which is indicative of coumarins.

The electrospray mass spectrometry data (operating on ESI^{+} mode with a NIST library match) showed a molecular ion peak at $[M]^{+}$ at *m/z* 192 and a very intense fragment peak at *m/z* 161 $[M\text{-}OCH_3]^{+}$. The mass spectrum also showed a fragment peak at *m/z* 121 due to $[M\text{-}C_3H_3O_2]^{+}$. This compound was identified as **7-hydroxy-6-methoxycoumarin,** also known as **scopoletin** with a molecular formula of

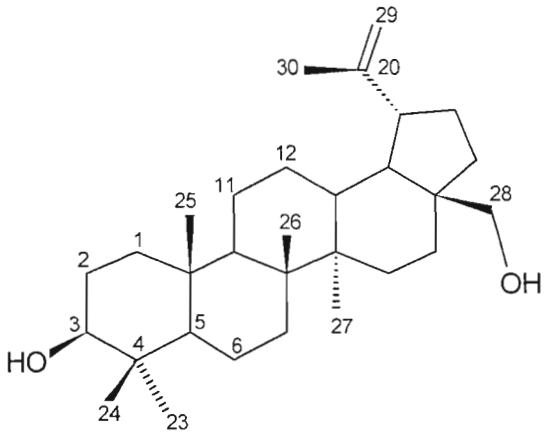
$C_{10}H_8O_4$. ^{13}C spectral data from published data for scopoletin also helped to confirm the structure of compound **4.2** (Table 4.2).

Table 4.2: ^{1}H, ^{13}C, HMBC data for compound **4.2** and ^{13}C of scopoletin[17] (δ in ppm, $CDCl_3$, 400 MHz)

Carbon number	δ_H (*J* in Hz) Compound **4.2**	δ_C Compound **4.2**	Functional groups Compound **4.2**	HMBC Compound **4.2**	δ_C scopoletin
2		161.5	O-C=O	H-4	160.0
3	6.23, *d*, *J*= 9.6	113.6	-CH		111.8
4	7.60, *d*, *J*= 9.6	143.4	-CH	H-5	143.2
4a		111.7	-C	H-3	110.6
5	6.90, *s*	107.7	-CH	H-4	108.5
6		150.5	-C	H-8	150.4
7	8.81, brs	149.9	-C-OH	H-5, H-9	149.0
8	6.80, *s*	103.4	-CH		102.3
8a		144.2	-C		144.0
9	3.81, s, (3H)	56.6	$-OCH_3$	H-5	55.3

17. L. Sun, W. Fu, J. Ren, L. Xu, K. Bi, M. Wang. *Arch. Pharm. Res.*, 2006, **29**, 135-139.

4.4.3 Structure elucidation of compound 4.3



4.3

Compound **4.3** was obtained as a white crystalline solid using flash chromatography and was crystallized from an ethyl acetate-hexane mixture. The structural elucidation of compound **4.3** was based on NMR spectroscopic methods and confirmed by mass spectral data. This compound showed a very intense pinkish colour upon spraying the TLC plate with vanillin and that is typical of triterpenoids. The ^{1}H and ^{13}C NMR spectral data (Table 4.3) of compound **4.3** displayed features characteristic of a lupane type of triterpenoid. The ^{13}C NMR spectral data showed thirty carbon peak resonances. The ^{1}H NMR data revealed six tertiary methyl signals (δ_H 0.94, 0.78, 0.80, 0.99, 0.98, 1.68) and in the HSQC spectrum, these signals correlated to δ_c 28.1 (C-23), 15.5 (C-24), 16.2 (C-25), 16.1 (C-26), 14.9 (C-27), 19.3 (C-30), respectively. The ^{1}H NMR signal at δ_H 2.31 is a multiplet which was assigned to proton at C-19.

The ^{1}H NMR spectrum showed two broad hydroxymethylene signals at δ_H 3.28 and 3.30, which correlated (HMBC) with a carbon signal at δ_c 60.7 and a hydroxylated methine at δ_H 3.14 (*s*) was correlated with a ^{13}C signal at δ_c 79.1. The ^{13}C NMR showed two carbon resonating at δ_c 109.8 and δ_c 150.6, corresponding to carbon-29 and 20, respectively. Proton signals at δ_H 4.63 and δ_H 4.64 are olefinic and were assigned to protons on carbon-29 (δ_c109.8). Mass spectral data obtained using positive ESI-MS displayed [M^+] at 442. The mass spectra also showed very intense fragments at *m/z* 411 due to a [M-CH_2OH], *m/z*

426 due to $[M-OH]^+$ and fragment peaks at *m/z* 235, 202, 193 are characteristic of the fragmentation pattern of a lupane-type of compound with an angular hydroxyl methylene group. Compound **4.3** was identified as **betulin [lup-20(29)-en-3β,28-diol]** with a molecular formula of $C_{30}H_{50}O_2$. ^1H, ^{13}C NMR data of compound **4.3** compares very well with authentic betulin[18,19].

18. S.B. Mahato, A.P. Kunda, *Phytochemistry,* 1994, **37**, 1517-1575.
19. K.S. El Deeb, R.A. Al-Haidari, J.S. Mossa, A-M. Ateya, *Saudi Pharm. J.*, 2003, **11**, 184-191.

Table 4.3: ^{1}H, ^{13}C NMR chemical shifts data of compound **4.3** and a reported ^{1}H and ^{13}C of betulin (δ, ppm, $CDCl_3$, Varian 400 MHz)

Carbon number	δ_H, 4.3	δ_C, 4.3	δ_H, betulin	δ_C, betulin
1	1.64, 0.84,*m*	38.9	1.67, 0.88,*m*	38.9
2	2.12,*m*	27.2	1.99,*m*	27.1
3	3.14,*tt*	79.1	3.13,*tt*	78.9
4		38.9		38.9
5	0.72,*m*	55.5	0.69,*m*	54.6
6	1.40, 1.35 *dd*, *J*=5.1, 12.1	18.5	1.48,1.32,*dd*	19.6
7	1.35,*m*	34.4	1.38,*m*	38.9
8		40.9		40.3
9	1.23,*t*	50.5	1.29,*t*	52.0
10		37.3		38.2
11	1.41,1.18,*dd*,	21.0	1.40,1.19,*dd*	19.6
12	1.63,1.07,*dd*	25.5	1.62,1.07,*dd*	26.9
13	1.63	37.3	1.62	36.6
14		42.7		42.5
15	1.70,1.08,*dd*, *J*= 4.8, 9.5	27.2	1.71,1.09,*dd*	27.0
16	2.0,1.23,*tt*	29.3	1.91,1.24,*tt*	29.6
17		47.8		48.1
18	1.63,*m*	48.9	1.62,*m*	47.7
19	2.31,*m*	47.8	2.30,*m*	47.6
20		150.6		157.4
21	1.96,1.41,*m*	29.9	1.95,1.40,*m*	29.8
22	1.86,1.11,*m*	34.1	1.86,1.12,*m*	34.0
23	0.94,*s*	28.1	0.96,*s*	28.7
24	0.78,*s*	15.5	0.76,*s*	15.5
25	0.80,*s*	16.2	0.81,*s*	17.0
26	0.99,*s*	16.1	1.07,*s*	17.0
27	0.98,*s*	14.9	0.98,*s*	14.8
28	*3.28, 3.30, brd,*	60.7	3.25,3.27,*dd*	60.3
29	4.63, 4.64 *dd*, J=1.1, 1.1	109.8	5.53,5.56,*dd*	109.6
30	1.68,*s*	19.3	1.68,*s*	19.1

4.5 Biological activity of isolated compounds

4.5.1 In vitro antimalarial activity

Traditionally, *S. umbellifera* has been utilized to treat ailments such as malaria, rheumatism, colic and insanity[2]. Crude ethanolic and ethyl acetate extracts of the bark and the leaves of *S. umbellifera* have been reported to inhibit *Plasmodium falciparum* significantly compared to other tested *Cussonia* species such as *C. spicata* and *C. paniculata*[4]. However, limited information is known about the antimalarial properties of *S. umbellifera*.

For this study, limited plant material led to the targeting of the three major compounds isolated from the active dichloromethane extract. The dichloromethane extract of *S. umbellifera* and the three isolated compounds, *ent*-kaur-16-en-19-oic acid (**4.1**), scopoletin (**4.2**) and betulin (**4.3**), were systematically evaluated *in vitro* against both the chloroquine-susceptible (D10) and chloroquine-resistant (K1) strains of *Plasmodium falciparum* for antimalarial activity. This *in vitro* evaluation was carried out at the Department of Pharmacology, University of Cape Town, following the Trager method (parasite lactate dehydrogenase) described in Chapter 3 (§3.5).

The dichloromethane extract exhibited an *in vitro* activity of 3.7 µg/mL (D10) and 5.5 µg/mL (K1), respectively, and chloroquine, the standard reference drug, gave 27.2 ng/mL (D10) and 34.4 ng/mL (K1) and this activity was classified as good relative to chloroquine activity. Any activity less that 1 µg/mL was regarded as potent while the activity less than 5 µg/mL was regarded as good and between 5 µg/mL and 10 µg/mL as moderate.

The three isolated compounds demonstrated the following antiplasmodial activity: compound **4.1** did not give any significant activity, IC_{50} 32.2 µg/mL (D10); compound **4.2** exhibited weak activity, IC_{50} 28.2 µg/mL (D10), while compound **4.3** exhibited significant activity (IC_{50} 3.2 µg/mL) against D10. Compound **4.3** was found to possess improved antimalarial activity compared to the crude extract and was identified as an active ingredient. This compound was previously isolated from

several other families such as Rhamnaceae *(Zizyphus vulgaris*) and Labiatae *(Zataria multiflora*) and showed moderate inhibition of different *P. falciparum* strains (IC_{50} <12 µg/mL and <27 µM)[20]. It was also isolated from a Tanzanian plant, *Uapaca nitida* and reported to be inactive at 500 µg/mL[21,22].

Table 4.4 Antiplasmodial activities of the crude *S. umbellifera*, *ent*-kaur-16-en-19-oic acid (**4.1**), scopoletin (**4.2**) and betulin (**4.3**)

Name	D10 IC_{50} (µg/mL)
Crude CH_2Cl_2	3.7
Compound **4.1**	32.2
Compound **4.2**	28.2
[redacted]	
Chloroquine	27.2 ng/mL

4.5.2 Other reported biological activities

Compound **4.1** (*ent*-kaur-16-en-19-oic acid) has been reported to be a cytotoxic agent[21] and inhibits growth in human cancer cells *in vitro*[22]. This compound and its glycosides were isolated from the leaves of *Cussonia racemosa* and *C. vantsilana* in Madagascar. In Japan, *Mikania hirsutissima* was a source of this compound and it showed weak cytotoxicity against leukaemia cells[23].

Compound **4.1** isolated from *Espeliti killipii*[24] *was* evaluated for antigiberellic activity where it gave an activity of 1:100 when compared to giberellic acid (GA-3) and antimicrobial activity against *B. subtilis* (16 mg/mL*) and B. aureus* (23 mg/mL) was shown by compound **4.1** isolated from *Espeletiopsis muisha* (Cuatr)[25].

20. H.L. Ziegler, H. Franzyk, M. Sairafianpour, M. Tabatabai, M.D.Tahrani, K.Bagherzadeh, H. Hagerstrand, D. Staerk, J. W. Jaroszewski., *Bioorg. Med. Chem.,* 2004, **12,** 119-127.
21. R.B. Badisa, S.K. Chaudhuri, E. Pilarihou, E.H. Walker, *Nat. Prod. Lett.*, 2002, **16**, 39-45.
22. G.E. Henry, L.S. Adams, J.C. Rosales, H. Jacobs, D. Heber, N.P. Seeram., *Cancer Lett.*, 2006**, 244,** 190-194.
23. E. Ohkoshi, M. Makino, Y. Fujimoto, *Chem. Pharm. Bull,* 1999, **47**, 1436-1438.
24. R.D. Torrenegra, A.N. Tellez, *Biochem. Systemat. Ecol.*, 1995, **23**, 449-450.
25. M.B. Martha, A.N. Tellez, R.D. Ruben, *Rev. Latinoamer. Quim.*, 2000, **28**, 119-124.

According to literature studies conducted, the antiplasmodial activity of *ent*-kaur-16-en-19-oic acid (**4.1**) has not been reported previously.

Compound **4.2** (scopoletin) is a well-known phytoalexin and is widely distributed in higher plants. A wide range of biological activity including anti-inflammatory, nitric oxide, antioxidant, bacterial, hepatoprotective, antiproliferative, cytotoxic, and hypocholesterolemic activity have been reported for this compound. Scopoletin was isolated from a sweet potato peel and showed antifungal properties[26]. The isolation of scopoletin from *S. umbellifera* has not been reported.

Compound **4.3** (betulin) has been shown to inhibit *P. falciparum* strains and an IC_{50} of 12 µg/mL was reported[14]. However, it has also been reported to be inactive against *P. falciparum* strains at 500 µg/mL[27,28]. Betulin has been isolated from the birch tree and exhibited a range of biological activities such as anticancer, antiviral, anti-inflammatory and antimalarial activity with low cytotoxicity in normal mammalian cells. In Russia, *Betula pendula* is a source of betulin and it showed good antiviral properties[29]. Although isolation and biological activity of betulin is well published, no information is known regarding its isolation from *S. umbellifera*.

