PHYTOCHEMICAL AND *IN VITRO* ANTI-MICROBIAL SCREENING OF *ECHINOPS HISPIDUS* FRESEN. AND *GREWIA SIMILIS* K. SCHUM.

BY

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I56/13098/2005

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE IN THE SCHOOL OF PURE AND APPLIED SCIENCES, KENYATTA UNIVERSITY

April, 2010

DECLARATION

Candidate

I declare that this thesis is my own work and it has not been submitted for any degree or examination in any other university and that all the sources used have been indicated and acknowledged by complete references.

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DEDICATION

My parents, brothers and sisters

and

My wife Elizabeth, my daughters Miriam and Ruth and my son, Samuel

AKNOWLEDGEMENTS

Glory be to God for the good health, inspiration and source of strength during this research work.

My deepest gratitude and thanks go to my supervisors, Dr. Alex Machocho, Dr. Alphonse Wanyonyi and Prof. Paul Tarus for their guidance, mentorship and encouragement throughout this work. Most sincerely I wish to thank all my supervisors for taking time to go through the draft. Special thanks go to Prof. Paul Tarus for organizing the funding of the project, Dr. Alex Machocho for his assistance with spectral data interpretations, Dr. Christine Bii of KEMRI for assisting me to do the anti-microbial asssays and Prof. Abiy Yesenewe of University of Nairobi for running the NMR spectra.

Thanks also go to all the technicians in the Chemistry Department, Kenyattta University especially the chief technician Mr. E. Maina for providing the chemicals, glassware and other apparatus that were used during the study.

Special gratitude to the following institutions; Kenyatta University for providing me with the necessary apparatus and giving me space in the research laboratory, University of Nairobi for the running of the NMR-spectra, Jomo Kenyatta University of Agriculture and Technology for running IR spectra and the University of Kwa Nzulu Natal, South Africa for running the NMR- and Mass-spectra.

Thanks to my wife for her support and patience through out this work. I would also like to thank my parents, sisters and brothers for their support, understanding and prayers.

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ABRREVIATIONS AND ACRONYMS

AIDS	Acquired Immunodeficiency Syndrome
ATTC	American Type Culture Collection
CAM	Complementary Alternative Medicine
CC	Column Chromatography
CD_3OD	Deuterated methanol
$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
COSY	Correlated Spectroscopy
d	Doublet
DCM	Dichloromethane
dd	Doublet of doublets
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulphoside
EtOAc	Ethylacetate
HIV	Human Immunodeficiency Virus
HMBC	Heteronuclear Multiple Bond Connectivity
HMQC	Heteronuclear Multiple Quatum Coherence
HSQC	Heteronuclear Single Quantum Coherence
IR	Infra-Red
J	Coupling constant in Hertz
KBr	Potassium bromide
MDRSA	Multidrug-resistant *Staphylococcus aureus*
MeOH	Methanol
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectroscopy
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
ppm	Parts per million
opb	Out of plane bend
PTLC	Preparative Thin Layer Chromatography
s	Singlet
str	Stretch
t	Triplet
TLC	Thin Layer Chromatography
TM	Traditional Medicine
TMPs	Traditional Medicinal Practisioners
TMS	Tetramethyl silane
UN	United Nations
UV	Ultraviolet
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization
µg	Micro gram
µm	Micro molar
m	Multiplet

ABSTRACT

Antimicrobial resistance is reported to be on the increase due to gene mutations of the disease causing pathogens. It is believed that, new antibiotics with activities and structures different from those in current use could be found through ethnobotanical route. This requires a follow up of promising leads with attempts to isolate and identify the active principles from the medicinal plants. Two Kenyan plants *Echinops hispidus* Fresen. (Asteraceae) and *Grewia similis* K. Schum. (Tiliaceae) were selected for this study because of their reputation in folklore medicine as antimicrobial agents. The antimicrobial screening of the crude extracts and the isolated compounds was done using the agar diffusion method. The ethyl acetate (EtOAc) root extract of *E. hispidus* showed a weak activity with an inhibition zone of 9 mm against *Staphyloccocus aureus.* The hexane/dichloromethane (DCM) root extract of *G. similis* exhibited a very strong activity with an inhibition zone of 15 mm. the hexane, DCM and EtOAc root extracts of *E. hispidus* showed a weak activity of 9mm, strong activities of 13 and 15 mm against *Cryptoccocus neoformans*, respectively. The hexane and DCM extracts of *E. hispidus* and the hexane/DCM extract of *G. similis* showed the minimum activity of 6 mm against *Pseudomonas aeroginosa* and *Escherichia coli*. Isolation and separation of the crude extracts were carried out using VLC, CC, TLC and centrifugation. This led to isolation of eight compounds whose structures were determined by Infrared, Ultraviolet, 1D- and 2D-NMR, MS and correlation with published data. The hexane/DCM extract of the whole root of *G. similis* led to isolation of 3β-sitosterol (**112)** and 3β-stigmasterol (**113)**. These compounds are being reported for the first time from this plant. The root hexane extract of *E. hispidus* led to isolation of six compounds; 3β-acetyl taraxerol (**114**), cameroonan-7α-ol (**115**), membrin-8α-ol (**116)** and membrinol-8β-0l (**117**), 4-[5-(penta-1,3-dieynyl) thien-2-yl] but-3-ynyl (**119)**. The dichloromethane extract of the whole root of *E. hispidus* yielded 4-[5-(penta-1,3-dieynyl) thien-2-yl] but-3-ynyl diol (**120**). Compounds **115**, **119**, **114**, **116**, **117**, **119** and **120** are being reported fore the first time from *E. hispidus.* The ethyl acetate root extract of *E. hispidus* and the hexane/DCM root extract of *G. similis* showed antibacterial activities against *Staphylococcus aureus* with inhibition zones of 9, 13 and 15 mm, respectively. The isolated compounds **112** , **113** and **120** showed mild antibacterial activities of 8 mm against *S. aureus*. Compound **119** showed a moderate antibacterial activity of 10 mm against *S. aureus*. The compounds **119** and **120** showed high antifungal activities of 33 and 27 mm against *C. neoformans*, respectively. The biologically active compounds are templates for synthesis of more potent and water soluble derivatives. These antimicrobial activities support the use of *E. hispidus* and *G. similis* for the treatment of antimicrobial related ailments by the Kipsigis and Maasai communities.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Traditional medicine comprises therapeutic practices which have been in existence for hundreds of years before the development of modern scientific medicine. It is still in use today without much documented evidence of adverse effects (WHO, 1978). The new health agenda in Africa focuses on the institutionalization of traditional medicine in parallel with orthodox medicine into the national health care scheme. This will ensure that the health agenda moves forward since effective health care cannot be achieved in Africa by orthodox medicine alone unless it has been complemented with traditional medicine (Elujoba *et al.,* 2005).

According to the World Health Organization, phytomedicine is defined as herbal preparations produced by subjecting plant materials to extraction, fractionation, purification, concentration or other physical or biological processes. These preparations may be produced for immediate consumption or as the basis for other herbal products. Such plant products may contain recipient or inert ingredients, in addition to the active ingredients (WHO, 2001). Traditional Medicine (TM) has maintained its popularity in all regions of the developing world and its use is rapidly spreading in industrialized countries (WHO, 2003).

In a constant attempt to improve their quality of life, humans have used plants as source of food, shelter, clothing, medicine, cosmetics, and for seeking relief from hardship of life. Some plants are known as medicinal because they contain active substances which cure diseases. Indeed plants are the oldest known sources of human and livestock healthcare, and an important component of global biodiversity. Sometimes the knowledge on medicinal plants means the only therapeutic resource of some communities and ethnic groups (Calixto, 2000).

The use plant extracts as medicine is an immemorial mode of health care in many parts of Africa and it is still the main vehicle of health care delivery today especially in the rural areas where conventional medical facilities are not within the reach of most people. Patronage of traditional medicine cuts across all social barriers with both educated and non-educated people embracing the practice (Makhubu, 2003). The first port of call for an average African when ill is the traditional medical practitioner (TMP) even if the modern health clinic is not far (Makhubu, 2003). This is because most Africans consult orthodox medical practitioners when traditional medicine had failed (WHO, 2003). The WHO estimates that 4 billion people (80%) of the world's population use herbal medicine for some aspect of primary health care (PHC) (Farnsworth *et al*., 1985; Calixto, 2000; Kokwaro, 1996; WHO, 2001). Indeed, well in the 20th century much of the pharmacopoeia of the scientific medicine was derived from the herbal lore of the native people. In the United States of America (USA) a quarter of the prescription drugs dispensed by community pharmacies contain one active ingredient derived from plants (Farnsworth *et al.,* 1985).

The increase in the patronage of herbal medicine is likely to continue because of global economic downturn and as bodies like the WHO-Africa region continue to advocate for its promotion and integration in the national health systems (WHO, 2003). Since such a large proportion of the population rely on herbal medicine, for their medical care, it is essential that information about the system should be preserved through proper documentation and the constituents of the phytomedicine analysed.

Scientific evidence from randomized clinical trials is only strong for many uses of acupuncture, some herbal medicines and for some of the manual therapies. Further research

is therefore needed to ascertain the efficacy and safety of several other practices and medicinal plants. Unregulated or inappropriate use of traditional medicines and practices can have negative or dangerous effects. For instance, the herb “Ma Huang” (*Ephedra*) is traditionally used in China to treat respiratory congestion (WHO, 2003). In the United States, the herb was marketed as a dietary aid, whose over dosage led to at least a dozen deaths, heart attacks and strokes (WHO, 2003). In Belgium, at least 70 people required renal transplant or dialysis for interstitial fibrosis of the kidney after taking a herbal preparation made from the wrong species of plant as slimming treatment (WHO, 2003).

In addition to patient safety issues, there is the risk that a growing herbal market and its great commercial benefit might pose a threat to biodiversity through the over harvesting of the raw material for herbal medicines and other natural health care products. These practices, if not controlled, may lead to the extinction of endangered species and the destruction of natural habitats and resources. Another related issue is that, at present, the requirements for protection provided under international standards for patent law and by most national conventional patent laws are inadequate to protect traditional knowledge and biodiversity (WHO, 2003).

The provision of safe and effective traditional medicine (TM) therapies could become a critical tool to increase access to health care. While China, the Democratic People’s Republic of Korea and Vietnam have fully integrated traditional medicine into their health care systems, many countries are yet to collect and integrate standardized evidence on this type of health care. Seventy (70) countries have a national regulation on herbal medicines but the legislative control of medicinal plants has not evolved around a structured model. This is

because medicinal products or herbs are defined differently in different countries and diverse approaches have been adopted with regard to licensing, dispensing, manufacturing and trading (WHO, 2003). The limited scientific evidence about TM/CAM's safety, efficacy and other considerations led the WHO to launch its first ever comprehensive traditional medicine strategy in 2002.

Infectious diseases account for one-half of all deaths in the tropical countries (Iw*u et al.,* 1999). Irrespective of the efforts made in curbing the incidence of epidermics, drug-resistant microorganisms and the emergence of hitherto unknown disease-causing microbes pose enormous public health concern. There is therefore need to continue the search for novel drugs which are devoid of these short comings. Phytomedicine has demonstrated its contribution to the reduction of excessive mortality, morbidity and disability due to diseases such as HIV/AIDS, malaria, tuberculosis, sickle-cell anemia, diabetes, mental disorders (Elujoba *et al*., 2005) and microbial infections (Iwu *et al*., 1999; Okigbo *et al*., 2005). It has reduced poverty by increasing the economic well-being of communities and develops health system by increasing health coverage to the people (Elujoba *et al*., 2005).

Phytomedicines are now very popular in developing countries with knowledge about the safety, efficacy and quality assurance of botanical medicine (Calixto, 2000). The medicinal plants should therefore be protected from going to extinct because a source of health and wealth lies in them. The people and orthodox practitioners therefore need to be given appropriate information on phytomedicine in order to use them and apply them in the health care delivery system. The integration or harmonization of phytomedicine should be developed in such a way to work hand in hand with orthodox medicine with minimum threat

to each other. China for example, is able to provide adequate and constantly improving health care coverage for its vast urban population because it harnesses the previous legacy of traditional medicine (Aregbeyen, 1983; Bodeker, 1994).

1.2 Bacterial infections

Bacteria may be classified as either Gram-negative or Gram-positive according to a staining technique devised by Christian Gram in 1884 (Baldry, 1976). On staining, those that retain the primary dye color are Gram-positive while those that change the color are Gram-negative. Some examples of Gram-positive are *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes* and *Actinomyces odontolyticu.* Examples of Gram-negative bacteria include; *Escherichia coli*, *Pseudomonas aeruginosa, Salmonella typhi, Vibrio cholerae* and *Bordetella pertussis.* The mammalian body provides a favorable environment for the growth of numerous microorganisms. This results into an unfavorable interaction usually causing diseases (Baldry, 1976).

1.2.1 *Staphylococcus aureus*

Staphylococcus aureus is a facultatively anaerobic, Gram-positive bacterium which is found living as a commensal on skin and often in the nose of healthy people. However, it is one of the commonest causes of acute pyogenic infections in man including boils, carbuncles and abscesses. It is also responsible for highly infectious type of bronchopneumonia (WHO, 1996). It can also cause food poisoning and its invasion into the blood causes septicemia. Although several anti-straphylococcal agents have been discovered, the organism is still a major threat to human health (Baldry, 1976).

Methicillin-resistant *S. aureus* (MRSA) is a bacterium responsible for difficult-to-treat infections in humans. MRSA is by definition a strain of *S. aureus* that is resistant to a large group of antibiotics called the beta-lactams, including methicillin, dicloxacillin, nafcillin, and oxacillin (UK Office for National Statistics Online, 2007). This is due to the altered penicillin-binding proteins (Barnes, 2000). MRSA is especially troublesome in hospital-associated (nosocomial) infections. In hospitals, patients with open wounds, invasive devices, and weakened immune systems are at greater risk for infection than the general public (UK Office for National Statistics Online, 2007).

1.2.2 *Escherichia coli*

Escherichia coli is a Gram-negative bacterium that is commonly found in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some, such as serotype O157:H7, can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls. The optimal growth of *E. coli* occurs at 37°C, but some laboratory strains can multiply at temperatures of up to 49°C (Kubitschek, 1990).

E. coli are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for fecal contamination (Hudault, 2001; Reid, 2001). As Gram-negative organisms, *E. coli* are resistant to many antibiotics that are effective against Gram-positive organisms. Resistance to beta-lactam antibiotics has become a particular problem in recent decades, as strains of bacteria that produce extended-spectrum beta-lactamases have become more common. These beta-lactamase enzymes make many, if not all, of the penicillins and cephalosporins ineffective as therapy (Paterson and Bonomo, 2005).

1.2.3 *Pseudomonas aeruginosa*

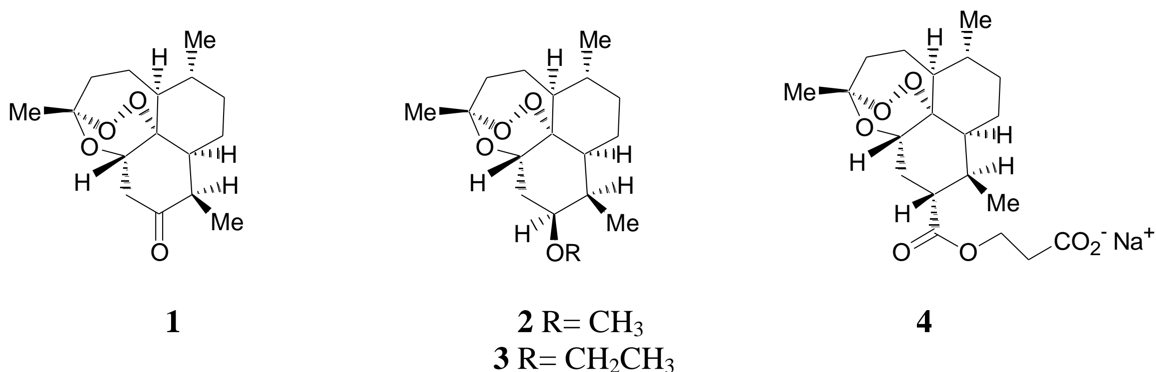
Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 µm by 1.5 to 3.0 µm. It is a common bacterium which can cause disease in animals and humans. It is found in soil, water, and most man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and has thus colonised many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or people with reduced immunity (Cornelis, 2008). Biofilms of *P. aeruginosa* can cause chronic opportunistic infections. These kinds of infections are a serious problem for medical care in industrialized societies; especially for immunocompromised patients and the elderly (Cornelis, 2008).

1.3 Phytomedicine and some naturally derived drugs

Apart from being the major components of ethnomedicines, medicinal plants have also been major sources of drugs used in the orthodox medical practice. It is reported that about 74% of plant-derived drugs which are currently in the market were actually derived from the indigenous knowledge of traditional people on ethnomedicines (Farnsworth *et al*., 1985).

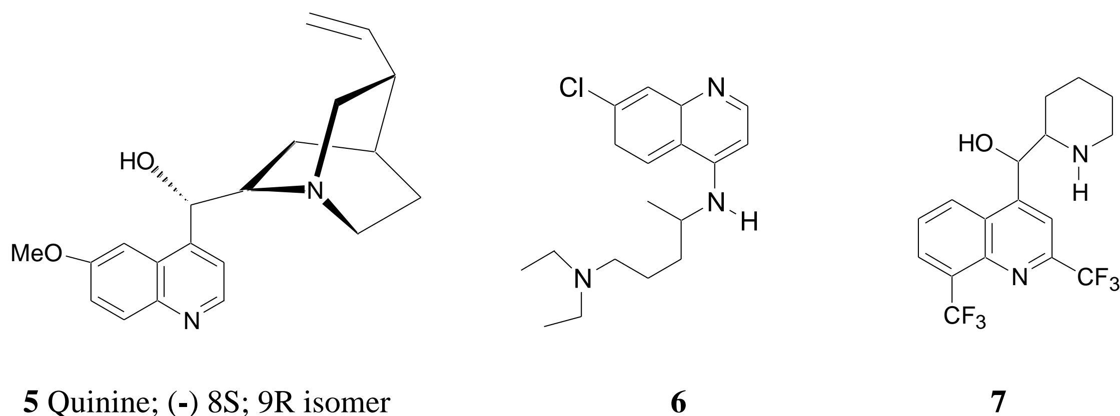
The first-generations of plant medicine were simple botanical materials employed in more or less crude form. Medicinal plants such as *Cinchona* spp.*, Opium* spp*, Belladonna* spp. and *Aloe* spp. were selected based on empirical evidence as gathered by traditional practitioners (Iw*u et al.,* 1999). The second-generation of phytopharmaceutical agents were pure molecules whose compounds differ from the synthetic therapeutic agent only in their origin, for example taxol from *Taxus spp.,* quinine from *Cinchona* spp. and reserpine from *Rauvolfia* spp. (Iw*u et al.,* 1999). In the development of third generation of plant medicine, the formulation is based on well-controlled clinical and toxicological studies of phytomedicine to improve the quality, efficacy, stability and the safety of the preparations (Akerere, 1993).

Numerous secondary metabolites have been isolated from different plants and have provided chemical templates for developing more potent analogs. A good example is the anti-malarial artemisinin (**1**) originally isolated from the Chinese plant *Artemisia annua* L. that enabled the synthesis of more potent and water soluble artemether (**2**), arteether (**3**) and sodium artesunate (**4**) (Peters, 1987).



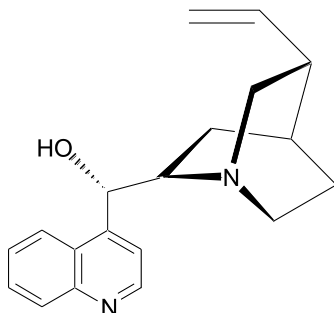
1 **2** R= CH_3 **4**
3 R= CH_2CH_3

Quinine (**5**) was first isolated from *Cinchona officianalis* L. barks in 1820 by Polletier and Coventou. This drug has been in use for many years as an antimalarial (Foye *et al*., 1989). Soon after the Second World War a large number of derivatives were synthesized and gave birth to more potent drugs such as chloroquine (**6**) and mefloquine (**7**). Other clinically useful natural products from this plant include quinidine (**8**), cinchonine (**9**) and cinchonidine (**10**).