4.6 *In vitro* anticancer activity

The anticancer properties of the isolated compounds were evaluated using the CSIR in-house cell lines namely renal (TK10), melanoma (UACC62) and breast (MCF7), following a method employed by the National Cancer Institute. This study was done for comparison with published information on the anticancer activities of these compounds, especially betulin (**4.3**). Scopoletin (**4.2**) was found to be inactive against all three cell lines with a total growth inhibition (TGI) of >100 ppm, while betulin inhibited the growth of all three cell lines with a TGI of 15.8 ppm. Betulin has previously been reported to exhibit strong cytotoxicity towards cancer

26. J. Peterson, H. Harrison, H.D. Jackson, *Hortscience,* 2002, **38**, 1129-1133.
27. J.C.P. Steele, D.C. Warhurst, G.C. Kirby, M.S.J. Simmonds, *Phytother. Res.,* 1999, **2**, 115-119.
28. A. Sami, M. Taru, K. Salme, Y. Jari., *Eur. J. Pharm. Sci.,* 2006, **29,** 1-13.
29. N.I. Pavlova, O.V. Savinova, S.N. Nikolaeva, E.I. Boreko, O.B. Flekhter, *Fitoter.,* 2003, **74**, 489-492.

cell lines (human lung carcinoma- A-529) with an IC_{50} 3.80 -13.8μM[30], but inactive against cancer cell lines such as melanoma (MEL-2), epidermiod carcinoma, leukaemia (HL-60, U937, K 562) and neuroblastoma (GOTO and NB-1) and also non-toxic up to 500 mg/kg body weight in mice[22]. It was previously found to possess antitumor activity against carcinoma[31]. Our experiments confirmed the published anticancer activity of betulin (**4.3**). Compound **4.1** showed no anticancer activity in all three cell lines.

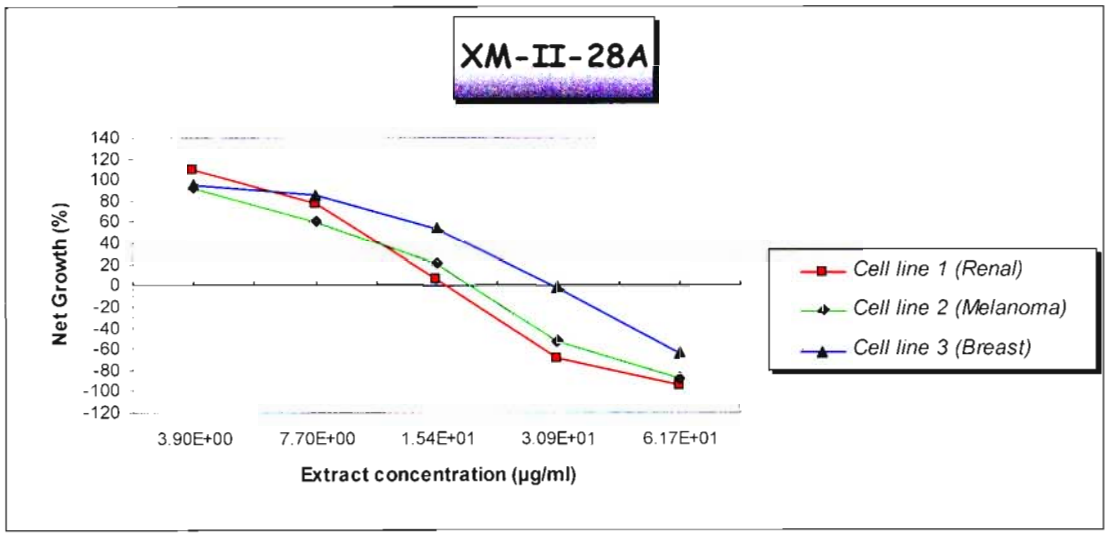


Figure 4.3: Anticancer activity of betulin (**4.3**)

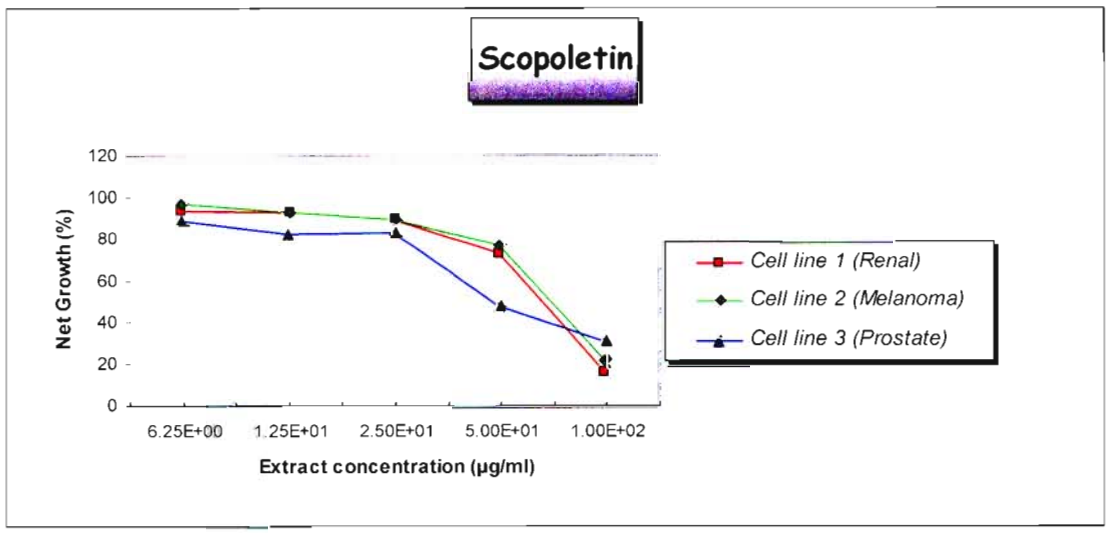


Figure 4.4: Anticancer activity of scopoletin (**4.2**)

30. C. Gauthier, J. Legault, M. Lebrun, P. Dufour, A. Pichette, *Bioorg. Med. Chem.*, 2006, **14**, 6713-6725.
31. J.A. Duke, *Amer. Bot. Counc.*, 1996, 96.

4.7 A comparative HPLC study of *S. umbellifera* extracts

Four *S. umbellifera* plants were randomly collected from different geographical sites of South Africa, namely Landgoed Farm-Georges Valley (Limpopo Province) Paradise (Limpopo, near Giyani), Northern side of Umtamvuna (KwaZulu-Natal) and South Coast-Trafalgar (KwaZulu-Natal). This was a comparative study to determine if there is any chemical variation between plants from different geographical areas or not. HPLC was chosen for this study to provide chemical fingerprints of the four extracts. The chromatograms would provide enough information on both minor and major compounds present in each species to make a conclusion. Methanol/dichloromethane crude extracts were prepared and dried and were assigned with different sample numbers.

Table 4.5 Collection sites and extract numbers

Collection site	Extract numbers
1. Landgoed Farm – Georges Valley (Limpopo Province)	P00245A (active against *P. falciparum)*
2. Paradise (Limpopo Province near Giyani)	P11505B
3. Northern side of Umtamvuna (KwaZulu-Natal)	P0966B
4. South Coast-Trafalgar (KwaZulu-Natal)	P19124B

4.8.1 Interpretation of chromatograms

The three isolated compounds **4.1, 4.2** and **4.3** were chromatographed as standard components for identification purposes in all four extracts. When all HPLC chromatographic conditions were optimized, compound **4.1** eluted at 35.6 minutes, compound **4.2** eluted at 11.9 minutes and compound **4.3** gave a retention time of 38.6 minutes.

Extract P00245A gave a distinct peak with a retention time of 39.1 minutes that corresponded to betulin with an 88.1% probability, denoted that this compound

could be present in this extract. The major peak at 35.5 minutes gave a library match with kaurenoic acid (*ent*-kaur-16-en-19-oic acid) with 62.7% probability and this affirms the possibility of this compound to be present in major quantities in the crude while a peak at 33.4 minutes corresponded to oleanolic acid with an 88.3% probability. A minor peak at 11.8 minutes was identified to be scopoletin(**4.2**).

P11505B extract gave major peaks at 33.1 minutes, 35.6 minutes, 38.3 minutes and 11.9 minutes which gave good library matches with oleanolic acid, kaurenoic acid (**4.1**), betulin (**4.3)**, and scopoletin (**4.2**) respectively.

P09665B showed major peaks at 33.9 minutes, 35.5 minutes, 38.4 minutes and 11.5 minutes which gave excellent library matches with oleanolic acid, kaurenoic acid (**4.1**), betulin (**4.3**) and scopoletin (**4.2**) respectively. Scopoletin was detected as a very minor component in all the spectra and in some cases, it was only identified using mass spectrometric data.

P19124B showed dominant peaks at 33.4 minutes, 35.5 minutes, 38.5 minutes and 12.0 minutes which gave good library matches with oleanolic acid, kaurenoic acid (**4.1**), betulin.(**4.3**) and scopoletin (**4.2**).

In the HPLC traces of all four crude extracts, the major peaks correspond to the compounds isolated from the active crude extract against *P. falciparum*. The major similarities of the chromatographed extracts were quite discernible even in the overlaid spectra (maxplot 200-600 nm). Scopoletin eluted as a minor peak at 12.0 minutes with a noticeable unique UV pattern with absorption maxima at 229, 297 and 344 nm which gave a good mass spectra with $[M]^+$ *m/z* 192. The two terpenoids did not show any UV absorption although their mass spectra showed intense molecular ion peaks $[M]^+$ m/z 442 (betulin) and $[M]^+$ *m/z* 302 (kaurenoic acid).

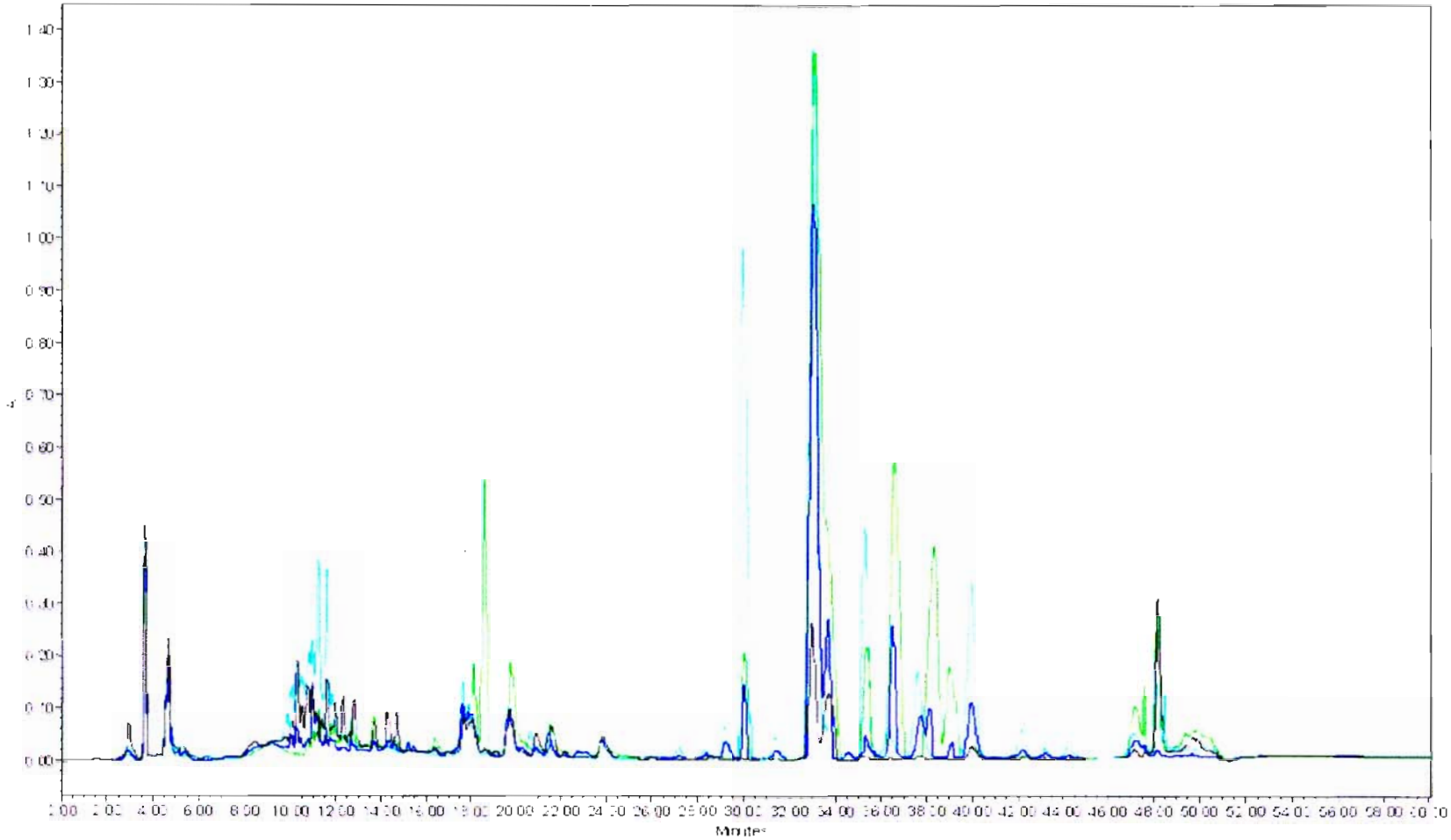


Figure 4.5: UV MAXPLOT (200-600 nm) of all four crude *S. umbellifera* extracts

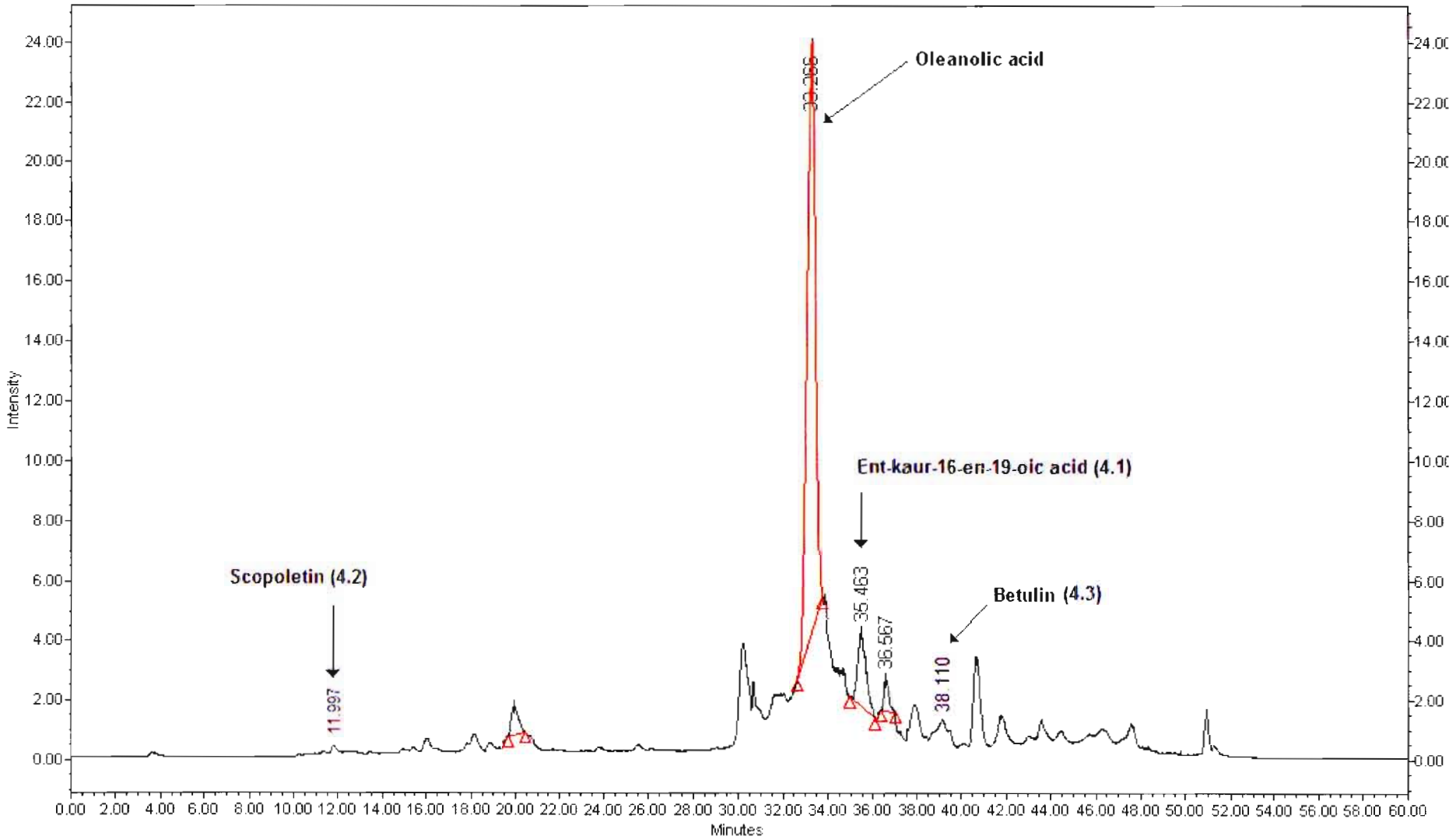


Figure 4.6: MS TIC with identified compounds from POO245A

4.8.2 Conclusion

The crude dichloromethane extract of the leaves of *S. umbellifera* showed good antimalarial activity. Column chromatography was used to fractionate the active crude extract and afforded three major compounds, *ent*-kaur-16-en-19-oic acid (**4.1**), scopoletin (**4.2**) and betulin (**4.3**). These compounds were tested against the *P. falciparum* strains and compound **4.3** (betulin) showed significant antiplasmodial activity against a susceptible strain (D10) and was regarded as the active ingredient. This compound could in future be tested against the resistant strain (K1) of *P. falciparum*. Betulin showed good anticancer properties against all three CSIR in-house cancer cell lines. Betulin derivatives such as betulinic acid have been reported to possess significant anticancer properties by inducing apoptosis in cells[32].

HPLC of four extracts of *S. umbellifera* collected in different geographical regions revealed that there is no significant difference between them. All three isolated compounds are present in all four extracts as labelled in Fig. 4.6 above.

4.9 Experimental

4.9.1 General

Materials and methods described in Chapter 3 were followed for all the experiments.

4.9.2 Plant material

6 Kg wet leaves of *S. umbellifera* were collected in April 2004, at Landgoed farm in Georges Valley, Limpopo Province. The plant was identified at the South African National Botanical Institute, Pretoria and a voucher specimen deposited at SANBI.

32. A. Sami, M. Taru, K. Salme, Y. Jari, *Eur. J. Pharm. Sci.*, 2006, **29**, 1-13.

4.9.3 Extraction and isolation

The 6 kg wet leaves were dried in an oven at 60 °C for overnight. 2.90 kg was recovered as dry matter and finely ground. 2.2 Kg of the dried, ground leaves were extracted using methanol/dichloromethane (1:1) for 12 hours, filtered and reduced to dryness using a rotovaporator. The methanol/dichloromethane extract was then partitioned between hexane, dichloromethane and water. The dichloromethane layer was dried and column chromatographed using silica gel eluted by 5% acetone/hexane followed by 100% acetone. This yielded 18 fractions (XM-II-13A-R). Some of these fractions were later combined on the basis of their TLC profiles. Fraction 13B (3.2 g) was further fractionated using flash silica with 2% EtOAc/hexane mobile phase and afforded a white pure compound (XM-II- 16A) (23 mg, 0.03 % yield). Fraction 13E (0.66 g) was further fractionated on flash silica gel using 40% EtOAc/hexane mobile phase and yielded a pure white crystalline compound (XM-II-28A) (16 mg, 0.02 % yield). Fraction 13J (0.1 g) was further purified on flash silica using 5% acetone/dichloromethane mobile phase and yielded a pure bright blue fluorescing compound (6 mg, 0.008 % yield).

HPLC starting eluent consisted of water (containing 10 mM trifluoroacetic acid) and acetonitrile (90:10) at a flow rate of 0.2 mL/min. All the chromatograms were recorded using the same experimental conditions. The gradient table is summarised in Table 6 below. The Waters Thermabeam system was operated in a positive scan mode (50-650 a.m.u.) with a gain of 10 while the nebulizer temperature was set at 70 °C. The isolated compounds, **4.1**, **4.2** and **4.3**, were used as references for the chromatographic study.

Table 4.6 Gradient conditions for Waters 2695.

Time	Flow	% water(C)	% acetonitrile (D)	Curve
0.0	0.20	90	10	6
1.0	0.20	90	10	6
10.0	0.20	30	70	4
40.0	0.20	10	90	6
41.0	0.25	0	100	6
45.0	0.25	90	10	2
50.0	0.25	90	10	3
58.0	0.20	90	10	6
59.0	0.20	90	10	6

The UV PDA detector was set to monitor peaks in the 200-600 nm range. Major peaks with their corresponding retention times were recorded and matched with the available GC/MS NIST library.

4.9.4 Physical data

4.9.4.1 COMPOUND 4.1

Systematic name: *ent*-Kaur-16-en-19-oic acid

Alternative name: Kaurenoic acid

Yield: 23 mg

Physical description: White crystals

Mass spectrum: HRESMS calcd for $C_{20}H_{30}O_2$ *m/z* 302.2246, found 302.4011, fragment peaks at *m/z* 288 $[M\text{-}CH_2]^+$ *m/z* 242 $[M\text{-}COOH\text{-}CH_3]^+$ $C_{20}H_{30}O_2$

Optical rotation: $[\alpha]^{20}_D$ -110° (c=3 in $CHCl_3$), Lit[15] $[\alpha]^{20}_D$ -110° (c=3 in $CHCl_3$),

Melting point 177-179 °C, Lit[33] 156-158 °C

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

33. R.D. Torrenegra, A. Nohem, A. Tellez, *Rev. Latinoamer. Qium.*, 1996, **24**, 1-6.

4.9.4.2 COMPOUND 4.2

Systematic name:	7-Hydroxy-6-methoxy-2*H*-1-benzopyran-2-one
Alternative name:	Scopoletin
Yield:	6 mg
Physical description:	Bright-blue fluorescing under UV, white powder under normal light
UV maxima	230, 255, 300, 345 nm
Melting point	199-201 °C, Lit[34] 181-183 °C
Mass spectrum:	EI/MS *m/z* 192, m/z 161$[M-OCH_3]^+$
^{1}H NMR:	See Table 4.2 ($CDCl_3$)
^{13}C NMR:	See Table 4.2 ($CDCl_3$)

4.9.4.3 COMPOUND 4.3

Systematic name:	Lup-20(29)-ene-3β,28-diol
Alternative name:	Betulin
Yield:	14 mg
Physical description:	White crystals (ethyl acetate)
Mass spectrum:	HRESMS calcd for $C_{30}H_{50}O_2$ *m/z* 442.3811, found: 442.3804 lupane fragments at *m/z* 411, 427, 235, 202, 193 $C_{30}H_{50}O_2$
Optical rotation:	$[\alpha]_D^{20}+20^0$ (c=3, $CHCl_3$), Lit[35] $[\alpha]_D^{20}+18^0$ (c=0.5, $CHCl_3$)
Melting point:	219-224 °C, Lit[15] 251-252 °C
^{1}H NMR:	See Table 4.3 ($CDCl_3$)
^{13}C NMR:	See Table 4.3 ($CDCl_3$)

34. H-M. Liu, X-Z. Feng, *Phytochemistry*,1995, **33**, 707-710.
35 L. Hui. *J. Chem. Soc., Perkin Trans.*, 1977, ***1***, 897-901.

Chapter 5

Bioassay-guided fractionation and isolation of components of *Elephantorrhiza elephantina* (Burch.) Skeels

5.1 Introduction

The CSIR's Bioprospecting Research Platform is in the process of scientifically validating traditional medicine and these developed products could be marketed as either herbals or pharmaceuticals to help combat prevalent diseases. The CSIR received anecdotal information through its collaboration with traditional healers on the use of a plant to help relieve urinary pains, reduce the enlargement of the prostate and incontinence. This type of condition is called benign prostatic hyperplasia. On receiving this plant, an urologist based in Pretoria evaluated the crude spray-dried extract for benign prostatic hyperplasia and a verbal positive result was reported to the CSIR.

According to a farmer from Limpopo Province, this plant is traditionally used to treat diarrhoea, dysentery, stomach disorders, sexually transmitted diseases, haemorrhoids and perforated peptic ulcers. It is also used for cleaning the womb after abortion. No published reports on the use of this plant as a treatment for benign prostatic hyperplasia were found. Traditionally, the rhizomes of this plant are prepared and drunk as a tea. A number of sites in different South African provinces were identified for the collection of this plant. The CSIR's Bioprospecting Research Platform thus decided to attempt to validate the medicinal properties of this plant. This was done by first identifying the correct plant species responsible for the reported activity and this was done in consultation with SANBI. Literature searches were then conducted on the identified plant species, suitable extracts

prepared and relevant *in vitro* and *in vivo* biological assays identified to validate this claim.

A bioassay-guided fractionation was conducted to isolate and identify the biomarkers and active ingredient(s). Seven compounds were identified and investigated for *in vitro* inhibition of the steroid 5α-reductase enzyme. Limited biological published information of these compounds led to their testing *in vitro* against other available biological targets. These compounds were therefore evaluated *in vitro* for different therapeutic applications such as anticancer, antimalarial, antioxidant and cytotoxicity.

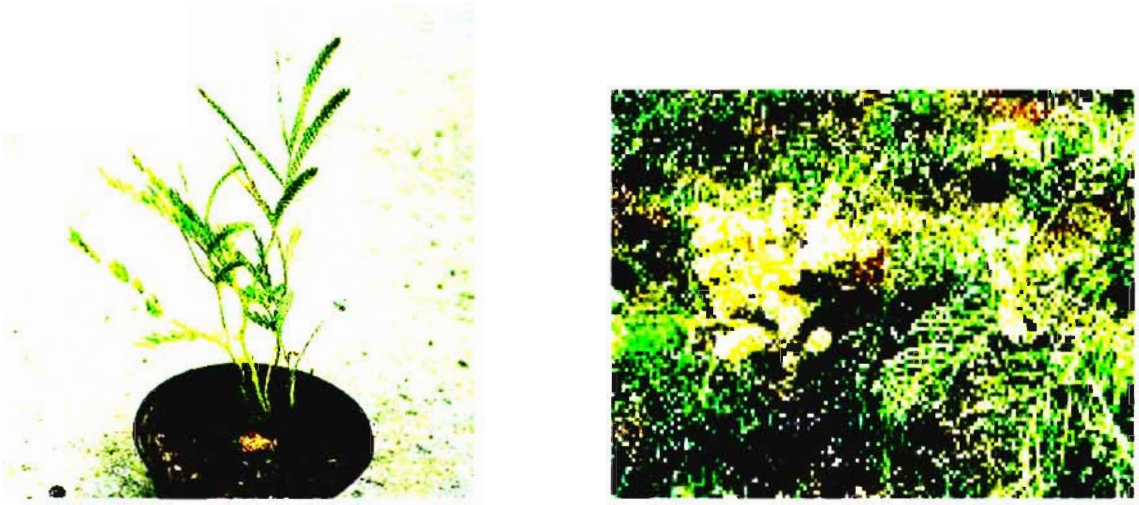
5.2 Botany

Plant specimens collected by the CSIR (with the help of a botanist) were sent to the South African National Botanical Institute for identification. The plant was identified as *Elephantorrhiza elephantina* (Burch.) Skeels which belongs to the Fabaceae family. Its vernacular names are eland's bean, elandswortel, elandsboontjie and intolwane[1]. Based on the assessment of a botanist, this plant is believed to be abundantly available in certain sites and this assessment was further supported by strong evidence published by scientists from the Department of Botany, University of Witwatersrand, Johannesburg[2]. This plant is widely distributed in southern African countries such as Mozambique, Lesotho, South Africa, Swaziland, Zimbabwe, Botswana and Namibia[1, 3].