5 Quinine; (**-**) 8S; 9R isomer **6** **7**

8 Quinidine (+) 8S; 9R isomer



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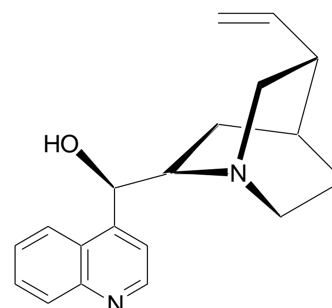

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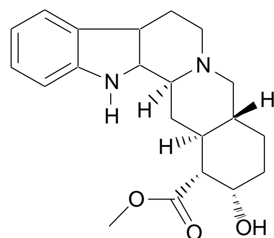
Africa is one of the main world producers of medicinal plants. Yohimbine (**11**), an indole alkaloid isolated from *Corynanthe pachyceras* K. Schum. (Elujoba *et al*., 2005) native to Ghana is a stimulant, an antiviral and is used to treat male impotence. The isoquinoline alkaloid, michellamine B (**12**) which was recently isolated from *Ancistrocladus abbreviatus* Airy Shaw. from Cameroon and Ghana has anti-HIV properties (Elujoba *et al*., 2005). *Agave sisalana,* a Tanzanian export plant is used in manufacturing of steroidal drugs like corticosteroids and oral contraceptive. *Prunus africana* is exported by Cameroon, Kenya and Madagascar and it is used for prostate gland hypertrophy (Elujoba *et al*., 2005).



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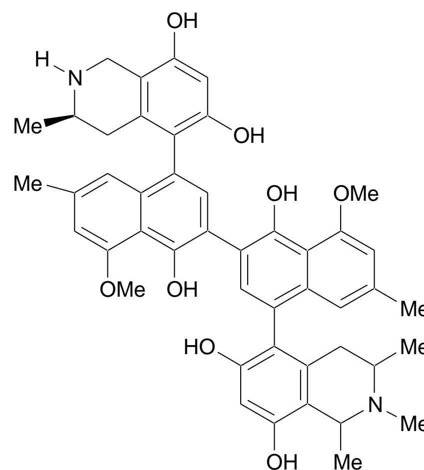

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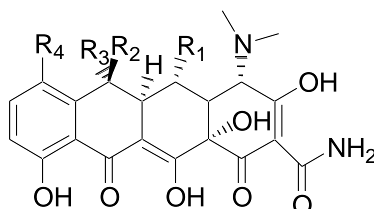
Vacines have not been developed for many of the bacterial infections. So far only Bacillus of Calmette and Guerin (BCG) vaccine against tuberculosis has been developed. This makes new chemotherapeutic agents necessary as many bacteria have developed resistance to many of the the antibacterials (WHO, 1996).

1.4 Antibacterial drugs

1.4.1 Tetracyclines

Tetracyclines (**13-18**) are are a group of broad spectrum orally active antibiotics produced by cultures of *Sacromyces species*. Tetracyclines act by inhibiting protein synthesis after uptake by active transport. However, many strains of organisms have become resistant to these agents hence decreasing their usefulness. Tetracyclines are known to chelate calcium and depositing in growing bones and teeth causing staining and sometimes dental hypoplasia. High doses of tetracyclines are also known to decrease protein synthesis in host cells (Tucker and Webster, 2005).



13 Tetracycline R_1=R_4=H, R_2=OH, R_3=Me

14 Doxytetracycline R_1=OH, R_2=R_4=H, R_3=Me

15 Methacycline R_1=OH, R_2= =CH_2, R_3=Me, R_4=H

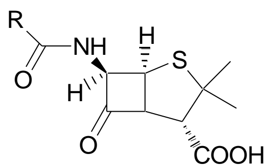
16 Chlorotetracycline R_1=H, R_2=OH, R_3=Me, R4= Cl

17 Oxytetracycline R_1= R_2=OH, R_3=Me, R_4=H

18 Democyclocycline R_1=R_3=H, R_2=OH, R_4=Cl

1.4.2 Penicillins

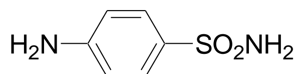
Penicillins (**19**-**24**) are β-lactam natural antibiotics which are mainly produced by *Penicillium notatum* and *P. chrysogenu* (Reynolds *et al*., 1999). The basic nucleus of penicillin is 6-aminopenicillanic acid where R can be substituted by different groups to give different properties. This is very important in the various *Staphylococcal* strains where their resistance has spread progressively. In developing countries at least 80% of *Staphylococci* now produce β-lactamase (Paterson and Bonomo, 2005). The drug has low permeability of the outer membrane of the organism hence reducing the ability of the drug to reach the target site.



	Chemical name	Other names	R
19	Pent-2-enyl penicillin	Penicillin - I or F	$-CH_2CH=CHCH_2CH_3$
20	Benzyl penicillin	Penicillin - II or G	$-CH_2$-Ph
21	p-Hydroxybenzylpenicillin	Penicillin - III or X	$-CH_2$-Ph-OH
22	N-Heptylpenicillin	Penicillin - IV or K	$-(CH_2)_6CH_3$
23	Phenoxymethylpenicillin	Penicillin - V	$-CH_2$-O-Ph
24	N-Amylpenicillin	Dehydro-F-penicillin	$-(CH_2)_4CH_3$

1.4.3 Sulphonamides

The chemically useful antibacterial sulfonamides are derived from sulfonamide by substitution on the amide moiety such sulphadiazine (**25**). Sulfonamides have some side effects such as headache, mental depression and vomiting (Tilles, 2001; Slatore and Tilles, 2004).



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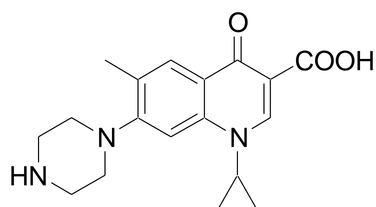
1.4.4 Fluoroquinolones

Fluoroquinolones are synthetic antibiotics, which are in clinical practice. These include the broad spectrum drugs such as ciprofloxacin (**26**), ofloxacin (**27**), norfloxacin (**28**) and pefloxacin (**29**). They are used for complicated urinary track infections in patients with gonorrhoea and bacterial prostatis (Knowles, 1997).



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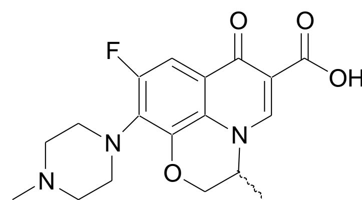

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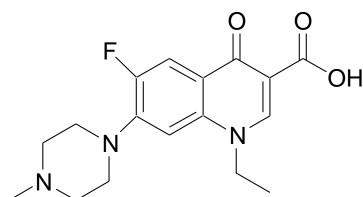

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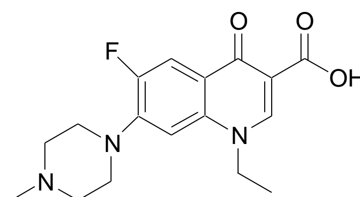

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Quinolones and fluoroquinolones are chemotherapeutic bactericidal drugs, eradicating bacteria by interfering with DNA replication. Quinolones inhibit the bacterial DNA gyrase or

the topoisomerase IV enzyme, thereby inhibiting DNA replication and transcription. Quinolones can enter cells easily via porins and therefore are often used to treat intracellular pathogens such as *Legionella pneumophila* and *Mycoplasma pneumoniae*. For many Gram-negative bacteria DNA gyrase is the target, whereas topoisomerase IV is the target for many Gram-positive bacteria (Owens and Ambrose, 2005; Ball *et al*., 1999).

Fluoroquinolones are generally well tolerated with most side effects being mild and serious side effects being rare. Some of the serious side effects common with these antibiotics include central nervous system (CNS), cardiac arryhythmias and tendon toxicity. Caffein, corticosteroids, theophylline, and nonsteroidal anti-inflamatory drugs enhance their toxicity (Owens and Ambrose*,* 2005; Ball *et al*., 1999).

1.5 Fungal infections

Fungi are classified in the kingdom fungi, which includes mushroom, moulds, yeast, slime moulds, rust and smut**.** The most common fungi responsible for systemic infections in humans are the species of *Candida* including *C. Albicans*, *C. tropicalis*. *C. guillermond* and *C. parapsilosis* (Baldry, 1976). With few exceptions pathogenicity among the fungi is not necessary for the maintenance or dissemination of the species. The ability of fungi to cause human disease appears to be an accidental phenomenon and the mycoses are primarily related to the immunological status of the host and environmental exposure, rather than to the infecting organism (Ellis, 1999).

Most fungi are unable to grow at 37 °C. Thus, thermo-tolerance to survive at this temperature is essential to fungal growth within human body. Similarly, as most fungi are saprophytic,

their enzymatic pathways function better at the redox potential of non-living substrates than at the lowered oxidation-reduction state of living tissue. However, many fungi prove to be able to surpass these two major physiologic barriers (Ellis, 1999).

Host defenses are of non-specific and specific in nature. The non-specific defenses include the antifungal activity of natural excretions, such as saliva and sweat; the protective effects of the endogenous normal microbiota of the skin and mucous membranes in competing for space and nutrients, thus limiting the growth of potential pathogens; and the mechanical barrier of the skin and mucous membranes preventing entry of fungi. Additionally, the body has the highly efficient non-specific inflammatory system to combat fungal proliferation involving the action of neutrophils, mono-nuclear phagocytes and other granulocytes. The specific or acquired immunity defending from fungal growth in tissue consist basically of the cell-mediated immunity regulated by T-lymphocytes. The role of specific antibodies or humoral immunity regulated by B-lymphocytes is not so clear (Ellis, 1999). The spectrum of fungal infections is different according to the major deficit in host defenses.

1.5.1 The genus *Cryptococcus*

The genus *Cryptococcus* is an encapsulated yeast-like fungus that can live in both plants and animals. Following its first identification in nature from peach juice samples, the major environmental sources of *Cryptococcus neoformans* have been shown to be either soil contaminated with pigeon droppings (*Cryptococcus neoformans* var. *neoformans*) or eucalyptus trees and decaying wood forming hollows in living trees (*Cryptococcus neoformans* var. *gattii*) (Callejas *et al*., 1998; Mahmoud, 1999). The genus *Cryptococcus* includes around 37 species. The species *C. neoformans* is composed of three variants: *C.*

neoformans v. gattii, *v. grubii*, and *v. neoformans*. *C. neoformans v. gattii* is found mostly in the tropics, but has also been confirmed on southern Vancouver Island on the southwestern coast of Canada (Levitz, 1991).

Among these, *Cryptococcus neoformans* is the only species that is pathogenic. *C. neoformans* is the causative agent of cryptococcosis. Given the neurotropic nature of the fungus, the most common clinical form of cryptococcosis is meningoencephalitis. Cryptococcosis may also involve the skin, lungs, prostate gland, urinary tract, eyes, myocardium, bones, and joints. The most commonly encountered predisposing factor for development of cryptococcosis is AIDS (Abadi *et al*., 1999). Less commonly, organ transplant recipients or cancer patients receiving chemotherapeutics or long-term corticosteroid treatment may develop cryptococcosis (Korfel *et al*., 1998).

The polysaccharide capsule and phenol oxidase enzyme of *Cryptococcus neoformans*, as well as its ability to grow at 37°C, are its major virulence factors. Recent data suggest that phospholipase enzymes may also play a role as one of the potential virulence factors. Phenol oxidase enzyme functions in production of melanin. The melanizing enzyme presumably prevents formation of toxic hydroxy radicals and thus protects the fungal cell from oxidative stress as well as the immune defense mechanisms of the host (Casadevall *et al*., 2000; Jacobson, 2000).

Fungi are one of the most neglected pathogens, as demonstrated by the fact that the amphotericin B, a polyene antibiotic discovered as long ago as 1956 (Casadevall *et al*., 2000; Jacobson, 2000), is still used as a gold standard for antifungal therapy. So far no antifungal

vaccine has been developed and hence new antifungal chemotherapeutic agents are necessary as many antifungals are no longer effective.

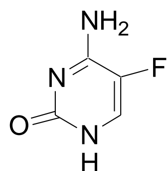
1.6 Antifungal drugs

The last two decades have witnessed a dramatic rise in the incidence of life threatening systemic fungal infections. The challenge has been to develop effective strategies for the treatment of candidiasis and other fungal diseases, considering the increase in opportunistic fungal infections in human immunodeficiency virus-positive patients and in others who are immune compromised due to cancer chemotherapy and the indiscriminate use of antibiotics.

Majority of the clinically used antifungals have various drawbacks in terms of toxicity, efficacy and cost, and their frequent use has led to the emergence of resistant strains. Additionally, in recent years public pressure to reduce the use of synthetic fungicides in agriculture has increased. Concerns have been raised about both the environmental impact and the potential health risk related to the use of these compounds. In the past few decades, a worldwide increase in the incidence of fungal infections has been observed as well as a rise in the resistance of some species of fungus to different fungicidals used in medicinal practice.

1.6.1 Flucytosine

Flucytosine (**30**) is a synthetic antifungal agent, which is active only to a few organisms, mainly those caused by yeast. When administered on its own, resistance is likely to develop; hence it is usually combined with amphotericin (**31**) for severe infections such as cryptococcal meningitis (Groll and Piscitelli, 1998). Another antifungal drug is diazole (**32**) among others.



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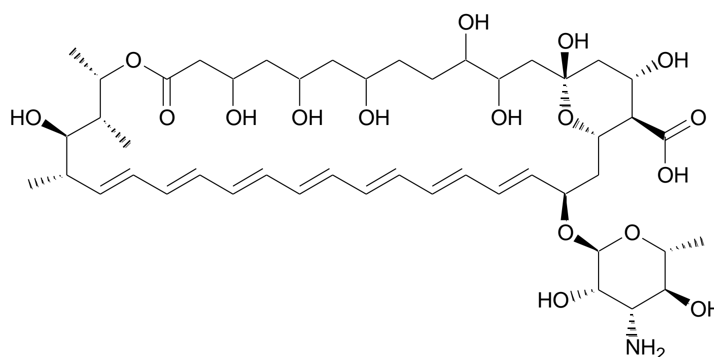

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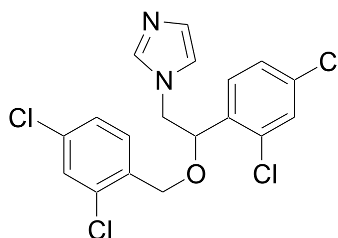

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1.6.2 Amphotericin

Amphotericin (**31**) is a macrolide antibiotic of complex structure and many carbon atoms. It acts by binding on the cell membranes hence interfering with permeability and transport functions. When administered orally amphotericin is poorly absorbed though it is the only route into the gastrointestinal tract. The commonest and most serious side effects of amphhotericin are renal toxicity, potassium loss, fever, and chills. Some degree of reduction of renal function occurs in more than 80 % of patients using the drug during the time of use (Schoffski *et al*., 1998).

1.7 Justification

Infectious diseases account for almost one-half of all deaths in tropical countries. In response to incidences of epidemics due to drug resistant micro-organisms and the emergence of unknown disease-causing microbes, there has been an enormous public health concern. Many commercially proven drugs in modern medicine were initially used in crude form in traditional medicine or for other purposes which suggested potential useful bioactivity. Phytomedicines have shown great promise in the treatment of infectious diseases including AIDS infections and cancer (Fauci, 1998).

Most of the sub-saharan population depends on traditional medicine for their primary health care (Kokwaro, 1996) and many of the drugs which are in use today were discovered through the ethnobotanical route (Farnsworth *et al*., 1985). This makes it necessary to have more research on medicinal plants so as to obtain more potent pharmacological agents (WHO, 2000). Although the phytochemical and antimicrobial screening tests have been done on the genera *Echinops* and *Grewia*, little has been reported on *Echinops hispidus* and *Grewia similis*. There is therefore need to carry out further studies on the two said plant species.

1.8 Hypotheses

The hypotheses of the study were;

i). Plants in the same genus or family have similar antimicrobial properties and compounds.

ii). The bioactive compounds are stable enough to be extracted, isolated and identified.

iii). The biological activity of medicinal plants is due to the secondary metabolites which remain bioactive after isolation from the plants.

1.9 Objectives of the study

1.9.1 General objective

The aim of this research was to extract, isolate, characterize and identify the bioactive compounds with anti-microbial properties from the plants *E. hispidus* and *G. similis*.

1.9.2 Specific objectives

i). To carry out sequential extraction of the roots of *E. hispidus* and *G. similis* using solvent extraction of increasing polarity (hexane, DCM and EtOAc).

ii). To carry out antifungal and antibacterial assays of the crude extracts of *E. hispidus* and *G. similis.*

iii). To isolate compounds from the crude extracts of *Echinops hispidus* and *Grewia similis* using chromatographic techniques (VLC, TLC, CC (silica gel), preparative-TLC and sephadex LH-20.

iv). To determine the structures of the isolated compounds using spectroscopic techniques (IR, UV, MS, ^{1}H-NMR and ^{13}C-NMR and 2D NMR for absolute assignment).

v). To carry out antifungal and antibacterial boiassays of the isolated compounds.

CHAPTER TWO

LITERATURE REVIEW

2.1 The family Asteraceae

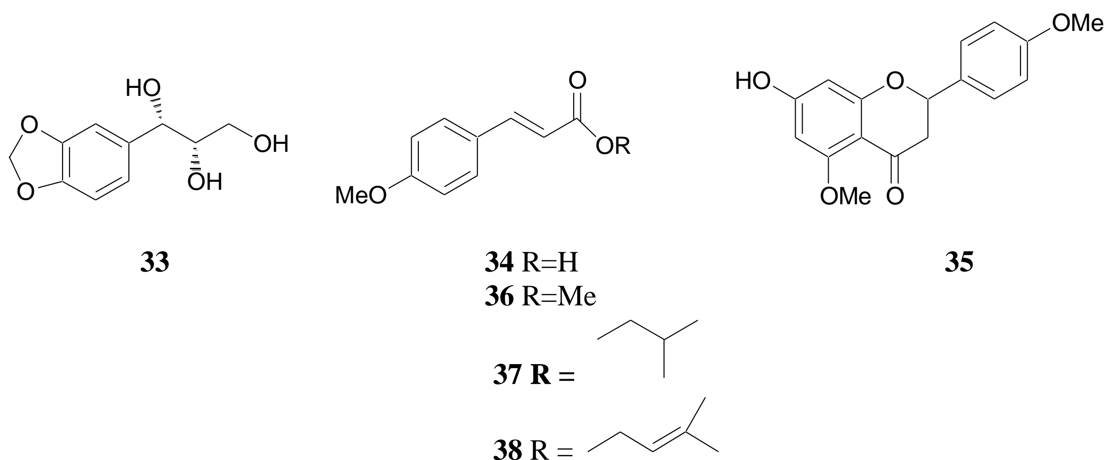
The family Asteraceae (previously Compositae) is one of the largest families of flowering plants with about 1100 currently accepted genera and 25000 species (Heywood, 1977). It is of worldwide distribution particularly in semi-arid region of the tropics and subtropics. Most members of the family are evergreen shrubs or sub-shrubs or perennial rhizomatous herbs; biennial and annual herbs are also frequent. It is generally accepted that Asteraceae is a "natural" family with well established limits and a basic uniformity of floral structure

imposed on all members by the common possession of characters such as the aggregation of the flowers into capitula and the special features of the Stamen and corolla (Zareh, 2005).

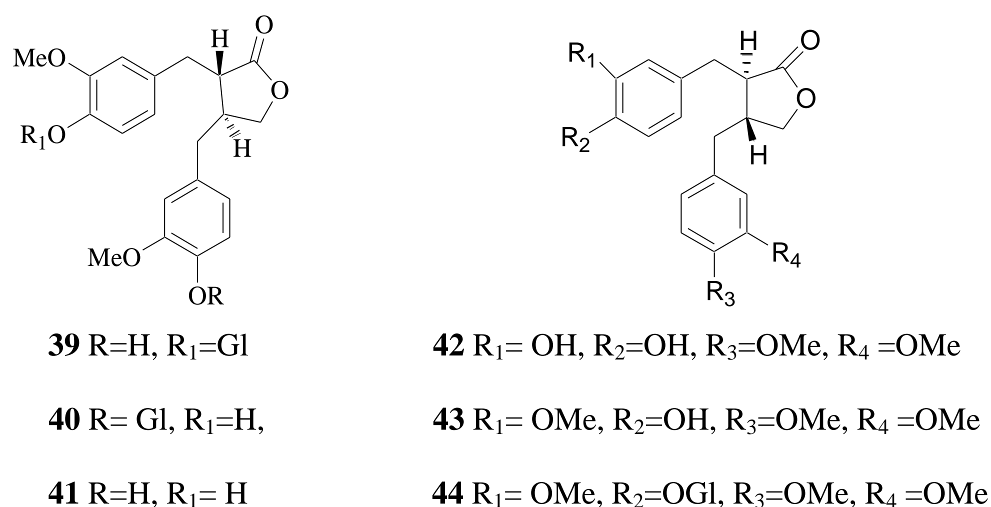
The economic value of the representatives of this family can be divided into various categories: food for man and other animals, poisonous, weeds, medicinal, wood, garden plants and cut flowers. The most well known food product of the family is certainly sunflower oil and kernels. Other well-known foods are Jerusalem and French artichokes, lettuce, chicory and herbal teas like camomile. Well-known medicinal plants are the African wormwood (*Artemisia afra*), wild rosemary (*Eriocephalus africanus*) and wild camphor bush (*Tarchonanthus camphoratus*).