E. elephantina is one meter in height, growing from an enormous underground rhizome as a native weed. It is very invasive and a non-climbing, perennial shrub, which is not a threatened species. The branched root system often forms extensive colonies of visible plants. Seeds can be used to propagate this plant species[4].

---

1. J.M. Watt, M.G. Breyer-Brandwijk, The medicinal and poisonous plants of Southern Africa, 2nd Edition, Edinburgh, 1962, 596-597.
2. W.N. Ellery, B.H. Walker, *S. Afr. J. Bot.* 1986, **52**,100-104.
3. I. Hedberg, F. Staugard , Traditional medicine in Botswana. Traditional medicinal plants. Ipelegeng publishers, Gabarone, p. 119-120.
4. H.P. Van der Schijff, L. Snyman, *Int. J. Plant. Sci.,* 1970, **76,** 114-128.



Source: www.bihrmann.com., www.fao.org / www.ecoport.org

Figure 5.1: *E. elephantina*

5.3 Ethnopharmacology

This plant is reported to be used for earaches, for cleaning of wombs after abortion, for blood diarrhoea in children, dysentery and sexual transmitted diseases[5]. In Botswana, roots are used to clean wombs after an abortion and in Zimbabwe, roots are used for abdominal pain, infertility in women and as aphrodisiacs[6]. This plant has also been proven scientifically to possess antimicrobial and antibacterial activity at the University of Botswana[3].

5.4 Reported phytochemical studies

The following compounds have previously been isolated from the dried rhizome extracts of *E. elephantina*: dihydrokaempferol, kaempferol, catechin, ethyl gallate, gallic acid, ethyl β-D-galactopyranoside and quercetin 3-*O*-β-D-glucopyranoside[5]. The *n*-butanol extract of *Elephantorrhiza goetzei* roots yielded two compounds, 3,3',4',5,6,7,8-heptahydroxyflavan and methyl gallate that were tested (with the

5. E. Aaku, M. Office, S.P. Dharani, R.R.T. Majinda, M.S. Motswaledi, *Fitoterapia*, 1998, **69**, 464-465.
6. A. Hutchins, A.H. Scott, G. Lewis, A.B. Cunningham, Zulu Medicinal Plants. An inventory. University of Natal Press, Pietermaritzburg, 1996, p. 126.

crude extract) for antifungal and antibacterial properties[7]. *E. elephantina* extracts showed good antioxidant activity in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay[8]. Literature reports reveal the presence of 5.8-22.3% tannins and 16.8% sugars in the extracts of the rhizomes[1].

5.5 Isolation and structural determination of the active compounds

The bulk rhizomes of *E. elephantina* were collected and identified by Ralph Peckover, crushed and oven dried at 60 °C. Both water and methanol/dichloromethane (1:1) extracts were prepared. Both extracts were tested *in vitro* at MDS Pharma in Taiwan in a steroid 5α-reductase enzyme assay at 100 µg/mL and exhibited very good activity (92% and 96% inhibition, respectively). The water extract was then spray-dried into a powder form, dissolved in water and partitioned between hexane, isobutanol and *n*-butanol. The solvents were removed from these fractions using a rotavaporator and a series of thin-layer chromatographic solvent systems were investigated for the chromatography of the isobutanol fraction, i.e. butanol:acetic acid:water (3:2:1), 5% MeOH/$CHCl_3$, chloroform:acetone:methanol (7:2:1) and toluene:methanol:acetone:pyridine (35:10:50:5). The last system gave a good TLC separation.

The complexity of these fractions and the crude extract is due to the reported tannins present in them and this led to the use of different chromatographic separating materials, namely polyvinylpyrrolidone (to trap the complex tannins), Sephadex G-25 and G-10, Sephadex LH-20, reversed-phase silica and normal silica gel.

---

7. F. Moyo, B.A. Gashe, R.R.T. Majinda, *Fitoter.*, 1999, **70**, 412-416.
8. V. Naidoo, MSc thesis, Screening of four plants commonly used in ethnoveterinary medicine for antimicrobial, antiprotozoal and antioxidant activity, University of Pretoria, 2004.

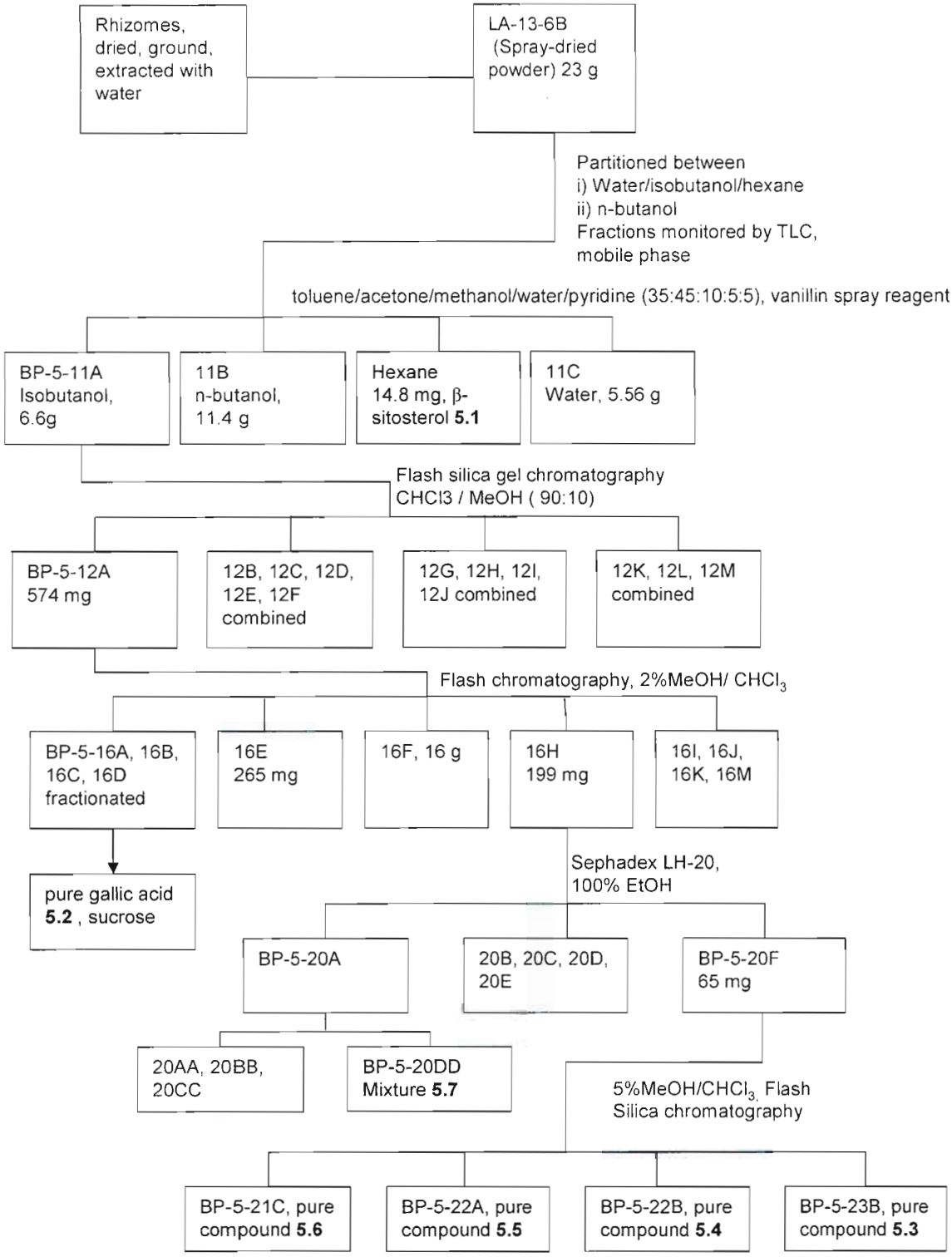
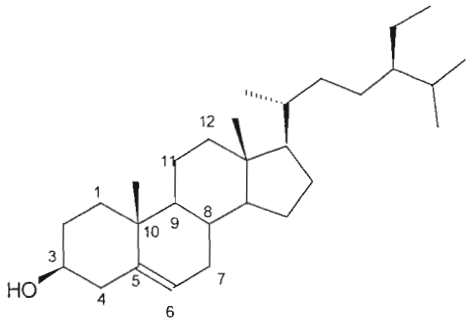


Figure 5.2: Flow diagram illustrating the fractionation of *E. elephantine* extract and isolation of the compounds (**5.1, 5.2, 5.3, 5.4, 5.5, 5.6** and **5.7)**

5.5.1 Structural elucidation of compound 5.1



5.1

Compound **5.1** was isolated as a white powder and crystallized from a mixture of hexane and chloroform. Silica gel chromatography (Sigma Aldrich, F_{254}) using a 10% EtOAc/hexane mobile phase was employed to purify this compound from the hexane fraction. TLC plates were used to monitor the separation process. This compound eluted as a mixture of two isomers and after several attempts a few milligrams of the pure compound was obtained.

The NMR data of **5.1** are collated in Table 5.1. ^{1}H NMR data revealed the following signals: δ_H 5.34 (1H, brs, H-6), 3.55-3.45 (1H, m, H-3), 2.26-1.08 (*m*), 0.99 (3H, s, H-19), 0.93 (3H, d, *J* = 6.4 Hz, H-21), 0.84 (3H, t, *J* = 7.2 Hz, H-29), 0.80 (3H, *d*, *J* = 6.6 Hz, H-27) and 0.68 (3H, *s*, H-18).

^{13}C NMR signals were assigned using the HSQC spectrum. Dominant signals include those at δ_c 140.7 (C-5), 121.4 (C-6), 71.8 (C-3), 18.9 (C-27), 19.4 (C-21), 23.1 (C-28) and 12.2 (C-18). GC/MS gave a molecular ion peak $[M]^+$ *m/z* 414, EI/MS also gave an intense $[M]^+$ peak at *m/z* 414, a fragment peak at *m/z* 396 which was due to the loss of a hydroxyl group on C-3 $[M\text{-}OH]^+$ and this is typical of sterols. Direct infusion using ESI/MS operating at ESI^- mode gave a $[M\text{-}H]^-$ peak at *m/z* 413. On TLC, a R_f of 0.75 (40% EtOAc/Hexane) was observed. After careful inspection of the spectral data of compound **5.1** and comparison with literature data, this compound was identified as **3β-stigmast-5-en-3-ol (β-sitosterol)**. This compound is well published[9,10].

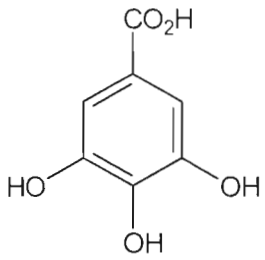
9. J.B. Stothers, Organic Chem., 1972, **24**, 75a.

Table 5.1: ^{13}C and ^{1}H NMR data (400 MHz for ^{1}H) in $CDCl_3$ of compound **5.1** and β-sitosterol[9, 10]

Carbon Number	δ_C Compound **5.1**	δ_H (*J* in Hz) Compound **5,1**	Lit: δ_C β-sitosterol[9,10]	Lit: δ_H (*J* in Hz) β-sitosterol[9,10]
1	37.3		37.3	
2	31.9		31.7	
3	71.8	3.55-3.45, *m*	71.8	3.55-3.45, *m*
4	42.5		42.3	
5	140.7		140.8	
6	121.4	5.34, *br s*	121.7	5.34, *br s*
7	31.7		31.7	
8	31.7		31.9	
9	51.3		50.1	
10	36.5		36.5	
11	21.2		21.1	
12	39.9		39.8	
13	42.2		42.8	
14	57.0		56.8	
15	24.3		24.3	
16	28.9		28.2	
17	56.0		56.1	
18	12.2	0.68, *s*	12.0	0.68, *s*
19	19.1	0.99, s	19.0	0.99, *s*
20	36.1		36.1	
21	19.4	0.93, *d*, *J*=6.4	19.4	0.93, *d*, *J*=6.4
22	34.0		33.9	
23	26.1		26.1	
24	45.9		45.8	
25	29.2		29.1	
26	19.1		19.0	
27	18.9	0.80, *d*, *J*=6.6	18.8	0.80, *d*, *J*=6.6
28	23.1	2.26-1.08, *m*	23.1	2.26-1.08, *m*
29	12.0	0.84, *t*, *J*=7.2	11.8	0.84, *t*, *J*=7.2

10. X. Zhang , P. Geoffroy, M. Miesch, D. Julien-David, F. Raul, D. Aoude-Werner, E. Marchioni, *Steroids,* 2005, **70**, *886-895.*

5.5.2 Structural elucidation of compound 5.2



5.2

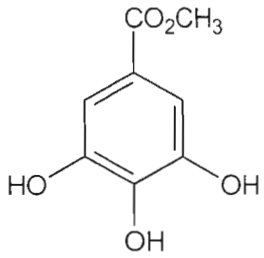
Compound **5.2** was isolated as yellowish-white crystals using flash chromatography with 2% MeOH/$CHCl_3$ as a mobile phase. The NMR data of **5.2** are summarised in Table 5.2. The ^{13}C NMR spectrum showed only five signals as C-2 and C-6 as well as C-3 and C-5 are chemically equivalent. ^{13}C NMR data showed the following signals: δ_c 121.0 (C-1, s), 110.0 (C-2, 6, d), 145.0 (C-3, 5, s), 137.0 (C-4, s), 169.9 (C-7, s). EI/MS gave a molecular ion $[M]^+$ *m/z* 170 and with TLC a R_f = 0.7 (2% MeOH/$CHCl_3$) was observed. Compound **5.2** was identified as **3,4,5-trihydroxybenzoic acid (gallic acid)** with molecular formula $C_7H_6O_5$. Data from the literature confirmed the structure[11,12].

Table 5.2: ^{13}C and ^{1}H NMR (400 MHz, $CDCl_3$) data for gallic acid (**5.2**)

Carbon number	δ_c Compound **5.2**	δ_H Compound **5.2**	Lit: δ_c Gallic acid[11,12]	Lit: δ_H Gallic acid[11,12]
1	121.0		121.4	
2,6	110.0	7.10, *s*	110.5	7.10, *s*
3,5	145.0		144.9	
4	137.0		135.9	
7	169.9		170.8	

5.5.3 Structural elucidation of compound 5.3

11. Y. Lu, L.Y. Foo, *Food Chem.*, 1999, **65**, 1-8.
12. K-J. Wang, C-R. Yang, Y-J. Zhang, *Food Chem.*, 2006, **101,** 365-371.



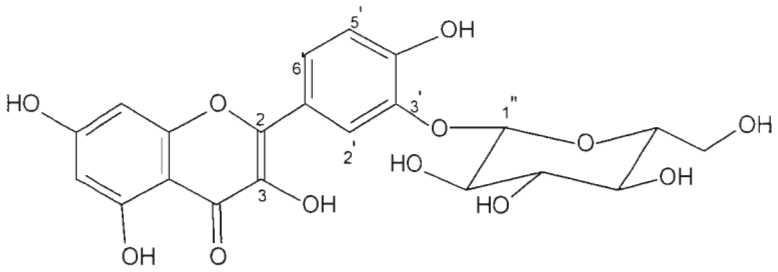
5.3

Compound **5.3** was isolated as white crystals first using Sephadex LH-20 (100% EtOH) and then flash silica gel chromatography (5% MeOH/$CHCl_3$). The NMR data of **5.3** is given in Table 5.3. The ^1H NMR spectrum showed a singlet peak integrating for two protons which almost resembles protons in compound **5.2,** δ_H 7.03 (2H, *s*, H-2, 6), a three-proton singlet at δ_H 3.80 that was assigned to a methoxy group. ^{13}C NMR showed six signals and most of these had similar chemical shifts to those of compound **5.2** except for one additional signal that appeared in the methoxy region (δ_c 52.3). EI/MS showed a very intense molecular ion peak at $[M]^+$ *m/z* 184, R_f = 0.72 (2%MeOH/$CHCl_3$). Compound **5.3** was identified as **methyl gallate** with molecular formula $C_8H_8O_5$. This was also confirmed by comparison with literature data[11,12].

Table 5.3: ^{13}C and ^1H NMR (400 MHz, $CDCl_3$) data for compound **5.3**

Carbon number	δ_c Compound **5.3**	δ_H **Compound 5.3**	Lit: δ_c Methyl gallate[12]	Lit: δ_H Methyl gallate[12]
1	121.0		121.4	
2,6	110.0	7.03, *s*	110.5	7.04, *s*
3,5	145.8		146.5	
4	137.0		135.9	
7	168.0		167.5	
8	52.3	3.80, *s*	52.3	3.80, *s*

5.5.4 Structural elucidation of compound 5.4



5.4

Compound **5.4** was isolated after chromatography on Sephadex LH-20 (100% EtOH) and further purified into a single compound using flash silica (5% MeOH/$CHCl_3$). Compound **5.4** was obtained as a yellow powder and crystallized from methanol as yellow needles. The compound showed UV absorption maxima at 268, 343, 275 and 314 nm, which are typical of flavonols. On TLC the R_f of **5.4** was 0.56 (toluene:acetone:methanol:water:pyridine, 35:45:10:5:5).

The structure of **5.4** was assigned after a series of careful inspections of all NMR data (1D and 2D) recorded from a Varian 400 MHz in CD_3OD using TMS as a reference (Table 5.4). The DEPT spectrum showed one CH_2 signal and ten CH signals but no CH_3 signals. In addition to this, the presence of a carbonyl group (δ_C 176.1), seven non-protonated oxygen-bearing sp^2 carbon atoms (δ_C 164.5, 161.2, 157.0, 149.1, 147.5, 145.4, 136.2) and two other sp^2 carbon atoms (δ_C 123.3 and 103.3) were observed in the ^{13}C NMR spectrum. The presence of a carbonyl carbon and fourteen aromatic carbons suggested that the compound is a penta-oxygenated flavonoid. In the ^1H NMR spectrum, doublets at δ_H 6.39 and 6.17 (J = 2.0 Hz) are characteristic of the *meta*-coupled H-6 and H-8 of the A ring of flavonoids. Three signals at δ_H 8.12 (d, J = 2.0 Hz), δ_H 7.75 (*dd*, J = 9.0 and 2.0 Hz) and δ_H 6.82 (*d*, J = 9.0 Hz) indicated the presence of a 3,4-disubstituted B-ring. Based on these results, compound **5.4** was identified as 3,3',4',5,7-penta-oxygenated flavone (a flavonol).

This assignment was corroborated by correlations observed in the HMBC spectrum: C-10→H-6, H-8; C-8→H-6; C-2→H-2′, 6′, C-1′→H-5′; C-4′→ H-2′, 6′, and C-3′→H-5′. The HMBC spectrum revealed that the anomeric proton of the sugar ring was correlated through three-bond coupling to C-3' of the B-ring, indicating the sugar substitution at the 3'-position. ^{13}C NMR data of compound **5.4** correlates well with the reported 3'-*O*-glycosylated flavonoid (Table 5.4). The ^{13}C NMR data for the 3-*O*-glycosylated flavonoid is quite different from the isolated compound **(5.4,** refer to Table 5.4) especially for C-2 which was reported at δ_c 158.4 for the carbon-3 glucosylated compared to δ_c 147.5 for the isolated compound **5.4.** The ^{13}C NMR signals for compound **5.4** correlates well with those reported for quercetin-3'-glucoside (Table 5.4).

The ^{1}H NMR spectrum also contained signals typical of a sugar moiety: δ_H 3.10-3.90 (H-2″, H-3″, H-4″, H-5″, Ha,b-6″). The anomeric proton resonating at δ_H 4.99 with a coupling constant of 7.2 Hz, indicated a β-glycoside. In the ^{13}C NMR spectrum sugar signals were observed at δ_c 103.1 (C-1″), 73.7 (C-2″), 76.6 (C-3″), 70.2 (C-4′), 77.2 (C-5″), 61.3 (C-6″), which are characteristic of a glucosyl group.

The HRMS gave an intense molecular peak at *m/z* 464 and a distinct fragment peak at *m/z* 307 resulted from the fragmentation of a sugar moiety from a flavonoid skeleton [M-sugar] and a fragment at *m/z* 447 is due to the loss of a hydroxyl group from the B-ring. After careful analysis of these data, compound **5.4** was identified as **quercetin 3′-*O*-glucoside.**

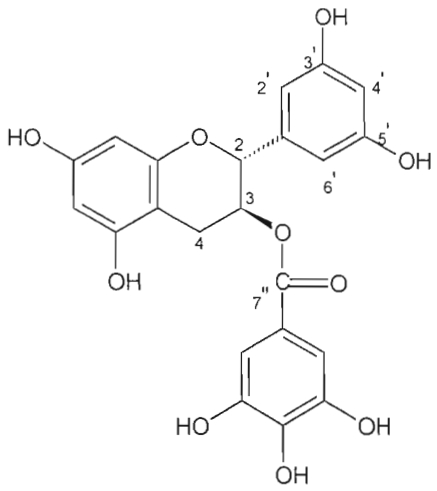
The assignment of the structure was corroborated by comparing the NMR data with literature data of the same compound[13]. This compound and other flavonol glycosides were reported to be crucial for UV defence of plants because they absorb light in the UV-B range and are also good antioxidants and free radical scavengers[13].

13. W.Oleszek, A. Stochmal, P. Karolewski, A.M. Simonet, F.A. Macias, A. Tava. *Biochem. Syst. Ecol.* 2002, **30**, 1011-1022.

Table 5.4: ^{13}C and ^{1}H NMR (400 MHz, CD_3OD) data for compound **5.4,** quercetin 3′-glucoside and ^{13}C NMR data for quercetin 3-*O*-glucoside[13]

Carbon number	δ_C Compound **5.4**	δ_H (*J* in Hz) Compound **5.4**	HMBC, Compound **5.4**	δ_C Quercetin 3′-*O*-glucoside[13]	δ_C Quercetin-3-*O*-glucoside[13]
2	147.5		H-2',6′	147.3	158.4
3	136.2			136.5	135.6
4	176.1			177.4	179.4
5	161.2			162.6	163.0
6	98.2	6.39,*d*,*J*=2.0	H-8	99.3	99.8
7	164.5			165.5	165.9
8	93.4	6.17,*d*,*J*=2.1	H-6	94.6	94.7
9	157.0			158.9	158.9
10	103.3		H-6,8	104.5	105.6
1′	123.1		H-5′	124.4	123.0
2′	116.9	8.12,*d*,*J*=2.0		118.1	116.0
3′	145.4		H-5′,H-1″	146.7	145.9
4′	149.1		H-2',6′	151.6	149.8
5′	115.9	6.82,*d*,*J*=9.0		117.1	117.5
6′	123.8	7.75,dd,*J*=9.0, 2.0		125.1	123.2
1″	103.1	4.99,*d*,*J*=7.2		104.4	104.3
2″	73.7	3.47		74. 8	75.7
3 ″	76.6	3.47		77.6	78.1
4″	70.2	3.41	H-6″	71.2	71.2
5 ″	77.2	3.76		78.4	78.3
6″	61.3	3.78, 3.54		62.4	62.5

5.5.5 Structural elucidation of compound 5.5



5.5

Compound **5.5** was isolated from fraction BP-5-20F using flash silica gel chromatography with 5% MeOH/$CHCl_3$ as the mobile phase and was crystallized from MeOH/$CHCl_3$/hexane as white-yellowish crystals. On TLC (T:A:M:W:Py, 35:45:10:5:5) the R_f was 0.46.

The NMR data of **5.5** are collated in Table 5.5. Twenty-two carbon atoms was observed in the ^{13}C NMR spectrum, which were identified as one carbonyl, eighteen aromatic carbons and three aliphatic carbon atoms. The DEPT spectrum showed one CH_2 and nine CH signals. In the ^{1}H NMR spectrum, the aliphatic protons were characteristic of a *trans*-flavan-3-ol moiety (δ_H 5.06, *d*, *J* = 6.0 Hz, H-2; 5.37, q, *J* = 5.5 Hz, H-3; 2.83, dd *J* = 16.5 and 4.7 Hz; 2.71, dd, *J* = 16.5 and 6.1 Hz, 2 x H-4). The low-field shift of H-3 indicated that an ester group is attached to this carbon. From the ^{13}C NMR data it was clear that apart from the flavan an addition aromatic ring was present. A two-proton singlet at δ_H 6.91 in the ^{1}H NMR spectrum is typical of a galloyl group and, therefore, a galloyl ester was attached to C-3. The *trans* coupling between H-2 and H-3 (*J* = 6.0 Hz) is smaller than that observed for catechin-type molecules (*J* = 8.0 Hz), but it may be possible that the two large substituents on C-2 and C-3 cause conformational changes of the C-ring of the flavonoid.

Two protons in *meta* position to each other were observed at δ_H 5.96 (J = 2.3 Hz) and δ_H 5.95 (J = 2.3 Hz), which are characteristic of a phloroglucinol-type A-ring of a flavonoid. The B-ring protons appear as a one-proton broad singlet at δ_H 6.84 and a two-proton doublet at δ_H 6.72 (J = 1.0 Hz), which is consistent with a 3',5'-dihydroxy substitution pattern. In an HMBC experiment, the following long-range correlations were observed: C-2→H-2′, 6′; C-5→H-4; C-6→H-8; C-9→H-4; C-1′→H-3; C-2′→H4′, 6′; C-4′→H-2′,H-6′; C-6′→ H-2′, 4′; C-7″→H-3.

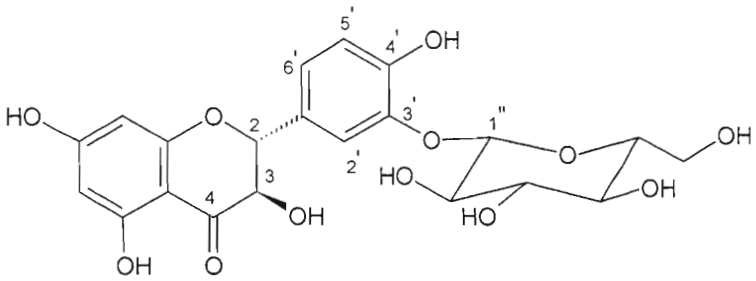
The HRMS gave a molecular ion peak at *m/z* 442. Direct infusion technique using the ESI/MS operating in an ESI^- mode gave a peak at *m/z* 441$[M-H]^-$. The structure of compound **5.5** was assigned as ***trans*-3-*O*-galloyl-3,3',5,5',7-pentahydroxyflavan** with molecular formula $C_{22}H_{18}O_{10}$. Literature studies revealed no published data for this compound, but the *cis*-3,3',5,5',7-pentahydroxyflavan has been isolated from *Humboldtia laurifolia*[14] and the 3-*O*-galloyl ester of the 2,3-*cis* compound was prepared synthetically and tested for antimicrobial activity against three resistant strains of *Staphylococcus aureus*[15]. With the exception of the coupling constant between H-2 and H-3, the NMR data of compound **5.5** were in good agreement with the published values of the 2,3-*cis* synthetic compound [14].

14. U. Samaraweera, S. Sotheeswaran, M. Sultanbawa, S. Uvais, *Phytochemistry*, 1983, **22**, 565-567.
15. J.C. Anderson, C.C. Headley, D. Paul, P.W. Taylor, *Tetrahedron*, 2005, **61**, 7703-7711.