2.1.1 Phytochemistry and pharmacology of the family Asteraceae

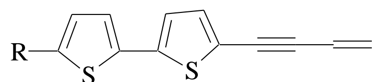
Many biologically active compounds have been isolated from the members of this family. Bioassay-guided purification of *Dendranthema zawadskii* Var. resulted in the isolation of (1*S*,2*S*)-1,2,3-trihydroxy-1-(3,4-methylenedioxyphenyl) propane (**33**), 4-methoxycinnamic acid (**34**), acacetin (**35)** and caffeic acid methyl ester (**36**) (Rahman and surk-sik, 2007). Compounds (**33**) and (**34**) showed significant activities against *Candida* species (inhibition zone: 9.5-13.0 and 9.0-12.0 mm, respectively (Rahman and Surk-sik, 2007). Compound (**34**) showed a moderately broad spectrum of antibacterial activity against both Gram-positive and Gram-negative bacteria, with inhibition zones in the range of 9.4-12.6 mm; whereas, compound (**35**) showed weak activities against both *Staphyloccocus aureus* and *S. sonnei* (Rahman and Surk-sik, 2007). Caffeic acid esters; isobutyl-3,4-dihydroxycinnamate (**37**) and 2-methyl-2-butenyl-3,4-dihydroxy cinnamate (**38)** are reported from the aerial parts of *Plazia daphnoides* Wedd. (Rahman and Surk-sik, 2007).



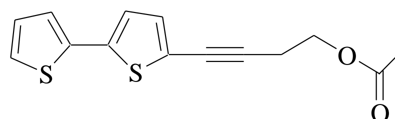
The lignans matairesinoside (**39**), arctiin (**40)** and Matairesinol (**41)** are reported from the genus *Centaurea* (Kamkaen *et al*., 2006). These compounds were reported to be toxic towards brine shrimps. The lignans 3'-dimethyl arctigenin (**42**), arctigenin (**43**) and arctiin (**44**) are reported from the seeds of *Arctium lappa* Linn. Compounds (**42)** and (**43)** exhibited 100% inhibition at a concentration of 50 μg/ml against *Helicobacter pylori* compared to standard amoxicillin 5 μg/ml (Kamkaen *et al*., 2006).



The thiophene derivatives 5-(3-butene-1-ynyl)-2,2'-bithiophene (**45**), 5'-methyl **-**5- (3-butene-1-ynyl) -2,2'-bithiophene (**46**) and 5-(4-acetoxy-1-butnyl)-2,2'-bithiophene (**47**) have been isolated from *Tagetes patula* L. Compound (**47)** is reported to have high % mortality of *Saccharomyces cerevisiae* (Margl, 2001).



45 R= H
46 R=Me



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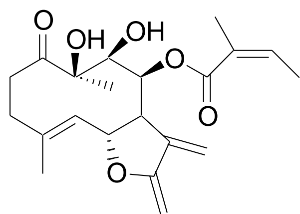
The extract of the flowers of *Euphantrium betonicaeforme* D.C. afforded the secondary metabolite 8β-angeloyloxy-9β,10β-dihydroxy-1-oxo-germacra-4*E*,11(13)dien-12,6α-olide (**48**), 3β-hydro- xyeicosan-1,5β-olide (**49**), 7-*O*-methyl kaempferol (**50**), kaempferol (**51**), and nepetin (**52**) are reported from the same plant (Agrawal *et al*., 1989; Ahmad *et al*., 1994). The hexane extract of the aerial parts of the same plant afforded taraxasteryl acetate (**53**) and α- amyrin (**54**) and β-amyrin (**55**) (Ahmad *et al*., 1994). The hexane extract of roots of the same plant yielded a mixture of β-sitosterol (**56**) and β-stigmasterol (**57)** (Agrawal *et al*., 1989)



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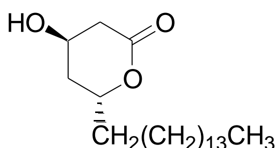

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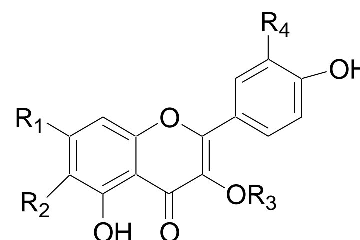
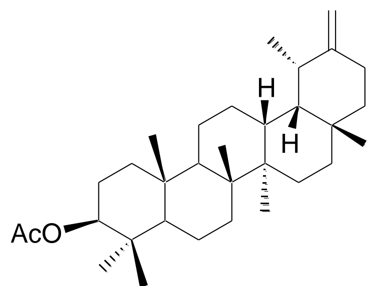


50 R_1=CH_3, R_2=R_4=H, R_3=H

51 R=R_2=R_4=H, R_3=H

52 R_1=R_3=H, R_2=CH_3, R_4=OH



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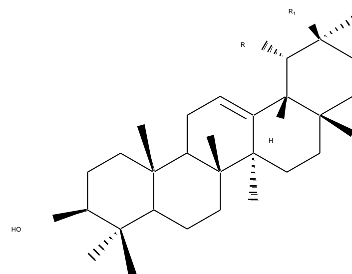
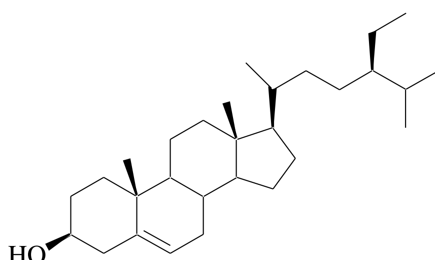


54 R=CH_3, R_1=H

55 R=H, R1=CH_3



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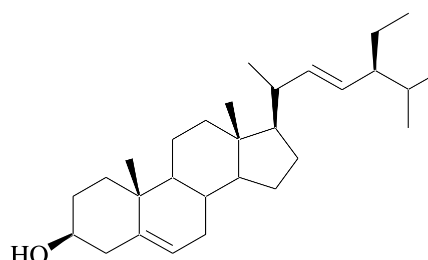

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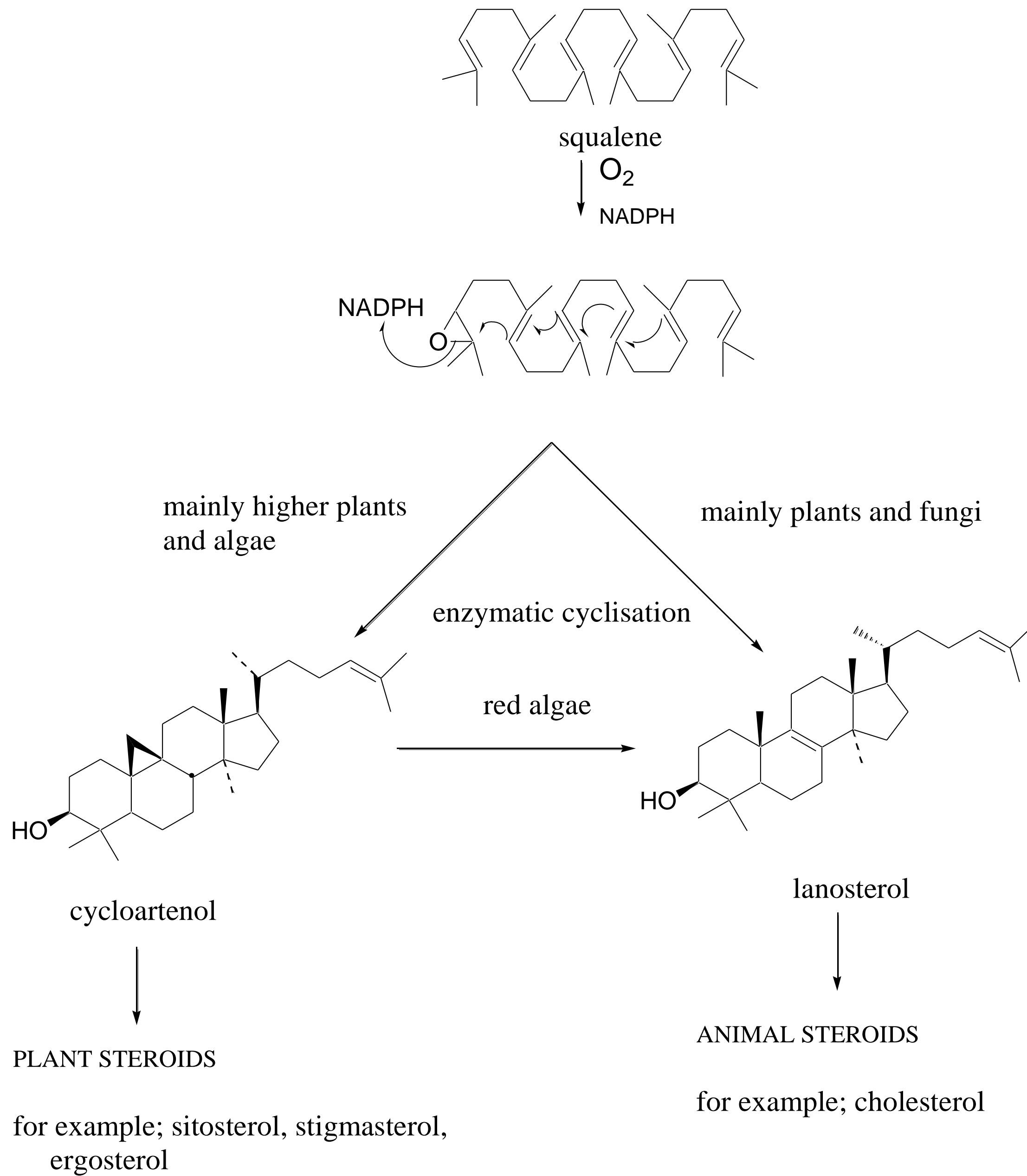
The 3β-sitosterol (**56**) and 3β-stigmasterol (**57**) isolated from the root extract of *Pluchea indica* Less. is reported to neutralize viper and cobra venom. The sterols (**56**) and (**57**) may play an important role, along with antiserum, in neutralizing snake venom-induced actions (Gomes *et al*., 2007). Both (**56**) and (**57**) from *Achillea ageratum* L. (Asteraceae), exhibited a high degree of growth inhibition (anti-tumour property) against Hep-2 cells and McCoy cells, compared with the solution used as a positive control. The anti-cancer activity of (**56**) was 8.39 µg/ml against Hep-2 cells and 7.19 µg/ml against McCoy cells while that of (**57**) was 7.08 µg/ml against Hep-2 and 6.14 µg/ml McCoy cells, respectively. The Dl50 for these two compounds were very near the accepted activity for natural products (6 µg/ml) (Gomez *et al*., 2001).

The isolated sterols from *Achillea ageratum* L. (**56**) and (**57**) with doses of 0.5 mg/ear significantly reduced the 12-*0*-tetradecanoylphorbol acetate (TPA)-induced mouse ear edema with doses of 1 and 3 mg (59% and 65% respectively). In the chronic model the anti-inflammatory effect generally was a more moderate one. The effect decreased to 36% with (**57**), and 40.6% with (**56**) chronic model (Gomez *et al.*, 2001).

2.1.2 Biosynthetic pathway of steroids

The natural steroids are derived by a series of chemical transformations, from the two parent triterpenes, lanosterol and cycloartenol. The biosynthesis of all the natural steroids is believed to proceed from acetic acid to lanosterol (or cycloartenol) through mevalonic acid and squalene. It is generally recognized that all steroids in animals originate from lanosterol, whilst cycloartenol is the precursor of the steroids in plants. The biosynthesis of 3β-sitosterol and 3β-stigmasterol is summarized in scheme 2.1.2 below (Swartz, 2006).



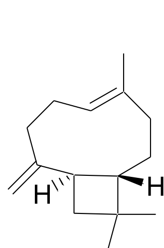
Scheme 2.1.2: Biosynthesis of lanosterol and cycloartenol (Swartz, 2006)

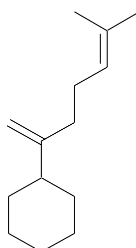
2.1.3 The genus *Echinops*

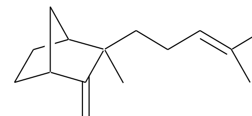
This genus belongs to the family Aesteraceae and comprises 120 species (Hymete *et al*., 2005). Members of this genus are widely used in Ethiopian herbal medicine for treatment of various diseases and illnesses such as migraine, diarrhoea, heart pain, intestinal worms and hemorrhoid (Hymete *et al*., 2005). The methanol extract of the leaf of *E. ellenbeckii* O. Hoffm. and the leaf and stem of *E. longisetus* A. Rich. are reported to have a strong inhibitory activity against *Staphylococcus aureus*. The activities were at 5 μg/μl for the leaf of *E. ellenbeckii* A. Rich. and 2.5 μg/μl for leaf and stem of *E. ellenbeckii* (Hymete *et al*., 2005). The flower extract of *E. ellenbeckii* is reported to have a strong inhibitory activity against *Candida albicans* (Hymete *et al*., 2005). The root and flower extracts of the two plants have shown lethal activity against earthworms. Moreover, the extracts of the roots of the two plants have shown molluscicidal activity of 100% at 20.25 and 45 μg /μl, respectively (Hymete *et al.*, 2005).

The volatile fractions of *E. grijsii* Hance. and *E. giganteus* A. Rich. are reported to contain tricyclic sesquiterpenes such as β-caryophyllene (**58**), β-bisabolene (**59**), β-santalene (**60**) and guaine **(61)**. Sesquiterpene lactones such as α-and β-caryophyllene epoxide (**61)** and **(62**), respectively, are also reported from this plant. Caryophylene is reported to have an anti-inflammatory, anti-bacterial (*Staphylococcus*), anti-fungal, anti-carcinogenic (MIC=1600 μg/ml, anti-edemic and anti-feedant (500 ppm) activity while bisabole has anti-viral activity, IC_{50} 1800 μg/l). Santalene is reported to have anti-inflamatory activity while caryophylene epoxide is reported to have anti-tumour activity (Weyerstahl *et al*., 1998). The major constituents reported from the essential oil of *E. giganteus* var. *lelyi* C.D. are the

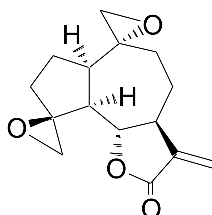
sesquiterpoenoids; (-)-silphiperfol-6-ene (26.9%) (**63**), (-)-presilphiperfol-7-ene (9.4%) (**64**), β-caryophylle- ne (**65**) and (-)-cameroonan-7α-ol (6.4%) (**66**) (Weyerstahl *et al*., 1998).

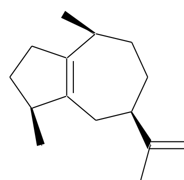


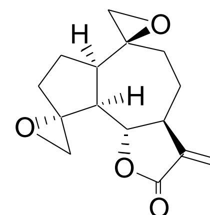




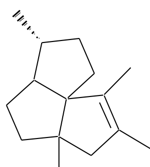
58 **59** **60**

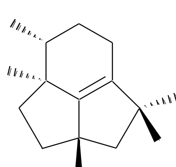


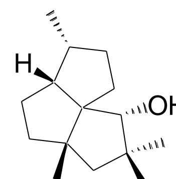




61 **62** **63**



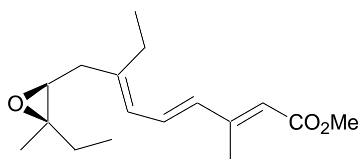


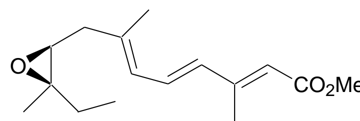


64 **65** **66**

Many sesquiterpenes, besides compounds which are toxic to mammals (such as poicrotoxin and its derivatives) or which have antibiotic or phytotoxic power (such as trichothecin), are extremely interesting from a physiological point of view. Phytohormones of plants and the juvenile hormones of insects are the two groups to be exemplified. The hormone regulated growth of the higher plants takes place through the balanced action of stimulating and inhibitory hormones. Such hormones are often terpenoids. The gibberellins stimulate such growth, whilst abscisic acid (ABA) and its derivatives all of which are sesquiterpenes are inhibitors. The physiological role of the insect juvenile hormones, the first such hormone to

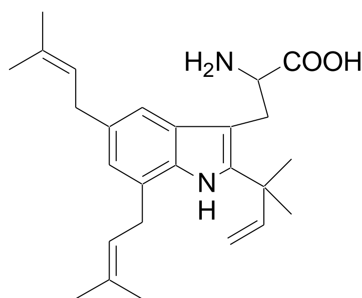
be isolated, which came from tens of thousands of male *Hyalophora cecrepia* L. butterflies, was neotenin (**66**), which is the methyl ester of 10,11-epoxy-7-ethyl-3,11-dimethyl-10,11-*cis*,2-*trans*,6-*trans*-tridecadienoic acid (JH). A second juvenile hormone is reported from *H. acropia* which has a methyl group instead of an ethyl group at C-7 (**67**) (Chaudhuri, 1992).

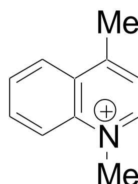


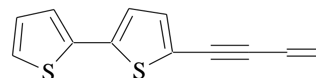


66 **67**

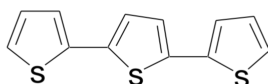
The alkaloids, echinine (**68)** has been reported from the seeds of *E. ritro* L. while echinorin (**69)** is reported from *E. ritro* and *E. sphaerocephalus* L. while Echinozolinone and 7–hydroxy echinozolinone with unknown structures are reported in *E. echinatus* Roxb. (Dopke and Fritsch 1969; Schroder and Luckner, 1968; Chaudhuri, 1992). The dichoromethane extract of the root of *E. ritro* of the Greek flora has shown antifungal activity and bioassay-guided fractionation of this extract led to isolation of thiophenes 5-(3- Buten-1-ynyl)-2,2'-bithiophene (**70)**, α-terthienyl (**71**) and 2-[pent-1,3-diynyl]-5-[4-hydroxybut-1-ynyl] thiophene (**72**) (Fokialakis *et al*., 2005). When assayed at 3 and 30 μM, respectively they are reported to be active against three *Colletotrichum* species, *Fasparium oxysporum, Phomopsis viticola*, and *P. obscuraus* (Fokialakis *et al*., 2005).

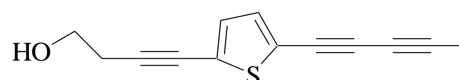






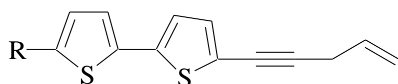
68 **69** **70**

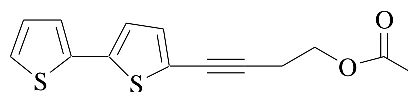




71 **72**

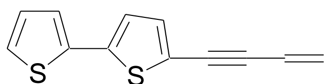
Echinops ritro L., *E. spinossimus* Turra., *E. albicaulis* Kar and Kir. and *E. transiliensis* Golosh. are reported to have termiticidal activity against *Coptotermes formosanus.* The bioactive compounds isolated were (**71**) and (**72**) with 100% mortality against *C. formosanus* termite at 1 and 2 % (wt/wt) concentrations, respectively (Fokialakis *et al*., 2005). The thiophene derivatives 5-(3-butene-1-ynyl)-2,2'-bithiophene (**72**), 5'-methyl (**-**5-(3-butene-1-ynyl)-2,2'-bithiophene (**73**) and 5-(4-acetoxy-1-butenyl)-2,2'-bithiophene (**74**) have been isolated from *Tagetes patula* L. (Margl, 2001). Compound (**74)** is reported to have high % mortality of *Saccharomyces cerevisiae.* 5'-(3-Buten-1-ynyl)-2,2'-bithiophene (**75)**, α-terthienyl (**71**), 2-[Pent-1,3-diynyl]-5-[4-hydroxybut-1-ynyl] thiophene (**76**) and thiophene (**77**) isolated from *Echinops ritro, E. spinossimus*, *E. albicaulis* and *E. transiliensis.*





73 R= H
74 R=Me

75



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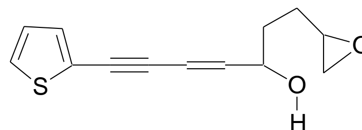
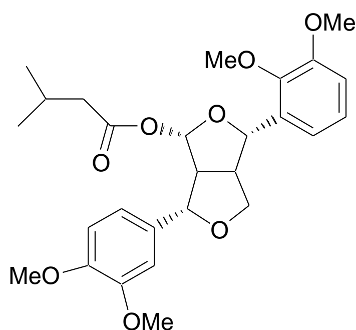


77

The lignans, (+)-4-(3-methylbutanoyl)-2,6-di(3,4-dimethoxy)phenyl-3,7-dioxabicyclo [3.3.0] octane (**78**) and (+)-4-hydroxy-2,6-di(3,4-dimethoxy)phenyl-3,7-dioxabicyclo [3.3.0] octane derivative (**79**) are reported from *E. gigateus* (Tene *et al*., 2004). The furofuran lignan pestaslignolide A (**80**) is reported from the leaves of *Petasites japonicas* Maxim. with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scarvaging activity, IC_{50} of 113.04 μg/ml while that of alpha-tocopherol was 25.65 μg/ml (Min *et al*., 2005). Pluviatilol (**81**) and (+)-syringaresinol (**82**) are reported from the roots of *Ainsliaeae acarifolia* Y. Harada. with cytotoxic activities of ED_{50} = 21.13 and 17.85 against SK-OV-3 human tumor cells, respectively (Choi *et al*., 2006).



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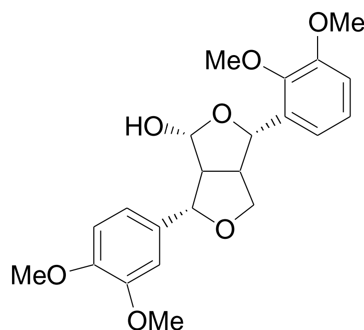

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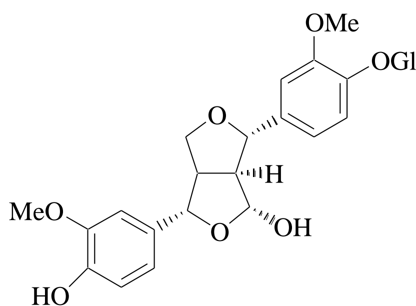
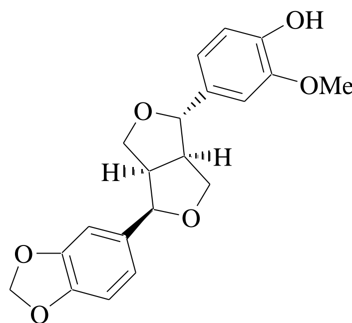
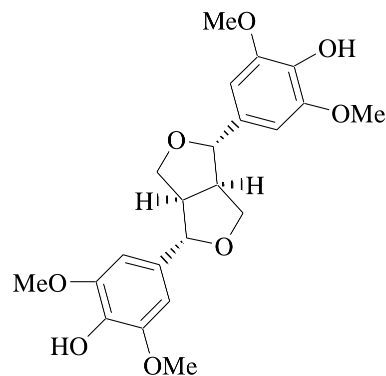
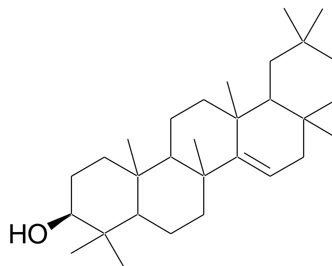






80 **81** **82**

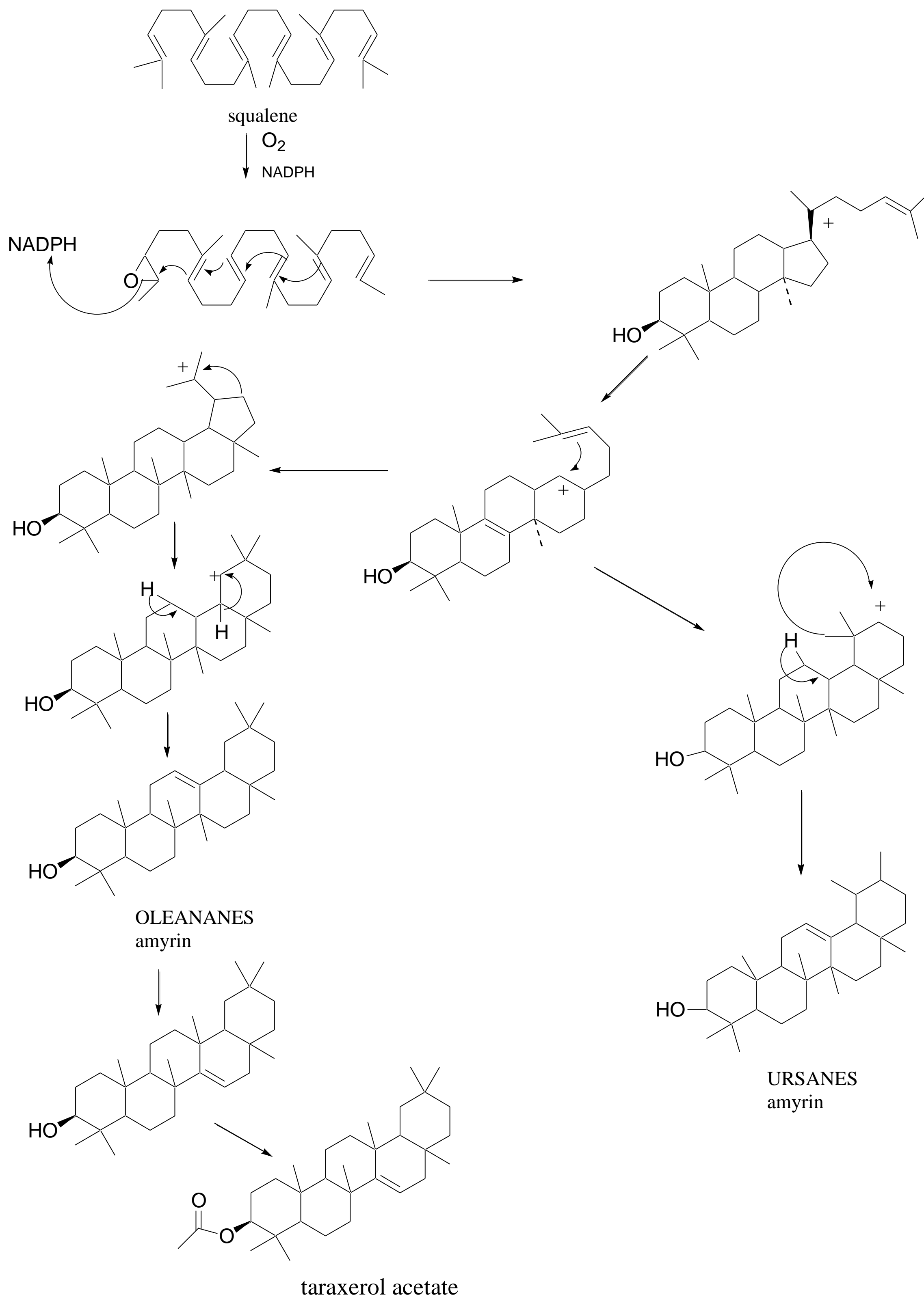
The triterpenoid, taraxerol (**83**) isolated from the roots of *Taraxacum japonicum* Koidz. exhibited significant inhibitory effects on Epstein-Barr virus early antigen (EBV-EA) induced by the tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), in Raji cells as a primary screening test for anti-tumor-promoters (cancer chemopreventive agents). It also exhibited potent anti-tumor-promoting activity in the two-stage carcinogenesis tests of mouse skin using 7,12-dimethylbenz[α] anthracene (DMBA) as an initiator and TPA as a promoter. The inhibitory effects of its acetate were weaker than those of taraxerol. These results strongly suggested that taraxasterol could be a valuable chemopreventive agent (Takasaki *et al*., 1999).



83

2.1.4 Biosynthetic path way of taraxerol acetate

The natural triterpenoids are derived by a series of sophisticated chemical transformations, from the two parent triterpenoids, lanosterol and cycloartenol. These reactions include the non-classical cations, molecular rearrangements hydride ion or methyl group shift and anti-Markownikoff's addition. The biosynthesis of acetyl taraxerol is summarized in scheme 2.1.2 below (Swartz, 2006).



Scheme 2.1.2: Biosynthesis of taraxerol acetate

2.1.5 Distribution and ethnobotany of *Echinops hispidus*

E. hispidus Fresen. is locally distributed between 1500-2200 m above sea level. It is common in the highlands of Kitale, Cherangani, Elgon, Tinderet, Kisii and Narok. The Kipsigis use it to treat venereal diseases. There is no work reported on the phytochemical and biological screening of the extracts of this plant.

2.2 The family Tiliaceae

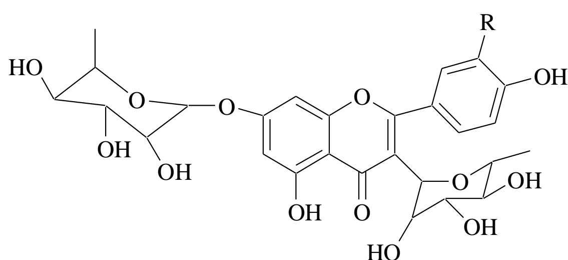
The family Tiliaceae (previously Malvaceae) comprises about 243 genera and 4225 species in the new phylogenetic circumscription (http://www.mobot.mobot.org). Members of this family are widespread in many regions of the world and particularly in tropical regions (Heywood, 1997). Many species of Tiliaceae are known by their different uses in folk medicine such as diuretic, in treatment of rheumatism, gastrintestinal disorders, snakebites, asthma and anti-inflammatory effects (Albuquerque da Costa *et al*., 2007).

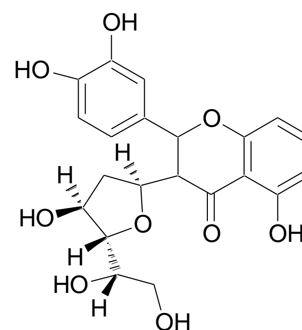
Plants belonging to this family are economically useful, as they provide an excellent source of naturally occurring fiber, like cotton from the genus *Gossypium*, and certain foods like okra, from *Abelmoschus esculentus*, which is used as a vegetable. The stem and roots of *Hibiscus tiawanensis* S.Y.Hu., native to Taiwan, have anti- inflammatory and antipyretic properties (Albuquerque da Costa *et al*., 2007). The dried leaf extract of *Hibiscus sabdariffa* acts as a anti-hypertensive by inhibition of angiotensin-converting enzyme (ACE). The plants of the *Hibiscus* sp. have been used as an antidote for chemical and wild mushroom poisoning as well as an Ayurvedic herbal shampoo in Indian medicine (Albuquerque da Costa *et al*., 2007).

Although some phytochemical and anti microbial studies have been done on this family, it has not been extensively studied yet (Toker *et al*., 2004). Lime or linden flowers, *Tiliae flos*, are of prominent importance in phytotherapy. They are stated to possess expectorant, diuretic, diaphoretic, antispasmodic, stomachic and sedative activities and have been used for the treatment of flu, cough, migraine, nervous tension, and ingestion problems, various types of spasms and liver and gall bladder disorders (Toker *et al*., 2004). The medicinal properties claimed for the drug have been attributed to its flavonoids, volatile oil and mucilage components. The use of the leaves as a remedy is not as common as that of the flowers, but they have been suggested to be employed as a diaphoretic. In European Pharmacopoeia (EP), the inflorescence of *Tilia platyphyllos* Scop. and *T. cordata* Miller. are considered official. *Tilia rubra* D.C. and *T. argentea* Desf. are widespread and are used for similar purposes in Turkish folk medicine (Toker *et al*., 2004).

2.2.1 Phytochemistry and pharmacology of the family Tiliaceae

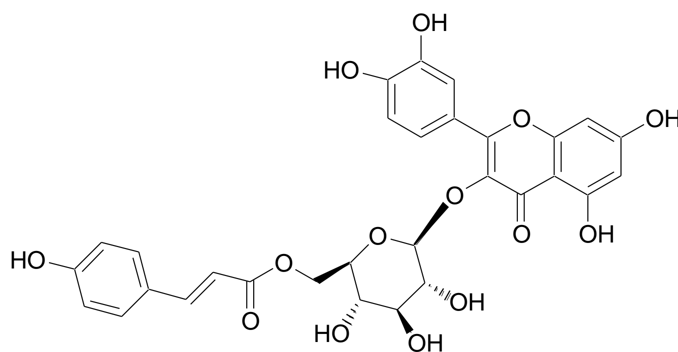
From the *T. argentea* Desf. leaves, flavonoid; kaempferol-3,7-*O*-α-L-dirhamnoside (**86**) and quercetin-3,7-*O*-α-L-dirhamnoside (**87**) were reported. The two flavonoid glycosides, showed signicant anti-inflammatory and antinociceptive activities in mice (Toker *et al*., 2004). These are also reported from *T. platyphyllos* Scop. and *T. rubra* D.C. bracts and leaves. The flavonoids; isoquatrin (**88**), rutin (**89**) and the insect antifeedant, tiliroside (**90**), were reported from the leaves of the same plant (Toker *et al*., 2004).





86 R= H
87 R= OH

88



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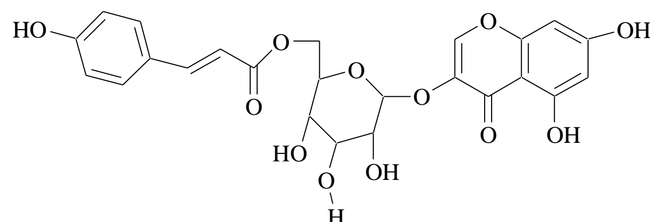
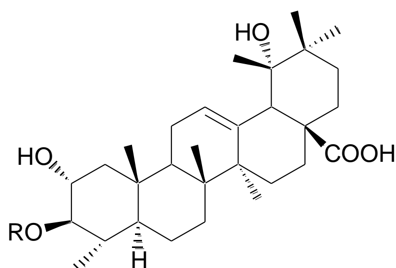
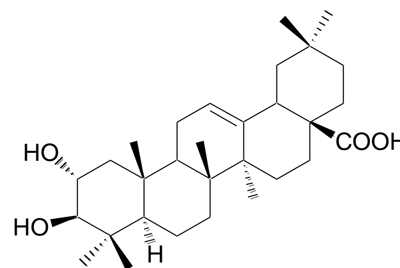


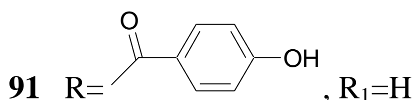
90

Luehea divaricata Mart. is a tree which grows in Brazil, Argentina and Paraguay. The *L. divaricata* is used in Brazilian folk medicine for different purposes. The leaves are used as diuretic and the stems as anti-inflammatory. The bark and aerial parts are used for healing skin wounds, pimples, and for vaginal washes. *Luehea divaricata* exhibited a broad spectrum of activity against dermatophytes. The aqueous extract of *L. divaricata* presented genotoxic

activity in the Ames test (*Salmonella*/microsome) with microsomal activation (Tanaka *et al*., 2003). Flavonoids, tannins and saponins are reported from the phytochemical screening of *Luehea divaricata.* The triterpene α-amyrin (**54**), 3β-*p*-hydroxyl benzoyloxy- tormentic acid (**91**) and its derivatives (**92** and **93**), maslinic acid (**94**) are reported from the methanolic extract of *L. divaricata* (Tanaka *et al*., 2003)





91 R= 

, R_1=H

94

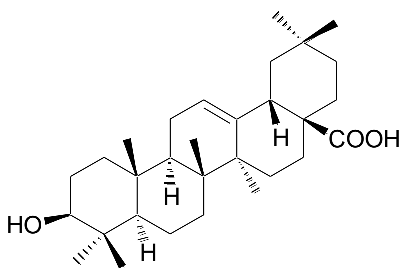
92 R=H, R_1= β-D-glucopyranosy

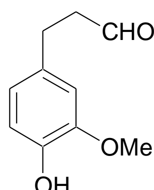
93 R=R_1=H

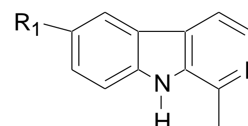
2.2.2 The genus *Grewia*

Grewia sp. (Tiliaceae) is a genus of flowering plants found in tropical and temperate regions as well and comprises of 29 species (Mulholland *et al*., 2002). Members of the *Grewia* species are used in the treatment of diarrhoea, gonorrhoea, urinary troubles, and irritation of the bladder, cancer, hookworms, snakebites, stomach pains, fever, dysentery, intestinal infestations and syphilis in many parts of Africa. In Indian this genus is used to cure pneumonia, bronchitis and urinary infections (Jaspers *et al.,* 1986; Badami *et al*., 2003).

Grewia asiatica Wild. was initially cultivated mainly for its sour fruits until its high medicinal value was discovered. The fruits of this plant are used as an astringent, an anti-inflammatory agent, for blood disorders, and a fever reducer. The bark is used medicinally for the treatment of diarrhoea. *Grewia occidentalis* L. is widely used for a wide range of medicinal and magical purposes, including the facilitation of childbirth in Natal, South Africa. The active compounds reported are oleanolic acid (**95**) and conififeraldehyde (**96**) (Mulholland *et al*., 2002).







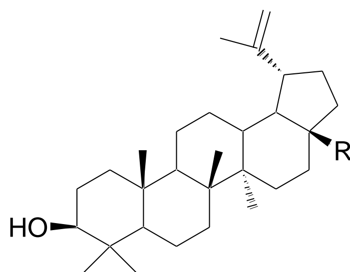
95

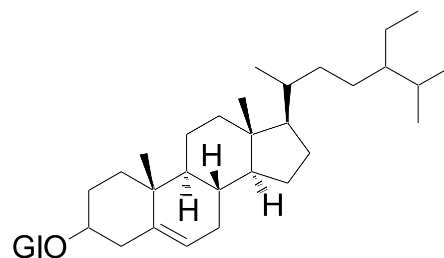
96

97 R_1= H
98 R_1= OMe
99 R_1= OH

The roots and bark of *G. villosa* Willd. have been used to treat diarrhoea, gonorrhoea, urinary troubles, irritation of the bladder and cancer (Jaspers *et al.,* 1986; Badami *et al*., 2003). *Grewia bicolor* Juss. is a part of Sudanese traditional medicine, and is used in the treatment of skin lesions and sometimes also as a tranquilizer (Yadav, 1999). The alkaloids; harman (**97**), 6-methoxy- harman (**98**) and 6-hydroxyharman (**99**) are reported from the methanol extract of *G. bicolar*. The methanol extract showed activity against Gram-positive and Gram-negative bacteria. It also exhibited a strong contraction of a rat uterus (Jaspers *et al*., 1986). The roots are used for the treatment of hookworms, snakebites, stomach pains, fever and dysentery while the barks are used for the intestinal infestations and syphilis. Chemical

investigation of the leaves of *G. damine* yielded lupeol (**100**), sitosterol β-D-glucoside (**101**), vitexin (**102**) and isovitexin (**103**) (Jayasinghe *et al.*, 2004).

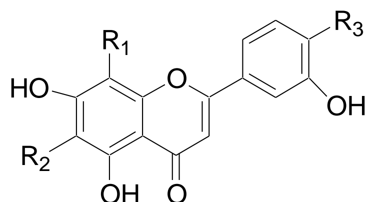


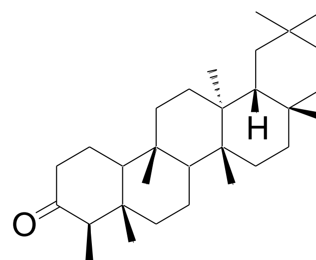


100 R = Me

104 R = CH_2OH

101 Gl = Glucosyl





102 R_1= Gl, R_2=R_3= H

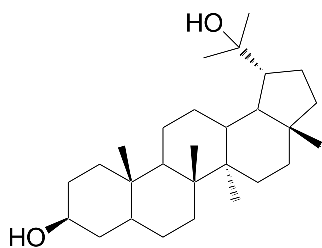
103 R_1= R_3=H, R_2= Gl

105

The triterpenoids, betulin (**104**), friedelin (**105**) and lupeol (**100**) are reported from *G. tiliaefolia* Vahl. barks. The cytotoxic activities (CT_{50}) for lupeol were 196, 330 and 302 μg/ml for Vero, $B_{16}F_{10}$ and HEP-2 cell lines (Badami *et al.*, 2003). The stem barks and roots of *Grewia tiliaefolia* are widely used in traditional Indian medicine to cure pneumonia, bronchitis, skin diseases, hypertension, ulcers, diarrhoea and urinary infections. Minimum inhibitory concentrations (MIC) of petroleum ether, chloroform and methanolic extracts are reported as 120, 150 and 210 μg/100 μl, respectively. The minimum inhibitory concentrations for the isolated steroids, β-sitosterol (**56**) and stigmasterol (**57**) and

triterpenoid, lupeol (**100**) are reported to be 80, 70 and 30 μg/100 μl, respectively. The petroleum ether extract and lupeol showed significant zones of inhibition in the cultures of *Pseudomonas aeruginosa* Gessard. and *Klebsiella pneumonia* Klebs. (Badami *et al*., 2003).

Grewia bilamellata Gagnep is reported to have *Plasmodium falciparum* activity. The chloroform fraction showed an anti-malarial activity with IC_{50} = 2.2 and 1.7 μM to the D6 and W2 clones of *P. falcifarum,* respectively. Insignificant cytotoxicity was observed against human oral epidermoid carcinoma cells (KB) (ED_{50}>20 μg/ml). Antimalarial bioassay directed fractionation of the chloroform-soluble fraction of the methanol extract from the combination of leaves, twigs and stems led to isolation of eight lignans, two triterpenoids, a quinol derivative and a sterol glucoside. Bio-assay results indicated that the isolates 3α, 20-lupandiol (**107**), grewin (**108**), nitidanin (**109**), 2α,3β-dihydroxy-olean-12-en-28-oic acid (**110**), 2,6-dimethoxy-1-acetonylquinol (**111**) possessed an *in vitro* antimalarial activity against D6 and W2 clones of *P. falcifarum*, respectively (Ma *et al*., 2006).