Table 5.5: ^{13}C and ^{1}H NMR (400 MHz, CD_3OD) data for compound **5.5** and the published 2,3-*cis*-isomer [14]

Carbon number	δ_c Compound **5.5**	δ_H (*J* in Hz) Compound **5.5**	HMBC, Compound **5.5**	Lit: δ_c 2,3-cis synthetic compound[14] ($(CD_3)_2O$)
2	79.5	5.06,*d*, *J*=6	H-2',4,6′	78.1
3	71.3	5.37,*q*, *J*=5.5	H-4,7″,10	69.2
4	24.5	2.83,*dd*,*J*=16.5,4.7 2.71,*dd*,*J*=16.5,6.1	H-2,9	26.6
5	158.8		H-4	159.2
6	96.7	5.96,*d*, *J*=2.3	H-8	96.6
7	158.3			157.8
8	95.8	5.95,*d*, *J*=2.3	H-6	95.8
9	156.6		H-2	157.5
10	99.8		H-3,7,8	99.1
1′	131.7		H-3	131.3
2′	114.6	6.72,*d*, *J*=1.0	H-2,4′,6′	114.9
3′	146.5			146.0
4′	116.4	6.84,*s*	H-2, 2',6′	119.4
5′	146.5			115.5
6′	119.4	6.72,*s*, *J*=1.0	H-2,2',4′	119.4
1″	121.5			121.8
2 ″, 6″	110.3	6.97,*s*		110.0
3 ″, 5″	140.0			141.0
4 ″	146.5		H-2″,6″	146.0
7 ″	167.7		H-2″,3,6″	166.1

5.5.6 Structural elucidation of compound 5.6



5.6

Compound **5.6** was isolated using flash silica chromatography with 5% MeOH/ $CHCl_3$ as the mobile phase. It was isolated as orange needles, crystallized using MeOH/CH_2Cl_2 with a TLC R_f value of 0.48 using T: A: M: W: Py (35:45:10:5:5) as the solvent system. Compound **5.6** was observed under UV and appeared orange in colour.

The structure of compound **5.6** was assigned after careful inspection of all NMR data (1D and 2D, 400 MHz) in CD_3OD (Table 5.6). Based on the DEPT spectra, the twenty-one carbon atoms observed in the ^{13}C spectrum were assigned as one CH_2, twelve CH and eight quaternary carbon atoms. The chemical shifts of the carbon atoms indicate the presence of a carbonyl group, twelve aromatic carbon atoms, five of which are bonded to oxygen atoms, a monosaccharide moiety and two additional oxygen-bearing, secondary aliphatic carbon atoms.

In the ^1H NMR the carbohydrate signals appear at δ_H 3.20- 3.81 with the anomeric proton resonating at 4.90 as a doublet with a coupling constant of J = 6.9 Hz. The ^{13}C NMR shifts are in agreement with a β-glucoside moiety. In the ^1H NMR spectrum two coupled doublets were observed at δ_H 5.05 (J = 10.2 Hz) and δ_H 4.59 (10.2 Hz). The signals were assigned to H-2 and H-3 and the large coupling constant indicated a 2,3-*trans* relationship with the C-2 phenyl and C-3 hydroxyl group in equatorial positions[16]. Two doublets at δ_H 6.13 and δ_H 6.15, (J = 2.2 Hz) are characteristic of the *meta*-coupled H-6 and H-8 protons of the A-ring of a

16. L.N. Lundgren, O. Theander, *Phytochemistry,* 1988, **27**, 829-832.

flavonoid. The signals observed for the B-ring (δ_H 7.40, *J* = 2.0 Hz; δ_H 6.87, d, *J* = 8.5 Hz; δ_H 7.45, *dd*, *J* = 8.5 Hz) define this ring as 3,4-disubstituted. NOE correlations were observed between H-2 and H-3 of the B-ring and between the protons of the sugar ring. The HMBC spectrum showed correlation between C-3′ of the B-ring and the anomeric carbon of the sugar which indicated substitution at C-3′ of the B-ring. HMBC showed the following other long-range couplings, C-6→H-8, C-4→H-2, C-1′→H-5′, C-2→H-6′, H-2′, C-10 →H-3, H-6, C-4′→H-6′,H-2′, C-3′ →H-1″. The carbon-13 chemical shifts of compound **5.6** were compared to those reported for the carbon-3 glycosylated flavonoid (Table 5.6). Major differences were observed in the ^{13}C NMR signals of C-3 (δ_c 76.3 as opposed to δ_c 72.3 for compound **5.6**) and C-6′ signals (δ_c 120.7 versus δ_c 124.2) which indicated towards a C-3′ substituted compounds.

In comparison with taxifolin-4′ glycosylated flavonoid, pronounced aglycone ^{13}C NMR signal differences were observed especially at C-2′ (δ_c 133.9[17] as opposed to δ_c 118.6 for compound **5.6**, C-5′ showed a signal at δ_c 118.5 whereas compound **5.6** gave δ_c of 115.9, C-6′ signal for a 4′substituted flavonoid was δ_c 120.7 whereas compound **5.6** gave a δ_c of 124.2). ^{13}C NMR signals for compound **5.6** correlates well with the reported taxifolin-3′-glucoside (Table 5.6) and therefore confirm that glycosylation occurred at C-3′ of the B-ring. The optical rotation of this compound (-26°) also compared well to the reported compound[16].

The ESI/MS direct infusion operating in an ESI$^-$ mode gave a molecular ion peak at *m/z* 465 (M-H), while the HRMS gave a distinct ion peak at *m/z* 466.1111. This corresponded to a molecular formula $C_{21}H_{22}O_{12}$.This compound was identified as **taxifolin 3'-*O*-glucoside.** This data is in good agreement with the values recorded for taxifolin-3'-*O*-glucoside previously isolated from *Pinus sylvestris* needles[13].

17 T. Fossen, A.T. Pedersen, Φ.M. Andersen, *Phytochemistry*, 1998,**47**, 281-285.

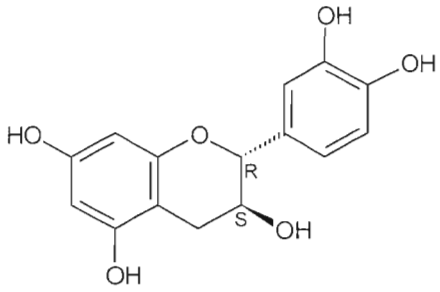
Table 5.6: ^{13}C and ^{1}H NMR, HMBC, DEPT (400 MHz, CD_3COCD_3) data for compound **5.6** and ^{13}C NMR of *trans*-taxifolin 3′-*O*-glucoside[13, 18]

Carbon number	δ_C Compound **5.6**	δ_H (*J* in Hz) Compound **5.6**	HMBC, Compound **5.6**	δ_C **Taxifolin 3'-*O*-glucoside**	δ_C **Taxifolin 3-*O*-glucoside**
2	83.5	5.05,*d*, *J*=10.2	H-2',6′	84.6	83.8
3	72.3	4.59,*d*, *J*=10.2		73.3	76.3
4	197.4		H-2	198.1	196.0
5*	163.4			165.2	165.4
6	96.5	6.13,s, *J*=2.2	H-8	97.3	97.4
7*	167.2			165.7	169.0
8	95.5	6.15,*s*, *J*=2.2	H-6	96.2	96.4
9	164.3		H-2	164.4	164.3
10	101.0		H-3,6	101.9	101.0
1′	128.9		H-3,5′	129.8	128.8
2′	118.6	7.40,*d*, *J*=2.0	H-2,6′	118.0	115.7
3′	145.2		H-5′, H-1″	146.3	146.5
4′	148.7		H-2',6′	149.1	147.4
5′	115.9	6.87,*d*, *J*=8.5		116.9	116.3
6′	124.2	7.45,*dd*, *J*=8.5,2.0	H-2′,2	124.6	120.8
1″	104.0	4.90,*d*,*J*=6.9		103.7	102.4
2″	73.9	3.48,s		74.7	73.1
3″	76.8	3.48,s		77.4	66.8
4″	70.6	3.41,s	H-6″	71.3	71.1
5″	77.3	3.76,s		78.0	78.0
6″	61.8	3.78, 3.54	H-4″	62.4	63.4

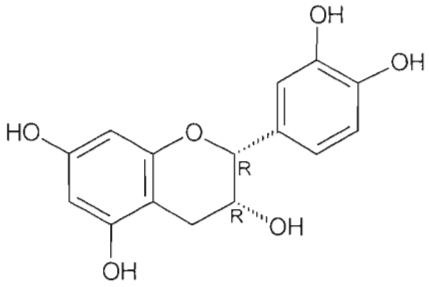
* May be interchanged

5.5.7 Structural elucidation of compound 5.7a and 5.7b

18.. E. Chosson, A. Chaboud, A.J. Chulia, J. Raynaud, *Phytochemistry*, 1998, **49**, 1431-1433.



5.7a (+)-catechin



57b (-)-epicatechin

Fraction **5.7** was isolated by using flash silica gel chromatography with 5% MeOH/$CHCl_3$ as the mobile phase and identified as a mixture of two structurally related compounds. Its R_f was 0.4 using T:M:A:Py (35:10:50:5) as a TLC developing solvent system. This mixture of compounds was UV active and also visible under normal light intensity.

In the NMR spectra two pairs of closely-related signals were observed. It was possible to separate the signals of the two different compounds and the NMR data of the two compounds are given in Table 5.7a and 5.7b.

^{1}H NMR data revealed four sets of doublets at δ_H 5.98, δ_H 5.99, δ_H 5.88, δ_H 5.85 all with *J* = 2.1 Hz, which were assigned to protons H-6 and H-8 of two phloroglucinol-type A-rings of flavonoids. The B-rings were characteristic of catechin and epicatechin, respectively. Two signals which were assigned to two H-2's were observed at δ_H 4.78 which appeared to be a singlet and at δ_H 4.49 as a doublet (*J* = 8.0 Hz). The coupling constants indicate that one compound has a *cis*-2,3 orientation (as in epicatechin), whereas the second compound has a 2,3-*trans*-relationship (as in catechin). Multiplets at δ_H 4.15 and δ_H 4.13 were assigned to the two H-3's. Proton signals between δ_H 2.01- 2.85 were due to four methylene protons and each set of two was coupling to each other. Two doublet of doublet signal at δ_H 6.78 and δ_H 6.81 were assigned to the one proton of each of the two molecules resonating at the same chemical environment [(2x H-6´ (*dd*, *J* = 2x 8.2 Hz)] of the B-ring. Two doublets observed at δ_H 6.62 were assigned to two H-5´ (*d*, *J* = 8.2 Hz) and these are in *ortho* positions to each H-6' protons. Two proton

singlets appeared at δ_H 7.01 and δ_H 7.02 were assigned to the proton on carbon-2 of the B-ring of each of the two molecules.

The DEPT spectra showed $2CH_2$'s and 14CH's in the ^{13}C NMR spectrum of the two compounds. The ^{13}C NMR spectrum showed the following signals which are also in close proximity to each other and were typical of two compounds of similar structure but with different stereochemistry. A-ring carbon signals were assigned as followed: δ_c 100.1, 99.9 (C-10), δ_c 157.0, δ_c 157.3 (C-5), δ_c 95.8, 95.6, (C-6), δ_c 158.3, 157.8 (C-7), δ_c 96.2, 96.3 (C-8), δ_c 158.0, 157.8 (C-9), B-ring signals were δ_c 132.7, 132.4 (C-1′), δ_c 115.4 (C-2′), δ_c 145.4, 145.8 (C-3′), δ_c 146.1, 145.5 (C-4′), δ_c 116.2, 115. 8 (C-5′), δ_c 120.0, 119.5 (C-6′), C-ring signals were δ_c 79.8, 82.8 (C-2), δ_c 28.7, 29.3 (C-4), δ_c 67.1, 68.5 (C-3). The data from HMBC spectra were not well resolved as the proton signals were congested in some areas of the spectra. COSY spectra showed proton–proton correlation between H-2, H-2′, H-6′and H-3.

The only substantial difference in the NMR spectra of the two compounds, is the difference in coupling constants between H-2 and H-3, and therefore, fraction 5.7 was identified as a 1:1 mixture of **catechin (5.7a)** and **epicatechin (5.7b).**

The mixture could not be separated due to the minute amount recovered. Direct infusion of compound **5.7** using ESI/MS operating in an ESI^- gave a distinct peak at *m/z* 289 $[M-H]^-$, which is a molecular weight of both catechin and epicatechin. The two compounds are well known and have been previously isolated from different plant species[19]. The most abundant flavan-3-ol derivatives in nature are (RS)-(+)-catechin and (RR)-(-)-epicatechin. We have obtained a specific optical rotation of -60° for the mixture, but since (RS)-(+)-catechin has a positive rotation

19. L.P. Foo, Y. Lu, H. Wong, *Phytochemistry*, 1998, **47**, 1137-1140.

(+17°) and (RR)-(-)-epicatechin has a negative optical rotation (-68°), we cannot confirm the absolute stereochemistry with optical rotation[20].