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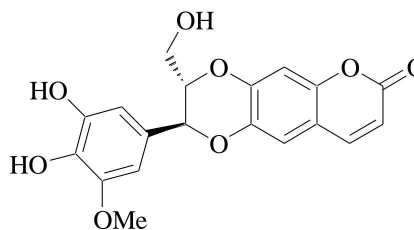

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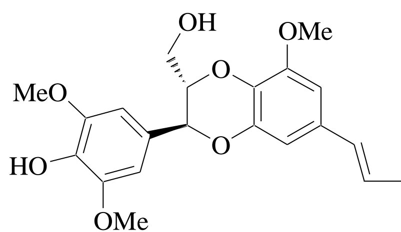
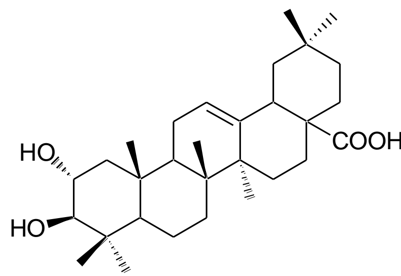
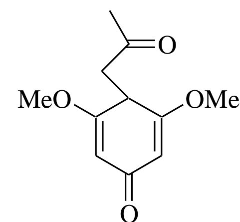






109 **110** **111**

2.2.3 Ethnobotany of *Grewia similis* K. Schum.

Grewia similis is widely distributed between 2000-7000 feet above sea level (Beentje, 1994). The Maasai use it for dental hygiene (Solio *et al*., 2006; Maundu *et al*., 2001). There is no work reported on the phytochemical and biological screening of the extracts of this plant.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant materials

The roots and aerial parts of *Echinops hispidus* Fresen. were collected in January, 2007 from near Eldoret town, Uasin-Gishu district of Rift-Valley province. The roots and aerial parts of Grewia similis K.Schum were colled from near Eldama-Ravine town, Keiyo district OF Rift-Valley province. The aerial parts were collected for the identification of the plants only. The plants were identified at the East Africa Herbarium, National Museum of Kenya and voucher specimens EH 001/2007 and GS 002/2007 were deposited at the National Museum Herbarium for future references.

3.2 General extraction

The roots of *E. hispidus* and *G. similis* were dried in an aerated room at temperatures between 15-20°C. Five kilograms (5 kg) of each plant material was then ground into fine powder using laboratory electric mill and then extracted thrice in 15 litres each of n-hexane, DCM and EtOAc starting from the least polar (hexane to pure EtOAc) for 48 hours with occasional swirling to ensure thorough extraction. The filtrates were concentrated and the solvents recovered by distillation using a rotary evaporator (Buchi Rotavapor R-205, England) under vacuum and temperature of about 45°C (Harborne, 1998). The concentrates were transferred to sample bottles, dried under vacuum and stored at – 20°C until required for bioassay and isolation.

3.3 Spray reagents

3.3.1 p-Anisaldehyde (for steroids, terpenes, flavanoids and sugars)

This was prepared by mixing 0.5 ml 4-methoxybenzaldehyde (p-anisaldehyde), 90 ml distilled ethanol (chilled for 3 hours), 5 ml (98%) sulphuric acid and 1 ml ethanoic acid.

3.3.2 Dragendoff's reagent (for alkaloids)

(a) Solution A

This was prepared by mixing 0.85 g bismuth (III) nitrate, 10 ml glacial acetic acid and 40 ml distilled water.

(b) Solution B

This was prepared by mixing 8 g potassium iodide and 20 ml distilled water.

Stock solution: This was prepared by mixing equal parts of solution A and solution B.

Spray solution: It was prepared by mixing 1 ml stock solution with 2 ml glacial acetic acid and 10 ml distilled water.

3.4 Chromatographic materials

Percolated thin layer chromatography (TLC) plates with fluorescent material were used (Alugram^{R} Sil G/Uv 254, Macherey-Nagel - Germany). Silica gel 60 (0.063-0.2 mm, Merch Chemicals Ltd - South Africa) was used for vacuum liquid chromatography (VLC) while silica gel 60 (70-230 mm, Merch Chemicals Ltd- South Africa) was used for column chromatography (CC). Filter gel (Sephadex LH-20) was used for the sephadex column.

3.5 Instrumentation

The developed TLC plates were viewed using UV lamp at 254 and 366 nm. Melting points were determined on a Gallenkamp-Sanyo (UK) machine. UV spectra were recorded on a Cecil CE 2041–2000 series spectrometer while IR spectra were recorded on a FTIR-8400 spectrometer, Shimadzu-Japan. ^{1}H-NMR and ^{13}C-NMR spectra were measured on an Oxford Varian Mercury YH-400 MHz and Jeol JNM-400 MHz spectrometers using deuterated solvents chloform and methanol as a solvents and tetramethylsilane (TMS) as an internal reference.

3.6 Antibacterial screening tests

This was carried out *in vitro* using the agar diffusion method (Chhabra and Uiso, 1992; McChesney *et al*., 1991). The screening consisted of 3 bacteria, standard test organisms, which included one Gram-positive *Staphylococccus aureu* ATCC 25833, and two Gram-negative; *Escherichia coli* ATCC 25837 and *Pseudomonas aeruginosa* ATCC 10729. All these were obtained from Kenya Medical Research Institute. The nutrient agar (14 g) was dissolved in 0.5 litre of distilled water. This was sterilized by autoclaving at 121°C and 7 kPa for 15 min. Rund filter paper MN 615 of 6 mm diameter was punched and the paper pieces , 6 mm, sterilized. Nutrient agar (15 ml) was poured into Petri dishes in a lamina flow apparatus under sterile conditions to 4 mm depth and 0.1 ml of bacterial solution was swabbed on the media surface. 2 mg of in 1ml DMSO and 10 μl of the solution were dispensed onto 6 mm sterile absorbent filter paper disc 6 mm (2 mg/disc) were then put on petri dish and finally incubated. The results were observed by measuring the diameter of the zone of inhibition from original 6 mm after 24 hours. The antibacterial test was done in triplicate for each test sample. Gentamycin was used as the standard antibiotic for positive

controls while DMSO was used asa negative control (Chhabra and Uiso, 1992; McChesney *et al*., 1991).

3.7 Antifungal screening tests

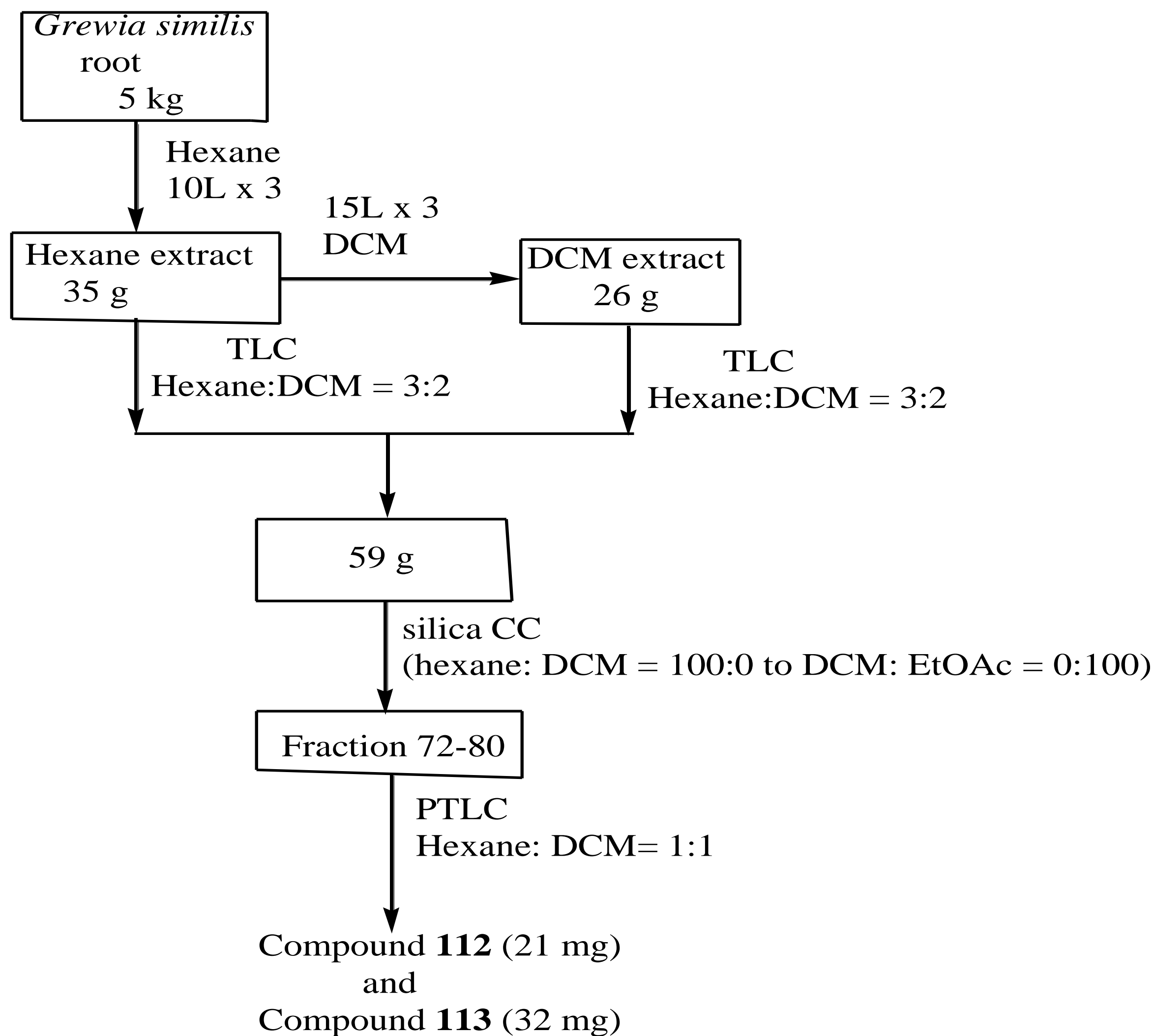
The agar plate diffusion method was used. *Cryptococcus neoformans* ATCC 37651 was used as the test organism and the standard antifungal agent amphotericin B was used as positive control (Chhabra and Uiso, 1992; McChesney *et al*., 1991). 2 mg of the extracts and isolated compounds were dissolved in 50 µl DMSO and the solution made to 1 ml with distilled water. Potatoes dextrose agar (PDA) autoclaved for 15 min at 121^{0} C and 7 kPa psi was poured into the petri dishes and holes of diameter 6 mm bored into the PDA, 0.1 ml of the drug solution was then added into the holes. DMSO solvent was used as a control for each petri dish. The petri dish was covered and then left at room temperature for fungus to grow. The results were taken on the third and fourth day by measuring the diameter of zone of inhibition from original diameter of 6 mm (Chhabra and Uiso, 1992; McChesney *et al*., 1991).

3.8 Isolation of the compounds

3.8.1 *Grewia similis* hexane and DCM extracts

The hexane and DCM root of extract of *G. similis* were combined based on the TLC profiles (hexane: DCM = 3:2) giving 61 g (scheme 3.8.1). 2 g of this extract was put in a vial and kept in a deep freezer for bioassays. The rest (59 g) was adsorbed on silica gel and subjected to column chromatography (diameter = 4 cm, length = 45 cm) over a column of silica gel 60 (Merch brand,70-230 mesh ASTM, 70g, Merch Chemicals Ltd. South Africa) using solvents

of increasing polarity from hexane through EtOAc (hexane: DCM; 100:0 to DCM: EtOAc; 0:100) to give 116 fractions (200 ml).



Scheme 3.8.1: Isolation of compounds **112** and **113** from the hexane/DCM root extract of *G. similis*

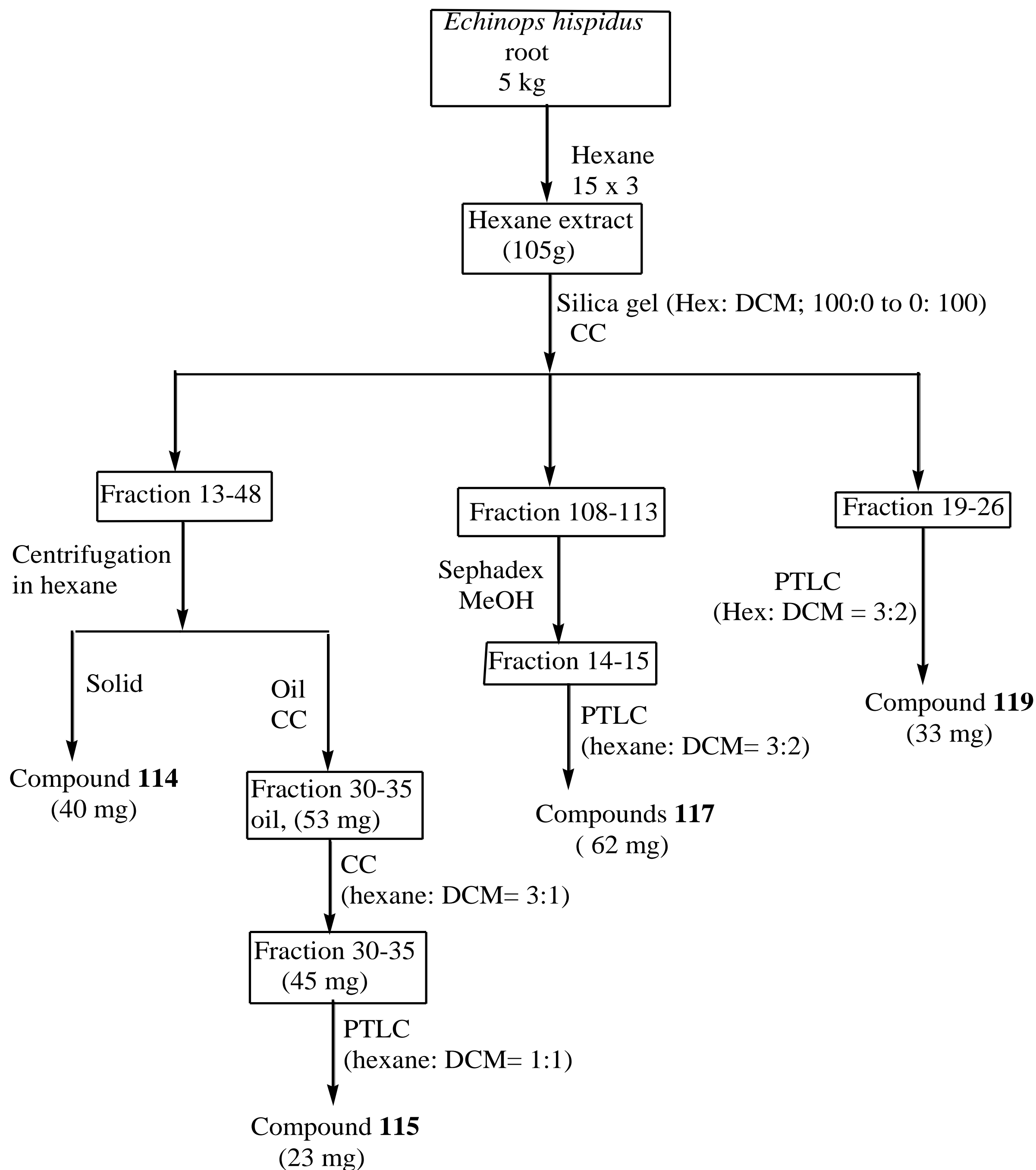
Fractions 72-80 (Hexane: DCM= 2:3) from the silica gel column gave two spots on the TLC plate (Hexane: DCM = 1:1) (Scheme 3.8.1). They were combined and subjected to preparative-TLC (hexane: DCM= 1:1) to yield two compounds **112** (21 mg) and **113** (32 mg) as colourles needles in acetone with melting points 131-133 and 174-176°C, respectively.

3.8.2 *Echinops hispidus* hexane extract

Analytical TLC (Alugram^{R} Sil G/Uv 254, Macherey-Nagel - Germany) was carried out on the hexane root extract of *E. hispidus* with the solvents hexane: DCM = 3:1, Hexane: DCM = 1:1 and Hexane: DCM = 1:3. The plates were sprayed with p-anisaldehyde and Dragendoff's reagents. The plates after spraying suggested the presence of terpenoids, flavonoids, thiophenes and alkaloids. 2 g of this extract were kept for bioassays and the rest (105 g) was adsorbed to silica gel 60 (70-230 mesh ASTM, 60 g, Merch Chemicals Ltd - South Africa) and loaded to a column packed with silica gel 60 (Merck brand, 70-230 mesh, 200 g). A small amount of silica gel just to cover the sample was then put on the top. The column was eluted with solvents of increasing polarity from hexane through EtOAc (hexane: DCM; 100:0 to DCM: EtOAc; 0:100) (Scheme 3.8.2). This afforded 127 initial fractions each of 100 ml.

The fractions showing similar spots on TLC were combined and the extracts from therein were subjected to further chromatographic separations over silica gel yielding 5 compounds. The extracts (400 mg) from the column fractions 13-48 (hexane: DCM= l:1) was spoted and gave four similar spots (Scheme 3.8.2). These were then combined and the solvent evaporated to give a colourless solid which was insoluble in hexane and yellow oil which was soluble in hexane. This was centrifuged and washed with hexane twice to afford compound **114** (40 mg) as colourless needles with a melting point 288-290°C. A small

amount was spotted and sprayed with p-anisaldehyde to giving a grey spot. 2 mg of this compound was put in a vial for biological assays and 15 mg packed in another vial for spectroscopic anlysis.



Scheme 3.8.2: Isolation of compounds, **114**, **115**, **117**, **119**, and **120** from *Echinops hispidus* hexane extract

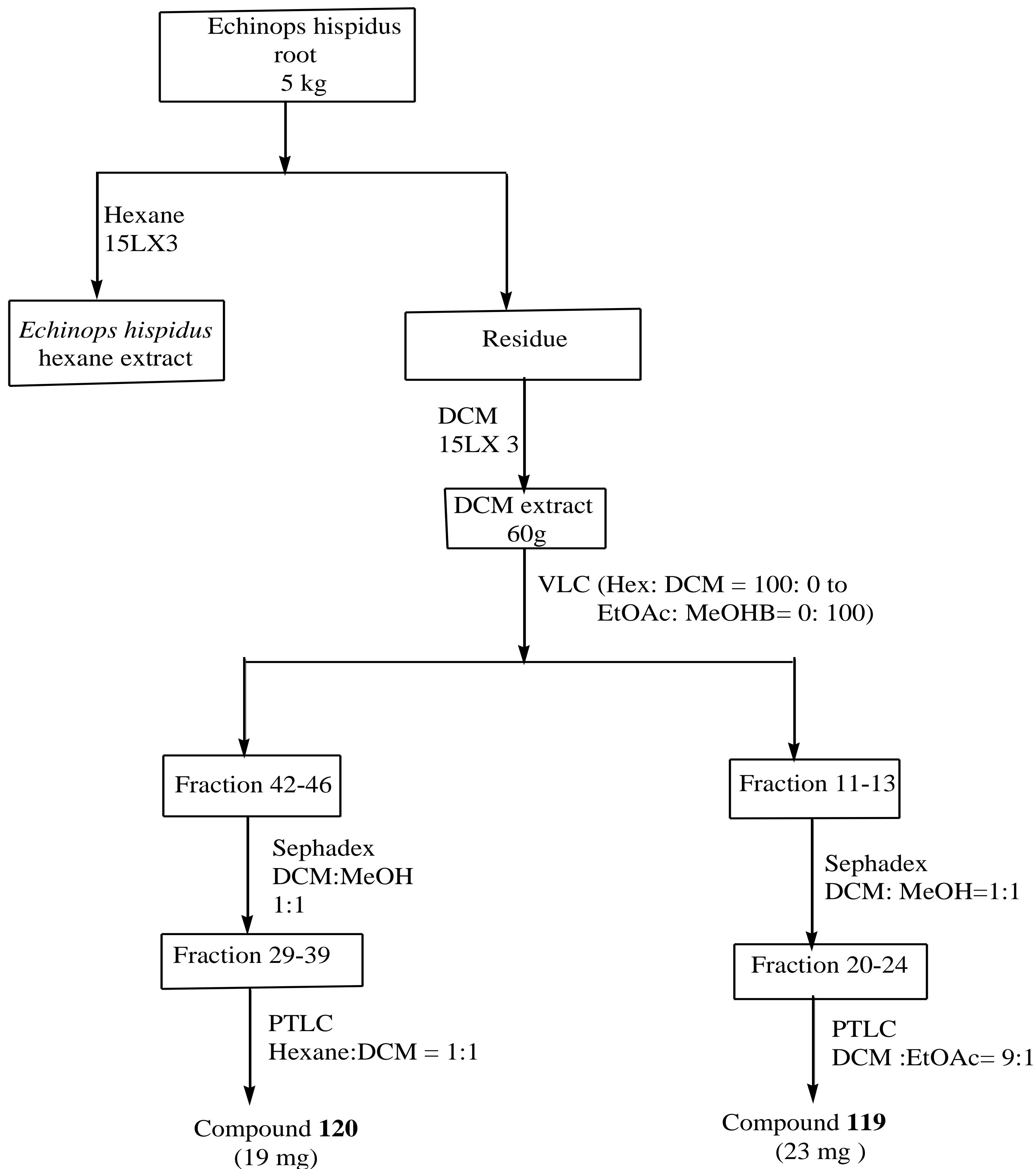
The yellow oil obtained after centrifugation of fraction 13-48 (n-hexane: DCM = l:1) was subjected to chromatographic separations (diameter = 2 cm, length = 40 cm) and eluted with solvents of increasing polarity. This afforded 45 fractions (Scheme 3.8.2). From the TLC profiles, fractions 30-35 (hexane: DCM =3:1) were combined and subjected to preparative-TLC (hexane: DCM = 1:1). This afforded compound **115** (53 mg) as a yellow oil with a heavy-floral odour. A small amount was spoted and sprayed with p-anisaldehyde. This gave a greenish spot indicating the presence of terpenoids (Dey and Harborne, 1991). 2 mg of this compound were put in a vial for biological assays and another 20 mg packed in another vial for spectroscopic anlysis.