Table 5.7a: ^{13}C and ^{1}H NMR, DEPT (400 MHz, D_2O) data for compound **5.7a** and catechin[19] (300 MHz, acetone-d_6)

Carbon number	δ_C, compound **5.7a**	δ_H *J* in Hz compound **5.7a**	δ_C, (+)-catechin	δ_H *J* in Hz, (+)-catechin
2	82.8	4.49,*d*,*J*=8.0	82.2	4.59, *d*, *J* 7.5
3	68.5	4.15,*m*	68.3	4.04,*m*,
4	28.7	2.53,*dd*, *J*=15.4, 7.9, 2.90, *dd* J= 16.1, 5.0	28.7	2.56,*dd*, J=16.1, 8.2 , 2.93, *dd*, *J* =16.1, 5.3
5	157. 5		157.1	
6	95.6	5.88,*d*,*J* =2.1	96.2	5.90, *d*, J= 2.2
7	157.8		157.6	
8	96.3	5.98,*d*,*J* =2.3	95.5	6.04, *d*, *J*= 2.2
9	157.8		156.8	
10	99.9		100.6	
1′	132.4		132.1	
2′	115.4	7.02,*m*	115.2	6.91, *d*, J 1.5
3′	145.8		145.6	
4′	145.5		145.6	
5′	115.8	6.62,*d*, *J* =8.2	115.3	6.81, *d*, *J* =8.1
6′	119.5	6.78,*d*, *J*=8.2, 1.6	120.0	6.77, *d*, *J* =8.2, 1.6

20. J. Buckingham, Dictionary of Organic Compounds, Chapman and Hall, 5th edition, 1982, p.1018.

Table 5.7b: ^{13}C and ^{1}H NMR, DEPT (400 MHz, D_2O) data for compound **5.7b** and epicatechin[19] (300 MHz, acetone-d_6)

Carbon number	δ_C, compound **5.7b**	δ_H J in Hz compound **5.7b**	δ_H J in Hz, (-)-epicatechin	δ_C (-)-epicatechin
2	79.8	4.78,*s*	4.88,*s*	79.0
3	67.1	4.13,*m*	4.24, *m*	66.6
4	29.3	2.47,*dd*, *J*=15.4, 7.9, 2.84, *dd*, *J*=16.1, 3.0	2.72,*dd*, *J*=16.7, 3.5, 2.87,*dd*, *J*=16.7, 4.5	28.5
5	157.0			157.0
6	95.8	5.85,*d*,*J*=2.1	5.96,*d*,*J*=2.3	96.1
7	158.3			157.2
8	96.2	5.99,*d*,*J*=2.1	6.06,*d*,*J*=2.3	95.4
9	158.0			156.6
10	100.1			99.6
1′	132.7			131.7
2′	115.4	7.01,*d*, *J*=1.6	7.05, *s*	115.0
3′	145.4			144.9
4′	146.1			145.1
5′	116.2	6.62,*d*,*J*=8.2	6.83,*m*	115.5
6′	120.0	6.81,*d*, *J*=8.2	6.83,*m*,	119.0

5.5.8 Discussion

Besides β-sitosterol, the compounds isolated above are mainly phenolic-type compounds including flavonoids and their glycosides (quercetin 3′-*O*-glucoside, gallic acid, methyl gallate, taxifolin 3'-*O*-glucoside, *trans*-3-*O*-galloyl-3,3',5,5',7-pentahydroxyflavan and a mixture of catechin and epicatechin). These compounds have been previously isolated from different plant families but nothing has been reported about their presence in the *E. elephantina* plant except for gallic acid[5] . However, compounds possessing some structural similarities have been isolated from *E. goetzei*[7]. *Trans*-3-*O*-galloyl-3,3',5,5',7-pentahydroxyflavan (**5.5**) has not

been described previously. The identification of a mixture of (-)-epicatechin and (+)-catechin was quite unequivocal after careful inspection of the NMR data (Tables 5.7a and 5.7b).

5.6 Biological activity evaluation

The isolated compounds were subjected to different *in vitro* screens to evaluate various biological activities. These include steroid 5α-reductase enzyme activity, anticancer activity, antioxidant activity, cytotoxicity and antimalarial activity.

5.6.1 *In vitro* steroid 5α-reductase activity

This assay was performed following the method described in Chapter 3 (§ 3.7). It should be noted that compounds **5.1, 5.2** and **5.3** were not subjected to this assay for the reasons stated below. B-Sitosterol (**5.1**) was reported to be an additional ingredient to saw palmetto to improve its effectiveness against enlarged prostate and was also reported to be the main constituent of *Hypoxias roper* (South African star grass or African potato) of which the extract is marketed as 'Harold' for the treatment of BPH[21]. *In vitro* studies have shown that Harold improves urinary flow rate thus reducing the BPH effect. Gallic acid (**5.2**) and its methyl ester (**5.3**) were reported to be ineffective when tested against the steroid 5α-reductase enzyme except when it was structurally attached to a catechin molecule[22].

Efficacy of each isolated compound as well as the crude spray-dried extract were expressed as percentage inhibitory activity and these compounds were tested at different concentrations. A mixture of four isolated flavonoids namely, **5.4, 5.5, 5.6** and **5.7** was prepared (Table 5.8) and the mixture tested at three different concentrations (100, 10 and 1 μg/mL). Due to financial constraints, the single compounds were tested at one concentration only.

21. J.P. Meyer, D.A. Gillatt, *BJU international*, 2002, **90**, 41-44.
22. R.A. Hiipakka, H-Z. Zhang, W. Dai, S. Liao, *Biochem. Pharm.*, 2002, **63**, 1165-1176.

Table 5.8: Preparation of the mixture

Compound name	Compound number	Mass percentage ratio (%)
Quercetin 3′-*O*-glucoside	**5.4**	25
Trans-3-*O*-galloyl-3,3',5,5',7-pentahydroxyflavan	**5.5**	25
Taxifolin 3′-*O*-glucoside	**5.6**	25
Catechin and epicatechin mixture (1:1 ratio)	**5.7**	25

The inhibitory effect of the crude extract, the isolated compounds and a mixture of the compounds on the steroid 5α-reductase was investigated and the results are given in Table 5.9.

Table 5.9: *In vitro* inhibition of steroid 5α-reductase enzyme by the crude extract, isolated compounds and a mixture thereof

Compound number	Test concentration	% inhibition
Spray-dried crude extract SM010119B	100	92
Compound **5.4**	1 μM	-9
Compound **5.5**	1 μM	-2
Compound **5.6**	1 μM	3
Compound **5.7** (mixture)	1 μM	-6
Mixture	1 μg/mL	-6
Mixture	10 μg/mL	-2
Finasteride*	0.025 μM	50

* Reference compound

It can be observed from Table 5.8 that the individual isolated compounds did not exhibit any significant activity when tested at 1 μM compared to the test compound finasteride which gave an IC_{50} of 0.025 μM. Only the crude spray-dried extract and

mixture showed good inhibition of the steroid 5α-reductase enzyme when tested at 100 μg/mL, these gave 92% and 80% inhibition respectively. The isolated compounds have structural similarities to those of some catechin derivatives isolated from green tea, which were tested against the steroid 5α-reductase enzyme and showed to be more effective when combined as opposed to when tested individually[17]. However, these specific compounds have never been tested for this biological activity.

When the mixture was screened at 1 μM and 10 μM, no improved activity was observed as compared to the compounds tested individually. When the mixture was screened at 100 μM, 80% efficacy was observed. Structurally, compound **5.5** only lacks a hydroxyl group at position C-4′ of the B- ring to resemble epigallocatechin gallate, which was tested against this enzyme at 5 μM concentration and showed a significant activity of 61% inhibition[17]. The presence of a gallate ester in the catechin molecule appears to be important for the inhibitory activity. Structural modifications of these compounds could still be carried out with the hope to improve the steroid 5α-reductase inhibitory activity. The mixture of catechin and epicatechin (compound **5.7**) did not exhibit any significant activity *in vitro.* This was also supported by reported literature where the two compounds were tested separately and showed no steroid 5α-reductase activity at concentrations of 5 μM and 1 μM[20].

5.6.2 *In vitro* anticancer activity

This work was carried out at the CSIR in-house anticancer screen using the method described in Chapter 3 (§ 3.7). All the compounds were tested *in vitro* against the three cancer cell lines namely melanoma, renal and prostate cell lines. The results were analyzed using the NCI protocol (§ 3.7.2).

Compound **5.1, 5.3, 5.4, 5.5, 5.6,** and **5.7** showed no anticancer activity against the three cell lines. Only compound **5.2** exhibited potent activity against the melanoma and prostate cell lines with a total growth inhibition (TGI) of 1 μg/mL. The two graphs below only represent the active compound (compound **5.2**) as well as one of the non-active (compound **5.1**).

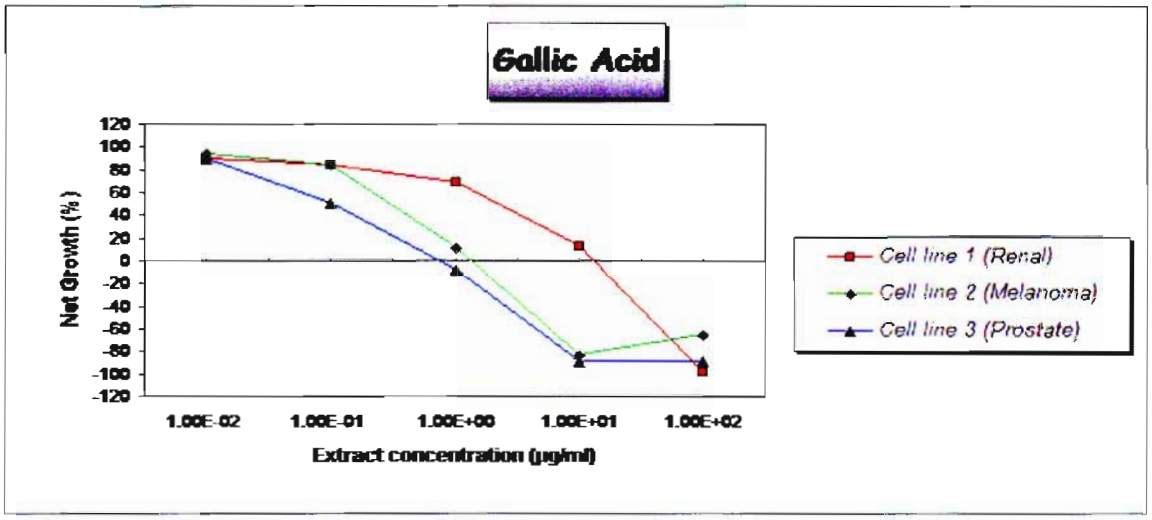


Figure **5.3**: Anticancer activity of compound **5.2**

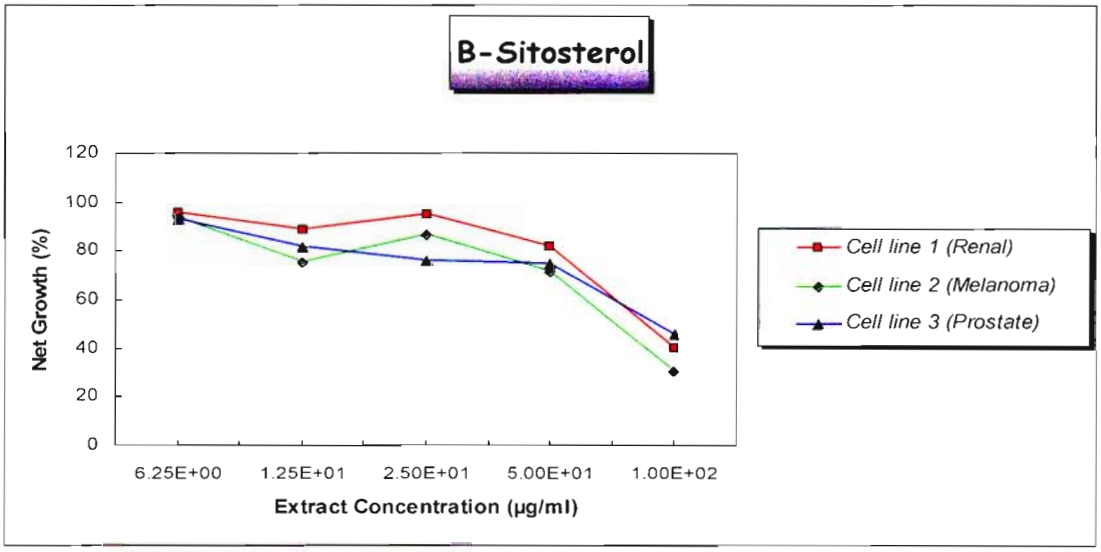


Figure **5.4**: Anticancer activity of compound **5.1**

The difference between the two graphs is quite remarkable as compound **5.1** did not show any growth inhibition even at 100 μg/mL as opposed to compound **5.2** where there was a clear TGI of 1 μg/mL. Although the anticancer activity of methyl

gallate (**5.2**) has been reported previously[23], some new derivatives could still be synthesized and tested against other cancer cell lines[24].

5.6.3 *In vitro* antimalarial activity

This work was carried out at the University of Cape Town, Department of Pharmacology. This was done following the procedure described in Chapter 3 (§ 3.7). The pure compounds were tested in duplicate against *Plasmodium falciparum* chloroquine-susceptible strain (D10) and chloroquine was used as a reference compound in all experiments. To our knowledge, no previous reports on the antimalarial activity of these compounds have been published.

Table 5.10: *In vitro* antiplasmodial activity of pure compounds against *P. falciparum* CQS D10 strain*

Compound	IC_{50} (μg/ml)
Compound **5.2**	6.7
Compound **5.3**	5.2
Compound **5.4**	14.1
Compound **5.6**	>100
Compound **5.7**	62.2
Chloroquine (ng/mL)	34.4

*Most active compound highlighted.

From Table 5.10, 3,3',5,5',7-pentahydroxyflavan 3-*O*-gallate (**5.5**), which is a new compound, showed the best antimalarial activity against a susceptible strain (D10) of *P. falciparum* although it was not as active as chloroquine.

5.6.4 Cytotoxicity

23. A.E. Bailey, R. Asplund, A.M.S. Owen, *J. Nat. Prod.*, 1986, **49**, 1149-1150.
24. N. Sakaguchi, M. Inoue, K. Isuzugawa, Y. Ogihara, K. Hosaka, *Biol. Pharm. Bull.*, 1999, **22**, 471-475.

In vitro evaluation of the cytotoxicity of the pure compounds against the mammalian cell line Chinese Hamster Ovarian (CHO) was conducted at the University of Cape Town, Department of Pharmacology. All compounds were evaluated in duplicate. The procedure followed was described in Chapter 3 (§3.7). Emetine was used a reference compound in all experiments. Cytotoxicity results were measured as 50% inhibitory concentration obtained from dose response curves fitting analysis Graph Pad Prism v.4 software. Five isolated phenolic compounds were tested against CHO cell line. β-Sitosterol (**5.1**) and gallic acid (**5.2**) were excluded as they were widely reported not to be cytotoxic against CHO cell lines even at high concentrations[25].