Fractions 108-113 (n-hexane: DCM = 1: 3) from the silica column were combined from the TLC profiles and rechromatographed through filter gel (Sephadex LH 20) using pure methanol as the eluant affording 26 fractions. From the TLC profiles, fractions 14-15 were combined and subjected to preparative-TLC (Alugram^R Sil G/Uv 254, Macherey-Nagel - Germany) with the solvent system (hexane: DCM = 3:2). This afforded compound **116** as colorless crystals (62 mg) of melting point 172-174°C. From the TLC profiles, fractions 19-26 (n-hexane: DCM = 1:4) from the silica gel column were combined and subjected to preparative-TLC (hexane: DCM = 3:2) to afford compound **119** as colorless needles (33 mg), with a melting point 118-120°C (Scheme 3.8.2).

3.8.3 *Echinops hispidus* dichloromethane extract

The DCM roots extract of *E. hispidus* (60 g) was obtaioned as a dark brown mass. Analytical Thin Layer Chromatography (Alugram^R Sil G/Uv 254, Macherey-Nagel - Germany) using hexane: DCM (3:2) was carried out on this extract. 2 g of this extract was preserved by freeze

drying to give the DCM extract for bioassays and the remaining material (58 g) was chromatographed through VLC using solvents of increasing polarity from n-hexane through methanol (hexane: DCM; 100:0 to DCM: EtOAc; 0:100) as eluants affording 78 fractions (scheme 3.8.3). The fractions 42-46 (DCM: EtOAc = 4:1) were combined and chromatographed through filter gel column (Sephadex LH 20) using (DCM: MeOH = 1:1) as eluant affording 45 fractions. Fractions 11-13 (Hexane: DCM= 4:1) from the VLC were combined since they had similar TLC profiles and chromatographed through filter gel column (sephadex LH 20) using (DCM: MeOH = 1:1) affording 24 fractions. Fractions 20-24 were combined and subjected to preparative-TLC (DCM: EtOAc = 9:1) furnishing compound **119** (23 mg) (Scheme 3.8.3). Fractions 29-39 were combined based based on the TLC profile and subjected to preparative-TLC (hexane: DCM = 1:1) affording compound **120** as white crystals (23 mg) (Scheme 3.8.3).



Scheme 3.8.3: Isolation of compounds **119** and **120** from *Echimops hispidus* DCM extract

3.8.4 Physical and spectroscopic data of isolated compounds

Compound 112 (21 mg)

White crystals in acetone; mp. 131-133°C; IR ν_{max} (KBr) 3445, 2830, 2857, 1337, 1257 cm^{-1}; ^{1}H-NMR ($CDCl_3$,, 400 MHz) δ 0.90 (9 H, s, H-21, 28, 29), 1.00 (3H, s, H-26), 1.06 (3H,s, H-18), 1.25 (6H, H-4, 19), 1.50 (2H,m), 3.22 (1H, m, H-3), 5.20 (1H, t, H-6); ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 14.1 (C-18), 14.1 (C-29), 17.5 (C-27), 18.0 (C-26), 21.4 (C-11), 23.3 (C-15, C-19, C-21, C-23), 23.4 (C-28), 26.2 (C-23), 28.1 (C-16), 29.4 (25), 31.1 (C-7), 31.3 (C-2), 31.7 (C-7), 31.9 (C-8), 33.8 (C-22), 34.8 (C-20), 37.0 (C-10), 37.1 (C-1), 39.7 (C-12), 41.5 (C-13), 42.1 (C-4), 46.9 (C-24), 47.7 (C-9), 55.2 (C-14), 59.1 (C-17), 79.1 (C-3), 124.4 (C-6), 139.6 (C-5).

Compound 113 (32 mg)

White crystals in acetone; mp. 174-176°C; The IR ν_{max} (KBr) 3423 cm $^{-1}$; ^{1}H-NMR ($CDCl_3$,, 400 MHz) δ 0.64 (3H,s, H-21), 0.92 (3H, s. H-29), 1.00 (3H, s, H-27), 1.02 (3H, s, H-26), 1.07 (3H, s, H-18), 1.25 (3H, m, H-19), 1.55 (m, H-2), 3.51 (1H, m, H-3), 5.05 (1H, dd, H-23), 5.15 (1H, dd, H-22), 5.35 (1H, m, H-6); ^{13}C-NMR ($CDCl_3$, 100M Hz) δ 11.9 (C-18), 12.2 (C-29), 19.4 (C-19), 21.1 (C-11, 21), 24.3 (C-15), 19.1 (C-27), 25.4 (C-28), 28.3 (C-16), 31.7 (C-7), 31.8 (C-8), 31.9 (C-2, 25, 26), 36.1 (C-20), 36.5 (C-10), 37.3 (C-1), 39.8 (C-12), 42.3 (C-4, 13), 50.2 (C-9), 51.3 (C-24), 56.0 (C-14), 56.9 (C-17), 71.8 (C-3), 121.7 (C-6), 129.3 (C-23), 138.3 (C-22), 140.8 (C-5).

Compound 114 (40 mg)

Colourless needles from $CHCl_3$; mp 289- 290^oC; IR ν_{max} (KBr): 3678 (C-OH), 1716 (C=O str), 1045 (C-O str), 2974, 1456, 1377 (aliphatic C-H str), 750-669 (olefinic C-H), 1047 and 928 (=C-H opb) cm^{-1} ; ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.80 (s, H-5, H-24), 0.89 (s, H-27), 0.82 (s, H-28), 0.96 (s, H-29), 0.91 (s, H-23, H-30), 0.95 (m, H-18), 0.98 (s, H-25), 1.01 (s, H-1, H-26), 1.30 (m, H-1, H-21), 1.45 (m, H-22), 1.57 (m, H-9, H-11), 1.60 (m, H-2), 1.62 (m, βH-6, H-12), 1.92 (d, J = 3.2, 11.2 Hz, H-16), 2.01 (H-7), 2.03 (H-2'), 4.41 (dd, J = 8.0, 4.8 Hz, H-3), 5.51 (dd, J =12.0, 4.4 Hz, H-15); ^{13}C-NMR ($CDCl_3$, 100 MHz): δ 15.5 (C-24), 16.6 (C-25), 17.5 (C-11), 18.7 (C-5), 21.3 (C-1'), 23.4 (C-27), 25.9 (C-26), 28.0 (C-2), 28.8 (C-23), 29.7 (C-20), 29.8 (C-28), 29.9 (C-30), 33.1 (C-21), 33.3 (C-29), 33.7 (C-12), 33.7 (C-13), 35.1 (C-22), 35.8 (C-17), 36.7 (C-19), 37.4 (C-10), 37.5 (C-1, C-16), 37.7 (C-13), 37.9 (C-4) 39.0 (C-8), 41.2 (C-7), 48.7 (C-18), 49.2 (C-9), 55.6 (C-5), 81.0 (C-3), 116.9 (C-15), 157.5 (C-14) , 171.0 (C-1').

Compound 115 (23 mg)

Yellow oil with a fruity odour; IR ν_{max} (KBr in chloroform): 3604, 3018, 2930, 1458, 667.3-769.5, 1215; MS: *m/z* (%) 222 $[M]^+$ (48), 207 (60), 189 (15), 165 (23), 151 (15), 137 (100), 126 (26), 95 (20), 91 (10), 81 (18), 79 (9), 77 (7), 67 (9), 55 (20); ^{1}H-NMR ($CDCl_3$, 400 MHz): δ 0.83 (3H, 9-Me), 1.15 (3H, 6β-Me), 1.18 (3H, 6α-Me), 1.26 (3H, 4-Me), 1.36 (2H, H-3), 1.45 (1H, H-11), 1.55 (4H, H-5, H-10), 1.64 (H-2), 1.73 (1H, H-1), 1.90 (2H, H-9), 3.66 (1H, H-7); ^{13}C-NMR ($CDCl_3$, 400 MHz): δ 19.4 (C-9-Me), 23.8 (C-6α-Me), 25.7 (C-4-Me), 29.0 (C-2), 32.5 (C-6β-Me), 36.1 (C-11), 35.4 (C-10), 38.7 (C-6), 43.8 (C-9), 47.6 (C-4), 40.0 (C-3), 51.6 (C-1), 52.6 (C-5), 67.1 (C-8), 89.7 (C-7).

Compounds 116 (62 mg)

White crystals in chloroform; mp. 172-174 ^{o}C; IR ν_{max} [(KBr in chloroform) 3500, 3018, 2930, 1458, 667-770, 2400, 1215, 1020 900 cm^{-I}]; UV ν_{max} ($CDCl_3$) 246 and 281 nm; ^{1}H-NMR (C_3D_6O, 400 MHz): δ 3.16 (1H, m, H-1, 5), 3.79 (1H, s, 4'-OMe), 3.80 (1H, s, 4''-OMe), 3.82 (1H, s, 3''- OMe), 4.02 (1H, m, H-4α), 4.22 (1H, m, H-4β), 4.84 (2H, d, J = 6.8 Hz, H-6), 4.92 (1H, d, J = 6.6 Hz, H-2), 5.56 (1H, d, J=3.2 Hz, H-8β), 5.69 (1H, d, J= 4.8 Hz, H-8α), 6.86 (1H, s, H-2'), 6.93 (1H, d, J=8.2 Hz, H-5'), 6.99 (1H, d, J= 8.2 Hz, H-6'), 7.03 (1H,d, J=8.0 Hz), 7.25 (1H, d, J= 8.0 Hz, H-2'', H-6''); ^{13}CNMR (C_3D_6O, 100MHz): δ 55.0 (C-5), 118.3 (C-3''), 62.9 (C-1), 72.1 (C-4), 83.5 (C-6), 87.4 (C-2), 101.9 (C-8), 110.7 (C-2'), 111.7 (C-2''), 112.0 (C-5'), 118.3 (C-3''), 118.7 (C-6'), 135.4 (C-1'), 136.5 (C-1''), 149.2 (C-4'), 149.7 (C-3'), 149.8 (C-4''),

Compound 119

White crystals in acetone; MS: *m/z* 216 $[M+2]^+$ (5 %), 214 $[M]^+$ (90 %), 196 $[M-CH_2OH]^+$ (3 %), 183 $[M-H_2O]^+$ (100 %); ^{1}H-NMR (MeOH, 400 MHz): δ 2.00 (3H, s, H-1''), 2.62 (2H, t, J = 7.2 , 13.2 Hz, H-3'), 3.29 dd (J = 6.6, 11.3 Hz, H-1'), 3.69 (2H ,t, J = 6.6, 11.3 Hz, H-1'), 6.98 (1H, d, J = 3.9Hz, H-4), 7.11 (1H, d, J = 3.8 Hz, H-3); ^{13}C-NMR (MeOH, 100MHz): δ 4.1 (C-5''), 24.5 (C-3'), 61.3 (C-4'), 64.1 (C-3''), 66.9 (C-2''), 74.8 (C-1''), 79.6 (C-1'), 84.2 (C-4''), 94.0 (C-2'), 123.5 (C-5), 127.4 (C-2), 132.4 (C-4), 134.9 (C-3).

Compound 120

White crystals in chloroform; mp. 118-220°C; MS: *m/z* (%) showed peaks at 230 $[M]^+$ (4 %), 216 [M-CH_3] (100 %), 173 [M-CC-CH_3 and $H_2O]^+$ (50 %), 159 [M- CC-CC-CH_3 and $CH_2OH]^+$ (64 %), 105 [M-CC-CC-CH_3 and –CH_2(OH)$CH_2OH]^+$ (15 %); ^{1}H-NMR (MeOH, 400MHz): δ 2.00 (3H, H-1’’), 3.67 (2H, t, J = 6.6 , 11.0 Hz, H-2’’), 4.59 (1H, d, J = 6.4 Hz, H-1’), 4.69 (1H, d, J = 6.4 Hz, H-1’), 7.14 (1H,d, J = 3.8 Hz, H-4), 7.29 (1H, d, J = 3.6 Hz, H-3); ^{13}C-NMR (MeOH, 100 MHz): δ 4.8 (C-5’’), 63.9 (C-3’), 64.1 (C-3’’), 66.2 (C-4’), 66.3 (C-2’’), 79.6 (C-2’’), 79.9 (C-1’), 84.4 (C-4’’), 91.4 (C-2’), 123.8 (C-5), 124.2 (C-2), 132.8 (C-4), 133.6 (C-3).

CHAPTER FOUR

RESULTS AND DISCUSSIONS

4.1 Yields of the extracts

The *Echinops hispidus* afforded 107 g (2.1%), 60 g (1.2%) and 68 g (1.4%) for hexane, DCM and EtOAc extracts, respectively. The *Grewia similis* afforded 35 g (0.7 %), 26 g (0.5%) and 37 g (0.7 %), for hexane, DCM and EtOAc extracts, respectively (table 3.1). The total extract of *E. hispidus* was 4.7% while that of *G. similis* was 1.9% (Table 4.1).

Table 4.1: The yields of the extracts

Plant	Extract	Mass (kg)	% yield
E. hispidus	Hexane	107	2.10
	DCM	60	1.20
	EtOAc	68	1.36
G. similis	Hexane	35	0.70
	DCM	26	0.50
	EtOAc	37	0.74

The results showed that the percentage yields decreased from hexane to DCM and then increased from DCM to EtOAC. These results imply that both plants are very rich in non-polar and polar metabolites.

The hexane and DCM root extracts of *E. hispidus* and hexane/ DCM root extracts of *G. similis* were spotted. On viewing the TLC at 254 and 366 nm on a UV lamp it gave strongly coloured spots. This suggested that the compounds present had chromophores. On spraying

the hexane and DCM root extracts of *E. hispidus* with p-anisaldehyde-sulphuric acid showed dark-pink, green and yellow spots on the TLC plate indicating the presence of thiophenes, lignans and flavanoids, respectively (Bijal and Shantu, 1990; Li-Hua *et al*., 2003; Harborne, 1998). When the hexane root extract of *E. hispidus* was sprayed with Dragendorff's reagent it resulted to an orange-yellow coloration indicating presence of alkaloids while the DCM extract gave negative results. The hexane/DCM extract of *G. similis* gave green and yellow spots on spraying with p-anisaldehyde-sulphuric acid indicated the presence of steroids and flavanoids, respectively (Harborne, 1998). No alkaloids were detected on spraying with Dragedorff's reagent.

4.2 Antibacterial activities of the crude extracts

The crude extracts were subjected to antibacterial assays using the agar diffusion method assay and the inhibition zones were measured in mm in triplicate (Section 3.6). The mean activities are as tabulated in Table 4.2.

Table 4.2: Antibacterial activities (mm) of the crude extracts

		Inhibition zone ± standard deviation		
Plant	Extract/standard drug	*S. aureus*	*P. aeruginosa*	*E. colli*
E. hispidus	Hexane	6 ± 0.02	6 ± 0.01	6 ± 0.02
	DCM	6 ± 0.04	6 ± 0.02	6 ± 0.03
	EtOAc	9 ± 0.07	6 ± 0.03	6 ± 0.01
G. similis	Hexane/DCM	15 ± 0.06	6 ± 0.01	6 ± 0.02
	EtOAc	6 ±0.03	6 ± 0.02	6 ± 0.01
Gentamycin (1000 µg/disk)		14 ± 0.01	14 ± 0.02	14 ± 0.03

The EtOAc root extract of *E. hispidus* exhibited a moderate activity with an inhibition zone of 9 mm compared to 14 mm of gentamycin. The hexane/DCM root extract of *G. similis* exhibited a strong activity of 15 mm against *S. aureus*. This activity was noted to be higher that of gentamycin. The EtOAc root extract of *E. hispidus* and hexane/DCM root extract of *G. similis* showed no activity against *P. aeruginosa* and *E. coli.* The hexane and DCM root extracts of *E. hispidus* and the EtOAc root extract of *G. similis* showed no activity against *P. aeruginosa*, *S. aureus* and *E. coli.*

These antibacterial studies showed that only the EtOAc root extract of *E. hispidus* and hexane/DCM root extract of *G. similis* had activity against Gram-positive bacteria but no activities against Gram-negative bacteria. This was expected as Gram-positive bacteria are more susceptible to antibiotics while G-negative bacteria are known to develop resistance and respond less to different antibiotics (Roshan *et al*., 2005). These antibacterial results support the the use of the two plants in traditional medicine against bacterial infections.

4.3 Antifungal activities of the crude extracts

The crude extracts were subjected to antifungal assays using the agar diffusion method assay and the inhibition zones were measured in triplicate in mm (Section 3.7). The mean activities are tabulated in Table 4.3.

Table 4.3: Antifungal activities (mm) of the crude extracts

Plant	Extract	Inhibition zone ± standard deviation
		Cryptococcus neoformans
E. hispidus	Hexane	9 ± 1.09
	DCM	13 ± 2.11
	EtOAc	15 ± 2.15
G. similis	Hexane	6 ± 1.16
	DCM	6 ± 1.03
AmphotericinB (1000 µg/disk)		38 ± 1.68

The hexane extract of *E. hispidus* showed a mild activity with an inhibition zone of 9 mm against *Cryptococcus neoformans.* The DCM and EtOAc root extract of *E. hispidus* showed strong activity of 13 and 15 mm against *Cryptococcus neoformans*, respectively. The hexane/DCM and EtOAc root extracts of *G. similis* had no activity against *C. neoformans*. Among all the crude extracts, the EtOAc root extract showed the highest activity of 15 mm against *C. neoformans.* These antifungal results support the the use of the *E. hispidus* in traditional medicine against fungal infections and necessitated further purification of the plant extracts to isolate the bioactive metabolites.

4.4 Structural elucidation of isolated compounds

On subjecting the hexane/DCM root extract of *G. similis* to chromatographic separations (Scheme 3.8.1) using solvents of increasing polarity it afforded two compounds **112** and **113.** The hexane root extract of *E. hispidus* afforded compounds **114**, **115**, **116** and **119** (Scheme 3.8.2). The DCM extract of *E. hispidus* was subjected to chromatographic separations and

afforded compound **120** (Scheme 3.8.3). The structures of the isolated compounds were elucidated by use of spectroscopic techniques and physical methods. Comparison with related documented spectral and physical data was also used to confirm the structures.

4.4.1 Compound 112

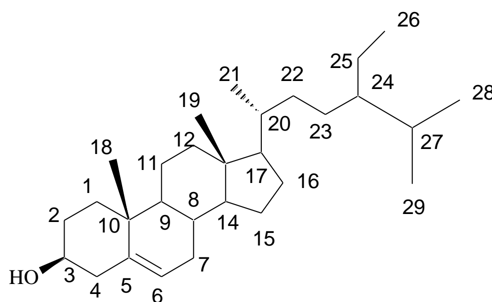
Compound **112** was obtained from the hexane/DCM extract of *G. similis* as colourless needles in acetone and had a melting point of 131-133°C. On the TLC the compound had a R_f of 0.6 in pure DCM. When the plates were spayed with p-anisaldehyde the spot turned purple and later green suggesting it was a triterpenoid (Dey and Harborne, 1991). The IR spectrum (appendix 1g) showed a broad band at 3423 cm^{-1} suggesting a hydroxyl group, intense bands at 2930 and 2857 cm^{-1} of a C-H stretch, a doublet of equal intensities at 1337 and 1257 cm^{-1} suggesting a C-H bending vibration of isopropyl (gem dimethyl groups) and a band at 1650 cm^{-1} due to carbon-carbon double bond (Dey and Harborne, 1991).

The ^{1}H-NMR spectrum (appendix 1a) displayed three regions namely aliphatic, hydroxylated and allylic regions and strongly suggested a triterpenoid structure (Dey and Harborne, 1991). The signal appearing as a triplet at δ 5.20 suggested the presence of a double bond at a quaternary carbon atom. A multiplet centered at δ 3.22 was characteristic of a proton geminal to a hydroxyl group at C-3 of triterpenoids was also observed. Six signals representing the methyl groups were observed at δ 0.90 (3 CH_3), 1.00, 1.06 and 1.25 which are characteristic of a triterpenoid (Ahmad and Rahman, 1996). The characteristic peaks were confirmed by the COSY correlations (Table 4.4).

Table 4.4: Some COSY, HMQC and HMBC correlations of 56

COSY	HMQC	HMBC
5.20 → 1.92	5.20 → 124.4	5.20 → 32.1
3.20 → 1.55	3.22 → 79.1	3.22 → 31.3, 42.1
	1.55 → 31.3	

The ^{13}C-NMR spectrum (appendix 1c) showed signals of olefinic carbon atoms at δ 139.6 and δ 124.4 which supported the presence of a double bond at the end of a fused ring between C-5 and C-6 (Ahmad and Rahman, 1996). This was confirmed by the HMQC experiment (appendix 1e) which showed correlation between δ 124.4 and δ 5.20. The HMQC experiment also confirmed that the hydroxyl group was at C-3 since the peak at δ 79.1 correlated to δ 3.22 (Table 4.4). The DEPT experiment (appendix 1d) showed six methyl, eleven methylene and nine methine carbon atoms. The methyl groups were assigned to the signals at δ 14.1, 15.5, 16.8, 18.0, 23.4 and 18.4. The overall spectral data of compound **112** (Table 4.5) compared closely with that of 3β-sitosterol, whose proposed structure is indicated below (Ahmad and Rahman, 1996). This compound is being reported from *G. similis* for the first time.



112

Table 4.5: ^{1}H- and ^{13}C-NMR data for **112** and reported data of 3β-sitoterol (Alam *et al.,*1996)

	112		3β-sitosterol	
No	δ_H ($CDCl_3$, J, Hz)	δc($CDCl_3$)	δ(MeOH, J, Hz)	δc(MeOH)
1		37.2		37.2
2	1.55 (m)	31.3	1.55 (m)	31.9
3	3.22 (m)	79.1	3.25 (m)	79.0
4	1.25 (d)	42.1	1.25 (d)	42.3
5		139.6		140.8
6	5.20 (1H, t)	124.4	5.31 (1H, m)	121.7
7		32.1		31.7
8		31.9		31.9
9		47.7		50.1
10		37.0		36.5
11		21.4		21.1
12		39.7		39.9
13		41.5		42.3
14		55.2		56.8
15		23.3		24.3
16		28.1		28.3
17		59.1		56.1
18	1.06 (3H, s)	14.1	1.16 (3H, s)	11.9
19	1.25 (3H, s)	18.4	1.20 (3H, s)	19.4
20		34.8		36.2
21	0.90 (3H, s)	16.8	0.91 (3H, s)	18.8
22		33.8		34.0
23		26.2		26.1
24		46.9		45.8
25		29.4		29.2
26	1.00 (3H, s)	18.0	1.01 (3H, s)	19.8
27		17.5		19.0
28	0.90 (3H, s)	23.4	0.98 (3H, s)	23.1
29	0.90 (3H, s)	15.5	0.96 (3H, s)	12.0

4.4.2 Compound 113

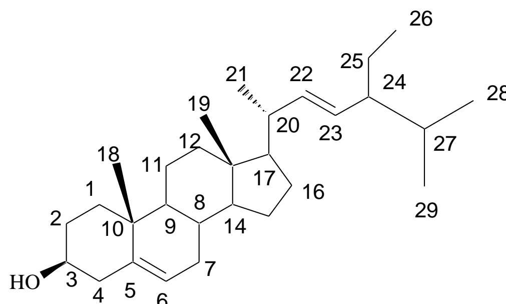
Compound **113** was obtained from the hexane/DCM extract of *G. similis* as white crystalline solid in acetone with melting point of 174-176°C. On the TLC the compound had a R_f of 0.7 in hexane-DCM (1:1). When the plates were spayed with p-anisaldehyde the spot turned purple suggesting that the compound was a triterpenoid (Dey and Harborne, 1991). The ^{1}H-NMR spectrum (appendix 2a) displayed several signals between 1.25 and 0.64 characteristic of a triterpenoid and closely compared to those of compound **112** discussed earlier. The diagonistic chemical shift values of the angular methyl protons for C-18 and C-19 appeared as singlets at δ 1.07 and 1.25, respectively. Four methyl groups appeared at δ 0.92, 0.93, 1.00 and 1.02 (Dey and Harborne, 1991). The multiplet at δ 3.51 suggested the presence of a proton attached to a hydroxylated carbon atom. A doublet at δ 5.35 suggested the presence of a double bond at a quaternary carbon atom. Two doublets of a doublet signals at δ 5.15 and 5.02 suggested the presence of a double bond on the side chain of a triterpenoid, which was lacking in compound **112**. The COSY spectrum correlations confirmed the proton assignments (Table 4.6).

Table 4.6: Some COSY, HMQC and HMBC correlations of compound **113**

COSY	HMQC	HMBC
5.35 → 2.00	5.35 → 121.7	5.35 → 31.7, 140.8
5.15 → 2.05, 5.02	5.15 → 138.3	5.15 → 31.7, 36.1
5.02 → 2.25	5.02 → 129.3	5.02 → 51.3, 138.3
3.51 → 1.55, 2.25	3.51 → 71.8	

The ^{13}C-NMR spectrum (appendix 2c) displayed twenty-nine carbon atoms confirming compound **113** was a modified triterpenoid. It showed signals at δ 140.8 and 121.7 confirming the presence of of olefinic carbons with the more deshielded signal assignable to the quartenary carbon at the bridge. The signal at δ 138.3 and 129.3 that lacked in the spectra of compound **112** represented the oleffinic carbons at the side chain. The peak at δ 71.8 was assigned to C-3 due to the presence of the hydroxyl group common to this class of compounds (Dey and Harborne, 1991). The three signals at δ 36.1, 36.5 and 42.3 were associated with the three quartenary carbon atoms. The main signals were correlated by the use of HMQC and HMBC experiments (appendix 2e and 2f). In the HMQC spectrum the signal at δ 121.7 corrrelated to δ 5.35 hence the signals were assigned to C-6 and H-6, respectively (Table 4.6). The peak at δ 71.8 showed correlation to δ 3.51 supporting the triterpenoid to be hydroxylated at C-3. The HMBC (Table 4.6) gave long range connectivity of the carbons to protons.

The spectral data (table 4.7) compared closely to that of 3β-stigmasterol, whose proposed structure is shown below (Alam et al., 1996). This compound is being reported from *G. similis* for the first time.



113

Table 4.7 ^{1}H- and ^{13}C-NMR data for 113 and reported data of 3β-stigmasterol (Alam *et al*., 1996)

	113		3β-stigmasterol	
No	δ($CDCl_3$, J, Hz)	δc($CDCl_3$)	δ(MeOHJ)	δc(MeOH)
1		37.3		37.2
2	1.55 (m)	31.9	1.43 (m)	31.9
3	3.51 (m)	71.8	3.25 (m)	71.8
4		42.3		42.3
5		140.8		140.8
6	5.35 (1H, d)	121.7	5.14 (1H, m)	121.7
7		31.7		31.7
8		31.8		31.9
9		50.2		50.1
10		36.5		36.5
11		21.1		21.1
12		39.8		39.9
13		42.3		42.3
14		56.0		56.8
15		24.3		24.3
16		28.3		28.3
17		56.9		56.1
18	1.07 (3H, s)	11.9	1.07 (3H, s)	11.9
19	1.25 (3H, m)	19.4	1.26 (3H, m)	19.4
20		36.1		36.2
21	0.64 (3H, s)	21.1	0.91 (3H, s)	18.8
22	5.15 (1H, m)	138.3	4.62 (1H, m	129.3
23	5.05 (1H, m)	129.3	4.61 (1H, m)	121.7
24		51.3		45.8
25		31.9		29.2
26	1.02 (3H, s)	21.2	1.01 (3H, s)	19.8
27	1.00 (3H, s)	19.1	1.00 (3H, s)	19.0
28		25.4		23.1
29	0.92 (3H, s)	12.2	0.97 (3H, s)	12.0

4.4.3 Compound **114**

Compound 112 was obtained from *E. hispidus* hexane root extract as colourless needles with a melting point of 289-290°C. On the TLC the compound had a R_f of 0.3 in hexane-DCM (7:3). When spotted on a TLC plate (hexane: DCM = 1:1) and sprayed with p-anisaldehyde, the spot turned purple then green suggesting a terpenoid skeleton (Dey and Harborne, 1991). The IR spectrum (appendix 3g) revealed the presence of a hydroxyl and keto-carbonyl functionality due to absorptions at 3678 and 1716 cm^{-1}, respectively.

The ^{1}H-NMR spectrum (appendix 3a) revealed signals of one olefinic proton at δ 5.51 (dd, J = 11.2, 3.2 Hz), one oxymethine deshielded proton at δ 4.41 (dd, J = 4.8; 8.0 Hz) and eight methyl singlets at δ 0.90 (12H), 0.97 (9H) and 1.00 (3H). The compound also showed the characteristics peak at δ 2.03 associated with the methyl protons of an acetyl group. These signals strongly suggested a pentacyclic triterpenoid (Mahato and Kundu, 1994). The acetoxyl group was assigned to C-3β because the resonance at δ 4.41 with large coupling constants (J = 4.8 and 8.0 Hz) instead of δ 3.19 for hydroxylated pentacyclic triterpanoid at C-3. The coupling constants of 4.8 and 8.0 Hz were attributed to the coupling of 3α-H with the two 2H-hydrogens (Chien *et al*., 2004). The COSY and NOESY spectrum was to confirm these assignments (Table 4.8).

Table 4.8: Some COSY, NOESY, HSQC and HMBC correlations of **114**

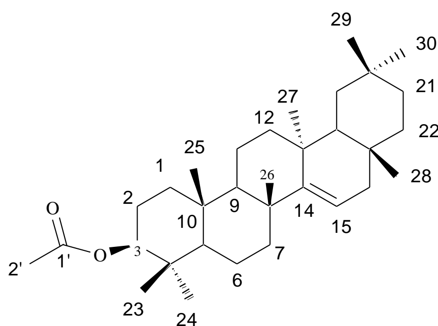
COSY	NOESY	HSQC	HMBC
5.50 → 1.62, 1.92	5.50 → 1.88, 1.92	5.50 → 116.9	5.50 → 37.5, 37.7, 39.0
4.41 → 0.80, 1.60	4.41 → 0.80, 0.97, 1.60	4.41 → 81.0	2.01 → 157.5
	2.03 → 0.97, 1.30, 1.60	0.95 → 48.7	1.92 → 157.5
	2.01 → 1.57, 1.62	0.80 → 55.6	1.62 → 157.5

The ^{13}C-NMR spectrum (appendix 3d) showed that compound **114** had thirty two carbon atoms. The signal at δ 171.0 which was attributed to carbonyl of the acetyl group further supported the presence of acetoxyl functionality (Mahato and Kundu, 1994). The presence of an acetoxyl group at C-3 was supported by the downfield shift of the signals at δ 81.0 instead of δ 79.1 for the hydroxylated pentacyclic triterpenoid at C-3 (Chien *et al*., 2004). The acetoxyl group at C-3 was confirmed by the HSQC experiment (apendix 3e) which showed connectivity between the proton at δ 4.41 and the carbon at δ 81.0 (Table 4.8). The HMBC experiment (appendix 3f) further confirmed this as the signal at δ 81.0 showed long range connectivity to the methyl protons at δ 0.80 (Table 4.8).

The ^{13}C-NMR spectrum (apendix 3d) also showed signals at δ 157.5 and δ 116.9 which suggested double bond functionality involving a quaternary carbon atom. The more deshielded signal was assigned to a quaternary carbon while the signal at δ 116.9 was assigned to a methine carbon. The highlighted peaks together with the ones between δ 55.6 and δ 15.5 attributed to methylenes and methyls strongly suggested that the compound was a pentacyclic triterpenoid. The ^{13}C-NMR also showed peaks at δ 15.5, 16.6, 23.4, 25.9, 28.8, 29.8, 29.9 and 33.3 which were assigned to the eight methyl groups. The HSQC experiment

(figure 3e) showed the olefinic proton at δ 5.50 was attached to δ 116.9. This indicated that the olefinic bond was located at a quaternary carbon atom. The HSQC experiment (appendix 3e) also showed connectivitties which led to assignment of δ 55.6, 37.9, 28.8 and 15.5 to C-5, C-4, C-23 and C-24, respectively. The HMBC experiment (appendix 3f) showed that δ 171.0 correlated with the protons at δ 2.03 supporting the acetoxyl group. The HMBC experiment also showed that δ 157.5 corrrelated to δ 0.95, 1.62 and 1.92 which suggested the olefinic bond to be between C-14 and C-15 on biogenetic grounds (Chien *et al*., 2004).

Comparisons of ^{1}H- and ^{13}C-NMR data with published data for taraxerol (Chien *et al*., 2004) were closely related except for the acetyl substituent (Table 4.9). The natural product was thus proposed to be 3β-acetoxyl taraxerol, whose proposed structure is shown below. This compound is being reported from *E. hispidus* for the first time**.**



114

Table 4.9: ^{1}H- and ^{13}C-NMR data for compound **114** and the reported data for taraxerol (Chien *et al*., 2004)

No	**114**		Taraxerol	
	δ_H ($CDCl_3$ J, Hz)	δ_C($CDCl_3$)	δ_H ($CDCl_3$, J, Hz)	δ_C($CDCl_3$)
1	1.30 m, 1.0 m	37.5	1.30 m, 1.1 m	37.8
2	1.60 m	28.0	1.60	27.2
3	4.41 dd (J = 8.0; 4.8 Hz)	81.0	3.19 dd (J = 8.4; 4.9 Hz)	79.1
4		37.9		38.8
5	0.80 m	55.6	0.80	55.6
6	1.62 m	18.7	1.60 m	18.8
7	2.01	41.2	2.03 m	41.4
8		39.0		39.0
9	1.57 m	49.2	1.45 m	49.3
10		37.4		37.6
11	1.57 m	17.5	1.45 m	17.5
12	1.62 m	33.7	1.60 m	33.7
13		38.0		38.1
14		157.5		158.1
15	5.51 dd (J = 3.2; 11.2 Hz)	116.9	5.53 dd	116.9
16	1.92 m	37.5	1.90 m, 1.70 m	37.8
17		35.8		35.8
18	0.50 m	48.7	0.95 m	48.8
19	1.62 m	36.6	1.60 m	36.7
20		29.7		28.8
21	1.30 m	33.1	1.30 m	33.1
22	1.45 m	35.1	1.40 m	35.2
23	0.97 s	28.8	0.97	28.1
24	0.97 s	15.5	0.80	15.4
25	0.97 s	16.6	0.92	15.5
26	1.00 s	25.9	1.09	25.9
27	0.90s	23.4	0.91	22.5
28	0.90 s	29.8	0.82	29.9
29	0.90 s	33.3	0.91	33.4
30	0.90 s	29.9	0.95	30.0
1’		171.0	-	-
2’	2.03 s	21.3	-	-

4.4.4 Compound 115

Compound **115** was obtained from the hexane root extract of *E. hispidus* as yellow oil with a fruity odour. On the TLC the compound had a R_f of 0.5 in hexane-DCM (1:1). When spotted on a TLC plate and sprayed with p-anisaldehyde, the spot turned purple then green suggesting a terpenoid skeleton (Dey and Harborne, 1991). Its IR spectrum (appendix 4h) showed a band at 3604 cm^{-1} indicating the presence of a hydroxyl group.

The ^{1}H-NMR spectrum (appendix 4a) showed four methyl signals at δ 0.83 (s), 1.15 (s), 1.18 (s) and 1.26 (s), four multiplets at δ 1.36, 1.55, 1.64 and 1.90 which were assigned to methylenes protons and one signal at δ 3.66 attributed to an oxygenated proton. The COSY correlations supported these assignments (Table 4.10).

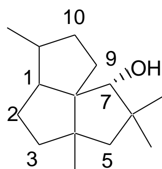
Table 4.10: Some COSY, HSQC and HMBC correlations of **115**

COSY	HSQC	HMBC
0.83 → 1.90	3.66 → 89.7	3.66 → 23.8, 25.7, 40.0, 52.6, 67.1, 89.7
	1.55 → 35.4, 52.6	1.55 → 52.6, 89.7
	1.15 → 32.5	
	1.64 → 29.0	
	1.36 → 40.0	
	0.83 → 19.4	

The ^{13}C-NMR spectrum (appendix 4d) showed fifteen peaks suggesting a sesquiterpenoid skeleton. The signal at δ 89.7 indicated the pressence of a hydroxylated carbon atom. This was supported by the HSQC experiment (appendix 4f) which showed that δ 89.7 correlated to δ 3.66 (Weyerstahl *et al.*, 1998). The peaks at δ 67.1, 47.6 and 38.7 were attributed to

quartenary carbons. This was confirmed by the DEPT (appendix 4e) and HSQC experiments (appendix 4f) since the signals showed no connectivity to any hydrogen atoms. The signals at δ 51.6, 43.8, 40.0, 35.4 and 29.0 in the ^{13}C-NMR spectrum (appendix 4d) were assigned to five methylene carbons. This was supported by the DEPT experiment (appendix 4e). The HSQC experiment (appendix 4f) showed these methylene carbons correlated to δ 1.73, 1.90, 1.36, 1.55 and 1.64, respectively (Table 4.10).

The DEPT spectrum (appendix 4e) showed the presence of four methyl carbons at δ 19.4, 23.8, 25.7 and 32.5, four methylene carbons at δ 29.0, 35.4, 36.1 and 40.0, three methine carbons at δ 43.8, 51.6 and 89.6, and one quaternary carbon at δ 67.1. The HMBC spectrum (figure 4g) showed that the peak at δ 3.66 correlated to δ 67.1; δ 1.64 correlated to δ 67.1; δ 1.55 correlated to δ 89.7 while δ 1.26 correlated to δ 89.7 (Table 4.10). This spectroscopic data were noted to be consistent with the structure of cameroonan-7α-ol isolated from *Echinops gigateus* (Weyerstahl *et al*., 1998). The compound was thus proposed to be cameroonan-7α-ol whose structure is shown below and it is being reported for the first time from *Echinops hispidus.*



115

Table 4.11: ^{1}H- and ^{13}C-NMR of compound **115** and the reported data for cameroonan-7α-ol (Weyerstahl *et al*., 1998)

No	**115**		cameroonan-7α-ol	
	δ_H(CDCl$_3$, J, Hz)	δ_C(CDCl$_3$)	δ_H(pyridine, J, Hz)	δ_C(pyridine)
1	1.73, m	51.6	1.72 q	51.7
2	1.64, m	29.0	1.33, m; 1.50, m	29.5
3	1.36, m	40.0	1.44, m	40.5
4		47.6		47.8
5	1.55 (4H, m)	52.6	1.54, m	53.2
6		38.7		39.1
7	3.66, s	89.7	3.67, s	89.6
8		67.1		67.6
9	1.90 (2H, m)	43.8	1.42, m	44.4
10	1.55, m	35.4	1.43, 1.63 m	35.8
11	1.45, m	36.1	1.53, 1.61 m	36.7
4-Me	1.26, s	25.7	0.98 s	26.0
6α-Me	1.18, s	23.8	0.97 s	24.3
6β-Me	1.15, s	32.5	1.04	32.8
11-Me	0.83, s	19.4	1.10	19.7

The structure of compound **115** was confirmed by the COSY spectrum (appendix 4b). The COSY spectrum (appendix 4b) showed correlations between δ 3.66 and 1.55 while δ 1.73 showed correlations to 0.83. The proton at δ 1.55 correlated to 1.73 and 1.64 while δ 1.18 correlated with 3.66 and 1.56. The NOESY spectrum (appendix 4c) showed correlations between δ 3.66 and 1.45. The proton at δ 1.73 correlated to 1.18 while that at δ 1.90 correlated to 1.73. The MS analysis (appendix 4i) showed the parent ion peak at *m/z* 222 (45 %) which was consistent with the molecular formular $C_{15}H_{26}O$. The peak at *m/z* 207 (60 %) was attributed to loss of a methyl group while that at *m/z* 189 (15 %) was due to loss of methyl group and a water molecule. The peak at *m/z* 165 (25 %) was assigned to loss of four

methyl groups while that at *m/z* 137 (100 %) was assigned to loss of three methyl groups and a $[CHOH]^{\cdot}$ group.