Table 5.11: *In vitro* cytotoxicity of compounds against Chinese Hamster Ovarian cell line*

Compound	IC_{50} (μg/mL)
Compound **5.3**	34.4
Compound **5.4**	>100
Compound **5.5**	>90
Compound **5.6**	>90
Compound **5.7**	>100
Emetine	0.07

*Cytotoxic compound highlighted

From Table 5.11 only methyl gallate (**5.3**) is cytotoxic against CHO cells and this might be due to the presence of the methyl group which transformed gallic acid moiety to methyl gallate. The other compounds were all non-cytotoxic and this proved their safety *in vitro*.

5.6.5 Antioxidant activity

This assay was carried out at the CSIR Bioprospecting Research Platform following the procedure described in Chapter 3 (§3.7). This assay measured the decolourisation of a purple colour to a yellow colour by a chromameter at different

25. M. Labieniec, T. Gabryelak, *Mut. Res.*, 2003, **539**, 127-135.

concentrations. When the purple colour disappears and a yellow colour is observed, it shows that the compound or an extract has antioxidant properties. The intensity of the yellow colour gives an indication of antioxidant activity. The compounds isolated above were tested and showed different levels of activities against DPPH. The antioxidant activity was expressed as percentage radical scavenging capacity (RSC). The crude extract (LA-10-80B) was tested at five (5) dose concentrations namely 100, 50, 25, 12.5 and 6.25 ppm and showed very good radical scavenging capacity (potent antioxidant).

At 6.25 ppm, the RSC was 82%. The epigallocatechin standard gave a 61% RSC at 1 ppm. Green tea was also tested at the same concentrations as *E. elephantina* and gave a %RSC of 41 at 6.25 ppm. This result showed that *E. elephantina* extract exhibited better antioxidant activity than green tea at the same test concentration. Isolated compounds showed good decolourisation of DPPH and were classified as moderate. Green tea was also tested at 20 ppm and gave a %RSC value of 68 whereas *E. elephantina* extract when tested at 12.5 ppm gave a %RSC value of 95[26]. These results confirm that *E. elephantina* extract has a better antioxidant activity than green tea. Compound **5.5** was not tested for this activity due to minute quantities obtained.

26. N.Y. Lee, C. Jo, S.H. Sohn, J.K. Kim, M.W. Byun, *J. Food Sci.*, 2006, **71**, C269-C274.

Table **5.12:** Shows antioxidant activity of crude *E. elephantina* extract, compound **5.4, 5.6** and **5.7** mixture), green tea and epigallocatechin gallate standard

Compound	Concentration (ppm)	% RSC
Crude *E.elephantina*	100	95
	50	95
	25	95
	12.5	95
Compound **5.4**	100	95
	50	95
	25	95
	12.5	77
	6.25	41
Compound **5.6**	100	21
	50	13
	25	10
	12.5	8
	6.25	10
Compound **5.7**	100	94
	50	94
	25	86
	12.5	48
	6.25	28
Green tea	100	96
	50	96
	25	96
	12.5	71
Epigallocatechin gallate	100	95
	10	95
	0.1	8
	0.01	8

5.7 Conclusion

With the exception of β-sitosterol (**5.1**), the compounds isolated from *E. elephantina* were mainly phenolic compounds with a flavonoid skeleton. Although these classes of compounds have been widely investigated for various biological activities, nothing has been reported on *E. elephantina* and its isolates to possess antibenign prostatic hyperplasia activity, antimalarial, anticancer, antioxidant activity, as well as their noncytotoxicity against the CHO cell line.

The compounds showed efficacy when tested at 100 µM against an *in vitro* steroid 5α-reductase inhibition assay and lost activity when tested separately. No efficacy was observed when the mixture of these flavonoids was tested at 10 µM and 1µM. For *in vitro* anticancer activity, the compounds did not show any activity against the three cell lines, except methyl gallate (**5.2**) that exhibited good activity but is known in the public domain for this activity. For *in vitro* antimalarial activity, only 3,3',5,5',7-pentahydroxyflavan 3-*O*-gallate (**5.5**) showed good inhibition against the chloroquine-susceptible strain (D10) of *P. falciparum*. Structural modification of this compound could lead to improved activity.

For *in vitro* cytotoxicity, the compounds showed that they are safe when tested *in vitro* against CHO cell lines except for compound **5.3,** a derivative of gallic acid which might have been formed as a result of the solvent used during the extraction process. *In vivo* toxicity of these compounds and the crude extract must still be pursued in future.

Some compounds isolated (**5.3, 5.5** and **5.7**) showed good antioxidant activity when tested as single entities but the crude *E. elephantina* extract showed excellent antioxidant properties even when tested at low concentrations. The crude extract proved to be a better antioxidant than green tea when tested at the same concentration. The crude extract as well as the compounds could be tested at lower concentrations and compound **5.5** could be used as an antioxidant.

5.8 Experimental

5.8.1 Plant material

The rhizomes of *Elephantorrhiza elephantina* were collected from the Limpopo Province in South Africa. The plant was identified at SANBI and its identification was further authenticated by a botanist, Ralph Peckover. The voucher specimens were deposited at SANBI.

5.8.2 Isolation of compounds

The bulk rhizomes of *E. elephantina* were sliced and dried overnight in an oven at 60 °C. The 5 kg oven-dried plant material was then ground and boiled in 25 litres of distilled water for 1 hour. The filtrate (tea) was cooled and decanted and separated from the plant material, filtered through a cheese cloth first and then through Whatman No.1 filter paper. The resultant extract was then spray-dried on a large scale and 50 g solid extract was recovered. Of this, 23 g was utilized for the fractionation process. A modified counter-current partitioning method was employed to purify the crude extract. This method entailed partitioning against (i) water/isobutanol/hexane, (ii) *n*-butanol. Four different fractions were obtained and coded BP-5-11A (isobutanol), BP-5-11B (*n*-butanol), BP-5-11C (water) and a hexane fraction. Fractionation of the hexane fraction yielded compound **5.1** (**β-sitosterol**). The other three fractions were investigated by TLC (toluene/acetone/methanol/water/pyridine, 35:45:10:5:5).

On TLC, the isobutanol fraction showed the presence of dominant UV active spots which were typical of phenolic compounds and hence it was further fractionated. The isobutanol fraction was purified using flash silica chromatography, (mobile phase $CHCl_3$/MeOH, 90:10) and afforded 13 fractions coded as BP-5-12 A-M. Some of these fractions were combined based on their TLC profile while fraction BP-5-12A (573 mg) was further purified using flash silica (2% MeOH/$CHCl_3$) to yield 13 fractions and these were coded BP-5-16 A to M. BP-5-16A, 16B, 16C and 16D were combined and further purified using flash silica to yield two pure compounds, namely **gallic acid** (compound **5.2**) and sucrose.

Fraction 16H (199 mg) was further fractionated using Sephadex LH-20, 100% EtOH and afforded six fractions (BP-5-20 A to F). Fraction BP-5-20A was further purified using flash silica gel (5% MeOH/$CHCl_3$) to afford compound **5.7**. Fraction BP-5-20F (65 mg) was purified using flash silica gel and afforded four pure compounds, namely compound **5.2, 5.3, 5.4, 5.5, 5.6** and a mixture of two structurally related compounds ((+)-catechin and (-)-epicatechin) represented as compound **5.7a** and **5.7b.**

5.8.3 Physical data

5.8.3.1 COMPOUND 5.1

Systematic name:	3β-Stigmast-5-en-3-ol
Alternative name:	β-Sitosterol
Yield:	15 mg
Physical description:	White powder
Mass spectrum:	MS (ES^-) *m/z*: 413 $[M-H]^-$ $C_{29}H_{50}O$
Optical rotation:	$[\alpha]_D^{20}$ -36 (c=1.0, $CHCl_3$)
	Lit: $[\alpha]_D^{20}$ -37 (c=1.2, $CHCl_3$)[27]
Melting point:	135-139 °C, Lit[10] 136-138 °C
^{1}H NMR:	See Table 5.1 ($CDCl_3$)
^{13}C NMR:	See Table 5.1 ($CDCl_3$)

5.6.3.2 COMPOUND 5.2

Systematic name:	3,4,5-Trihydroxybenzoic acid
Alternative name:	Gallic acid
Yield:	60 mg
Physical description:	Yellowish white crystals
Mass spectrum:	MS (ES^+) *m/z*: 171 $[M+H]^+$, $C_7H_6O_5$
UV maxima:	210, 270 nm

27. S.B. Mathur, *Phytochemistry*, 1972, ***11***, 449-450.

Melting point:	249-250 °C, Lit[28] 256-258 °C
^{1}H NMR:	See Table 5.2 ($CDCl_3$)
^{13}C NMR:	See Table 5.2 ($CDCl_3$)

5.8.3.3 COMPOUND 5.3

Systematic name:	3,4,5-Trihydroxybenzoic acid methyl ester
Alternative name:	Methyl gallate
Yield:	15 mg
Physical description:	Yellowish white crystals
Mass spectrum:	MS *m/z*: 184 $[M]^+$ $C_8H_8O_5$
UV maxima:	210, 250, 270, 300, 340 nm
Melting point:	202-205 °C, Lit[29] 198-200 °C
^{1}H NMR:	See Table 5.3 ($CDCl_3$)
^{13}C NMR:	See Table 5.3 ($CDCl_3$)

5.8.3.4 COMPOUND 5.4

Systematic name:	3,5,7-Trihydroxy-2-[4-hydroxy-3-(3,4,5-trihydroxy-6-hydroxymethyltetrahydropyran-2-yloxy)-phenyl]-chromen-4-one
Alternative name:	Quercetin 3´-glucoside
Yield:	58 mg
Physical description:	White powder
Mass spectrum:	*MS(ES$^+$) m/z*: 465 $[M+H]^+$, fragment peak *m/z* 307 loss of sugar moiety, *m/z* 447$[M-OH]^+$ $C_{21}H_{20}O_{12}$
Optical rotation:	$[\alpha]_D^{20}$ -40 (c=0.2 in MeOH) Lit[30]: $[\alpha]_D^{20}$ -63.5 (c= 0.33 g/100mL in MeOH)
Melting point:	239-241 °C, Lit[31] 240-241 °C
UV maxima:	255, 350 nm

---

28. A.K. Batta, S. Rangaswami, *Phytochemistry,* 1973, **12**, 214-216.

29. G.D. Manners, L. Jurd, *Phytochemistry*, 1979, **18,** 1037-1042.
30. A.E. Polovinko, G.P. Yakovlev, *Chem. Nat. Compd.*, 1985, **21**, 252-253.
31. H-S.Z. Wagner, *Physiol.Chem.*, 1964,**335**, 232-239.

^{1}H NMR:	See Table 5.4 (CD_3OD)
^{13}C NMR:	See Table 5.4 (CD_3OD)

5.8.3.5 COMPOUND 5.5

Systematic name:	3,4,5-Trihydroxybenzoic acid 2-(3,5-dihydroxy-phenyl)-5,7-dihydroxy-chroman-3-yl ester
Alternative name:	3-O-Galloyl-3,3',5',5,7-pentahydroxyflavone
Yield:	9 mg
Physical description:	White-yellow crystals
Mass spectrum:	HR/MS *m/z* 442.2120, calcd for $C_{22}H_{18}O_{10}$, ???? MS (ESI^+) *m/z*: 443 $[M+H]^+$
Optical rotation:	$[\alpha]_D^{20}$ -49 (c=0.2 in MeOH)
Melting point:	211-212 °C
^{1}H NMR:	See Table 5.5 (CD_3OD)
^{13}C NMR:	See Table 5.5 (CD_3OD)

5.8.3.6 COMPOUND 5.6

Systematic name:	(2*R*,3*R*)-,2-[3-(β-D-glucopyranosyloxy)-4-hydroxy-phenyl]-2,3-dihydro-3,5,7-trihydroxy-,4*H*-1-benzo-pyran-4-one
Alternative names:	3,3',4',5,7-Pentahydroxyflavanone 3'-*O*-glucoside Taxifolin 3'-*O*-glucoside
Yield:	38 mg
Physical description:	Fine orange needles
Mass spectrum:	HRMS *m/z* 466.111 calcd for $C_{21}H_{22}O_{12}$, ????? MS (ESI^-) *m/z*: 465 $[M-H]^-$
Optical rotation:	$[\alpha]_D^{20}$ -26 (c=0.2 in MeOH), Lit[32]. $[\alpha]_D^{21}$-26 c=0.3 in MeOH)
UV maxima	290, 327 nm

32. L.Y. Foo, J.J. Karchesy, *Phytochemistry*, 1989, **28**, 1237-1240.

Melting point:	185-187 °C, Lit[33] 203-295 °C
^{1}H NMR:	See Table 5.6 (CD_3COCD_3)
^{13}C NMR:	See Table 5.6 (CD_3COCD_3)

5.8.3.7 COMPOUND 5.7a

Systematic name:	(+)- Catechin
Yield:	43 mg
Physical description:	Yellow crystals
Mass spectrum:	MS (ESI^-) *m/z*: 289.01 $[M-H]^-$ $C_{15}H_{14}O_6$
^{1}H NMR:	See Table 5.7a (D_2O)
^{13}C NMR:	See Table 5.7a (D_2O)

5.8.3.8 COMPOUND 5.7b

Systematic name:	(-)- Epicatechin
Yield:	43 mg
Physical description:	Yellow crystals
Mass spectrum:	MS (ESI^-) *m/z*: 289.01 $[M-H]^-$ $C_{15}H_{14}O_6$
^{1}H NMR:	See Table 5.7b (D_2O)
^{13}C NMR:	See Table 5.7b (D_2O)

33. G. Hergert, J. Joceah, *J. Org. Chem.*, 1958, **23**, 700-704.