4.4.5 Compounds 116

Compound **116** was obtained from the hexane root extract of *E. hispidus* as white crystals with a melting point of 172-174°C. On the TLC the compound had a R_f of 0.4 in hexane-EtOAc (2:3) and was UV active at 254 and 366 nm giving a blue colour. This indicated aromaticity in the compound (Li-Hua *et al*., 2003). When the TLC plate was sprayed with p-anisaldehyde, the spot turned green-blue suggesting that the compound was a furofuran lignin (Li-Hua *et al*., 2003). The IR spectrum (appendix 5h) showed a sharp peak at 3500 and 1058 cm^{-1} suggesting the presence of a hydroxyl group. The UV spectrum showed peaks at 246 and 281 nm suggesting the presence of a furofuran lignan (Min *et al*., 2005).

The ^{1}H-NMR spectrum (appendix 5a) showed three singlets at δ 3.79, 3.80 and 3.82 suggesting three aromatic methoxy groups. It also showed five signals at δ 6.86 (1H), 6.93 (1H), 6.99 (1H), 7.03 (2H) and 7.25 (2H) which indicated the presence of seven aromatic signals. These aromatic signals showed two AB parts of two ABX systems that suggested a 1’,3’,4’ tri-substituted and a 1’’,4’’ di-substituted phenyl groups of a furofuran lignan (Shuyun *et al*., 2007). The ^{1}H-NMR spectrum also showed two methine protons at δ 3.16 (m), two benzylic oxymethine protons at δ 4.84 (d, J = 6.8 Hz, 1H) and 4.92 (d, J = 6.6 Hz, 1H), an oxygenated methylene multiplets at δ 4.02 and 4.22, oxymethine protons at δ 5.56 (d, J = 3.2 Hz, 1H) and 5.69 (d, J = 4 Hz, 1H). These characteristic peaks suggested a tri-substituted lignanolide skeleton (Min *et al*., 2005). The presence of a furofuran lignanolide skeleton was also supported by the COSY spectrum (appendix 5b) which showed the

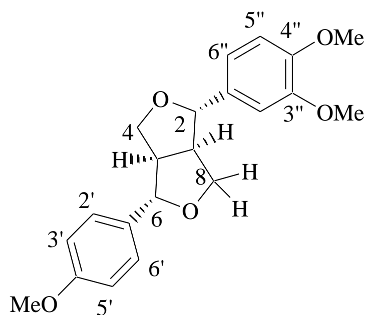
^{1}H-NMR signals at δ 2.87, 3.12 and 4.83 correlated to δ 4.92 (Table 4.12). The large coupling constants of δ 4.92 (1H, d, J = 6.6 Hz) and 4.83 (1H, d, J = 6.8 Hz) indicated both protons were pseudoaxial (Shuyun *et al*., 2007).

Table 4.12: Some COSY, NOESY and HSQC of **116**

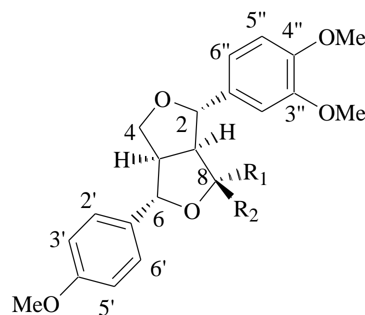
COSY	NOESY	HSQC
7.25 → 7.03	7.24 → 3.80, 4.92	7.25 → 118.7
7.03 → 6.99	7.03 → 3.82, 4.92	6.99 → 118.7
6,93 → 6.86	5.56 → 4.92	7.03 → 112.0
3.16 → 4.02, 4.22, 4.84, 4.92, 5.56, 5.69	3.16 → 4.22, 5.56, 5.69	5.69 → 101.9
		4.92 → 87.4
		4.84 → 83.5
		4.22 → 72.1
		4.02 → 72.1
		3.82 → 55.5

The ^{13}C-NMR spectrum (appendix 5d) supported the presence of furofuran lignan by the oxygenated methine carbon at δ 101.9, four tri-substituted carbons at δ 87.4, 83.5, 62.9 and 55.0 and an oxygenated methylene at δ 72.1. This was further supported by the DEPT experiment (figure 5e) which showed one methylene carbon atom at δ 72.1 and a hydroxylated methylene at δ 101.9 of the furofuran lignolide. The DEPT experiment also showed methyl carbons at δ 54.1, 55.3 and 55.5 and five quaternary signals at δ 149.8, 149.7, 149.2, 136.5 and 135.4. This further suggested a 1’, 3’, 4’ tri-substituted and a 1’’, 4’’ di-substituted phenyl groups (Shuyun *et al*., 2007).

The furofuran lignan skeleton was confirmed further by the HSQC and HMBC experiments. The HSQC (appendix 5f) showed that the protons at δ 4.02 and 4.22 correlated to δ 72.1; δ 5.69 correlated to δ 101.9; δ 6.86 correlated to δ 110.7 and 117; δ 6.93 correlated to δ 118.3; δ 7.25 correlated to δ 110.7 while δ 6.98 correlated to δ 118.7. The HMBC (figure 5g) showed δ 3.92, 7.00 and 7.25 correlated to δ 149.8 while δ 6.98 and 7.00 correlated to δ 16.5. These HSQC and HMBC correlations (Table 4.12) supported the 1', 3', 4' tri-substituted and a 1'', 4'' di-substituted phenyl groups (Shuyun *et al*., 2007). These spectroscopic data compared closely with the one reported for membrin **118** and petaslignolide A (Estrada-Reyes *et al*., 2002; Min *et al*., 2005). The compound was therefore proposed to be a mixture of two epimers **116** and **117**.



118



116 R_1= OH, R_2= H
117 R_1= H, R_2= OH

Table 4.13: ^{1}H- and ^{13}C-NMR data of compound **116** and reported data for membrin (Estrada-Reyes *et al*., 2002)

No	**116**		Membrin	
	δ_H (Acetone-d_6, J, Hz)	δ_H(Acetone-d_6)	δ_H ($CDCl_3$, J, Hz)	δ_H($CDCl_3$)
I	3.16 m	62.9	2.73 t (J = 7.2 Hz)	62.0
2	4.92 d (J = 6.6 Hz)	87.4	4.76 d (J = 7.2 Hz)	87.4
4α 4β	4.02 m 4.22 m	72.1	3.91dd (J = 8.8, 2.6 Hz) 4.12 dd (J = 8.8, 5.6 Hz)	71.4
5	3.16 m	55.0	3.03 m	54.2
6	4.84 d (J = 6.8 Hz)	83.5	4.76 d (J = 7.2 Hz)	82.5
8α 8β	5.69 d (J = 4.0 Hz) 5.56 d (J = 3.2 Hz)	101.9	3.84 d (J = 4.0 Hz) 4.20 d (J = 3.0 Hz)	71.4
1’		135.4		135.9
2’	6.86 s	110.7	6.95 d (J = 2.0 Hz)	110.3
3’		149.7		149.0
4’		149.2		148.6
5’	6.93 d (J = 8.2 Hz)	112.0	6.84 d (J = 8.4 Hz)	111.0
6’	6.99 d (J = 8.2 Hz)	118.7	7.06 d (J = 8.4 Hz)	118.8
1’’		136.5		134.0
2’’	7.25 d (J = 1.8 Hz)	111.7	7.10 d (J= 2.0 Hz)	110.7
3’’		118.3		118.8
4’’		149.8		148.6
5’’	7.03 d (J= 8.0 Hz)	112.0	6.71 d (J = 8.4 Hz)	113.0
6’’	7.25 dd (J = 1.8 Hz)	118.7	6.84 d (J = 8.4 Hz)	118.8
3’’-OMe	3.82 s	55.5	3.81 s	55.3
4’’-OMe	3.80 s	55.3	3.88 s	55.4
4’-OMe	3.79 s	54.1	3.90 s	56.0

The H-8 (δ 5.69, d, J = 4.8 Hz and δ 5.56, d, J = 3.2 Hz) and C-8 (δ 101.9) signals of compounds **116** and **117** were more deshielded compared to that of membrin (Table 4.14) as C-8 is oxygenated by a hydroxyl. The configurations of the hydroxyl group at C-8 of **116** and **117** were deduced from the coupling constants of the two ^{1}H-NMR signals at δ 5.69 (d, J =

4.8 Hz) and δ 5.56 (d, J = 3.2 Hz). The coupling constant of J = 4.8 and 3.2 Hz suggested H-8α and H-8β hydrogens, respectively (Min *et al*., 2005). This in turn suggested a β- and α-orientations of the C-8 hydroxyl groups, respectively (Estrada-Reyes *et al*., 2002).

Table 4.14: ^{1}H-NMR of compounds **116**, **117** and reported data for membrin at C-8 (Estrada-Reyes *et al*., 2002)

No	**116** 8β-epimer	**117** 8α-epimer	**118**
H-8	5.69 d (J = 4.8, 8α-H)	5.56 d (J = 3.2, 8β-H)	3.84 d (J = 4.0, 8α-H) 4.20 d (J = 3.2, 8β-H)

4.4.6 Compound 119

Compound **119** was obtained from the hexane and DCM root extracts of *E. hispidus* as white crystals with a melting point of 110-112°C. On the TLC the compound had an R_f of 0.7 in hexane-EtOAc (2:3). When spoted on a TLC plate and sprayed with p-anisaldehyde, the spot turned pink-red suggesting aromaticity (Bijal and Shantu, 1990).

The ^{1}H-NMR signals (appendix 6a) showed two aromatic proton doublets of an AB system at δ 7.11 (d, J = 3.8 Hz) and δ 6.98 (d, J = 3.9 Hz) which suggested a thiophene skeleton (Fokialakis *et al*., 2005). The triplet at δ 2.62 was assigned to a methylene proton while the signal at δ 3.29 dd (J = 6.6, 11.3 Hz) and δ 3.67 (t, J = 6.6 and 11.3 Hz) suggested a hydroxylated methylene carbon atom. The singlet at δ 2.00 implied a methyl group attached to a carbon-carbon triple bond (Fokialakis *et al*., 2005). Both the COSY (appendix 6b) and NOESY spectrum (appendix 6c) showed that the peak δ 2.62 correlated to 3.69. The COSY

spectrum confirmed the aromatic protons as δ 7.11 correlated to δ 6.98. The thiophene structure was further supported by the COSY and NOESY spectra (Table 4.15).

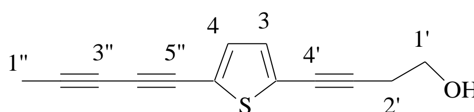
Table 4.15: Some COSY, NOESY, HSQC and HMBC correlations of **119**

COSY	NOESY	HSQC	HMBC
7.11 ➔ 6.98,2.62	7.11 ➔ 3.29, 3.67	7.11 ➔ 134.9	7.11 ➔ 134.9,61.3,
3.67 ➔ 2.62	6.99 ➔ 3.29	6.98 ➔ 132.4	6.98 ➔ 74.8,132.4
3.29 ➔ 2.62	3.67 ➔ 2.67	3.67 ➔ 61.4	3.67 ➔ 94.0
		2.62 ➔ 24.5	2.67 ➔ 127.4,132.4,134.9
		2.00 ➔ 4.8	

The ^{13}C-NMR spectrum (apendix 6d) indicated the presence of four aromatic signals at δ 134.9, 132.4, 127.4 and 123.5. The former two were protonated while the later were quaternary. It also showed seven alkyne singlets at δ 94.0, 84.2, 79.6, 74.8, 66.9, 64.1 and 61.2, one oxygenated methylene at δ 61.3, one methylene at δ 24.5 and one highly shielded methyl singlet at δ 4.8. This information further suggested the presence of a thiophene ring substituted at both C-2 and C-5, where the substituents contain three alkyne groups (Fokialakis *et al*., 2005). This was supported by the DEPT, HSQC and HMBC experiments (Table 4.15). The DEPT experiment (appendix 6e) showed one methyl signal at δ 4.8, one methylene signals at δ 24.5 and one oxygenated methylene signal at δ 61.3, two methine signals at δ 132.4 and 134.9 and two quaternary signals at δ 123.4 and 127.4. The HSQC (appendix 6f) showed the signal at δ 7.11 correlated to δ 134.9 while δ 6.98 correlated to δ 132.4. The signal at δ 3.69 and 3.29 correlated to δ 61.3; δ 2.62 correlated to δ 24.5 and δ 2.00 correlated to δ 4.8. The HMBC spectrum (appendix 6g) showed that δ 7.11 correlated to δ 79.6 while δ 94.0 correlated to δ 123.5. The signal at δ 7.11 correlated to δ 66.9 and 79.6

while δ 123.5 correlated to δ 3.67. These HMBC correlations further supported a thiophene ring substituted at C-2 and C-5

The ^{1}H-NMR and ^{13}C-NMR data closely resembled that of 4-[5-(penta-1,3 dieynyl)thien-2-yl]-3-ynyl alcohol (table 4.10) which is reported from *E. ritro* (Fokialakis *et al*., 2005). The compound was therefore proposed to be 4-[5-(penta -1,3 dieynyl)thien-2-yl]-3-ynyl alcohol whose structure is shown below. This compound is being reported from *E. hispidus* for the first time.



119

Table 4.16: ^{1}H-NMR and ^{13}C-NMR data of compound **85** and reported data for 4-[5-(penta -1,3 dieynyl)thien-2-yl]-3-ynyl alcohol (Fokialakis *et al*., 2005)

No	**119**		4-[5-(penta-1,3-dieynyl)thien-2-yl]2-chlorobut-3-ynyl alcohol	
	δ_H (MeOD, J, Hz)	δ_C (MeOD)	δ_H ($CDCl_3$, J, Hz)	δ_C
2		127.4		125.0
3	7.11 d (J = 3.8 Hz)	134.9	7.08 d (J = 3.9 Hz)	133.7
4	6.98, d (J = 3.9 Hz)	132.4	7.05 d (J= 3.9 Hz)	133.1
5		123.5		123.1
1’	3.29 dd (J = 6.6, 11.3 Hz) 3.67 t (J= 6.6, 11.3 Hz)	66.9	3.30 dd (J = 6.0, 11.2 Hz) 3.70 dd (J = 6.0, 11.2 Hz)	66.5
2’	2.62 t (2H, J = 7.2, 13.2 Hz)	24.5	4.92 t (J = 6.0 Hz)	24.8
3’		94.0		94.4
4’		79.6		80.1
1’’	2.00 s	4.8	2.00 s	4.8
2’’		84.2		83.9
3’’		64.1		64.2
4’’		66.9		66.3
5’’		74.8		75.0

The structure of **119** was confirmed by the MS spectrometry analysis (appendix 6h) which showed the characteristic $[M+2]^+$ peak at m/z 216 (5%). This was attributed to the typical isotope pattern (^{32}S, 95.6% and ^{34}S, 4.16%) (Margl *et al.***,** 2001). The peak at *m/z* 214 (90 %) was attributed parent ion $[M]^+$ which was consistent with the molecular formular $C_{13}SH_{10}O$ and molecular mass 214. The peak at *m/z* 196 (3 %) was attributed to loss of a $-CH_2OH$ group while the one at m/z 183 (100 %) was possibly due to loss of a water molecule.

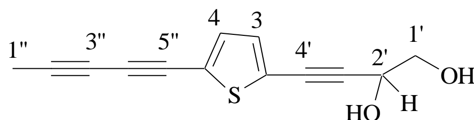
4.4.7 Compound 120

Compound **120** was obtained from the DCM root extract of *E. hispidus* as colourless needles with a melting point of 118-120°C. On the TLC the compound had a R_f of 0.6 in hexane-EtOAc (2:3). When spoted on a TLC plate and sprayed with p-anisaldehyde, the spot turned pink-red and later yellow suggesting aromaticity in the compound (Bijal and Shantu, 1990). The ^1H-NMR signals (appendix 7a) showed two proton doublets at δ 7.29 (d, J = 3.6 Hz) and δ 7.14 (d, J = 3.8 Hz) suggested an AB system characteristic of an aromatic system. The two deshielded aliphatic protons at δ 3.67 (t, J = 6.6, 11.0 Hz), 4.59 (t, J = 6.4 and 11.0 Hz) indicated the presence of a hydroxylated methylene and hydroxylated methine hydrogens. The three proton singlet at δ 2.00 implied a methyl group attached to a carbon-carbon triple bond.

The ^{13}C-NMR spectrum (appendix 7b) revealed two methine singlets at δ 133.6 and 132.8 and six alkyne singlets δ 91.4, 84.4, 79.6, 79.9, 66.3 and 64.1. It also showed peaks at at δ 63.9 and 66.2 which suggested a deshielded oxygenated methylene and hydroxylated methane carbons, respectively. One highly shielded singlet at δ 4.8 confirmed the presence of a mehyl group attached to a carbon-carbon triple bond (Fokialakis *et al*., 2005). This

information suggested the presence of a thiophene ring substituted at both C-2 and C-5, where the substituents contain three alkyne groups (Fokialakis *et al*., 2005).

The ^{1}H-NMR data was similar to that of **119** except δ 3.69, 4.59 and 4.69 were deshielded (Table 4.17). The ^{13}C-NMR data was also noted to be very close to that of **119** except δ 63.9 and 66.2 were more deshielded (table 4.9). This was due to the additional hydroxyl group at C-2'. From the above physical and spectroscopic data the compound was proposed to be 4-[5-(penta-1,3-dieynyl) thien-2-yl] but-3-ynyl diol whose structure is shown below. This compound is being reported for the first time.



120

Table 4.17: ^{1}H- and ^{13}C-NMR data of compound **120** and 4-[5-(penta-1,3 dieynyl)thien-2-yl]-3-ynyl alcohol

No	**120**		4-[5-(penta-1,3-dieynyl)thien-2-yl]-2-chlorobut-3-ynyl alcohol	
	δ_H (MeOD, J, Hz)	δ_C (MeOD)	δ_H ($CDCl_3$, J, Hz)	δ_C
2		124.2		127.4
3	7.29 (d, J = 3.6 Hz)	133.6	7.11 (d, J = 3.8 Hz)	134.9
4	7.14 (d, J = 3.8 Hz)	132.8	6.98 (d, J = 3.9 Hz)	132.4
5		123.8		123.5
1'	3.69 (t, J = 6.6, 11.0 Hz)	63.9	3.67 (t, J= 6.6, 11.3 Hz))	61.3
2'	4.59 (t, J = 6.4, 11.0 Hz)	66.2	2.62 (2H, t, J = 7.2, 13.2 Hz)	24.5
3'		91.4		94.0
4'		79.9		79.6
1''	2.00 s	4.8	2.00 s	4.8
2''		84.4		84.2
3''		64.1		64.1
4''		66.3		66.9
5''		77.6		74.8

The deshielding of the signals at δ 4.59 and 3.69 in the ^{1}H-NMR spectrum and δ 63.9 and 66.2 in the ^{13}C-NMR spectrum were due to the additional hydroxyl group at C-2'. The structure of compound **120** was confirmed by the GC-MS spectrometry analysis (apendix 7c) which showed the parent ion ion peak at *m/z* 230 $[M]^{\cdot}$ (4 %) consistent with molecular formular $C_{13}H_{10}SO_2$. The base peak appeared at *m/z* 216 (100 %), possibly corresponded to loss of $[-CH_3]^{\cdot}$ group while the peak at *m/z* 173 was attributed to loss of $[-C{\equiv}CCH_3]^{\cdot}$ and H_2O. The peak at m/z 159 (40 %), was possibly corresponding to loss of $[-C{\equiv}C\text{-}CH(OH)CH_2OH]^{\cdot}$ while the one at *m/z* 135 (64 %), was possibly due to loss of

$[-C \equiv C-C \equiv C-CH_3]^{\cdot}$ and $[-CH_2OH]^{\cdot}$. The peak at *m/z* 105 (15%) was attributed to loss of $[-C \equiv C-C \equiv C-CH_3]^{\cdot}$ and $[-CH_2(OH)CH_2OH]^{\cdot}$ groups.

4.4.8 Antibacterial activity of isolated compounds

The isolated compounds were subjected to bacterial assays using the diffusion method assay and the inhibition zones in mm measured after 24 hours, 2 mg/disk (diameter 6 mm) (Chhabra and Uiso, 1992). The antibacterial activities were tested in triplicates against one Gram-negative S*taphylococcus aureus* ATCC 25833, and two Gram-negative organisms, *Escherichia coli* ATCC 25837 and *P. aeroginos*a ATCC 10729. The mean activities were tabulated as in Table 4.18.

Table 4.18: Antibacterial activities (mm) isolated compounds

Compounds (2 mg/disk)	*S. aureus*	*E. coli*	*P. aeroginosa*
Compound **112**	8 ± 1.34	6 ±1.03	6± 1.45
Compound **113**	8 ± 1.56	6 ±1.01	6± 1.01
Compound **114**	7 ±1.06	6 ±1.09	6± 1.33
Compound **115**	6 ±1.44	6 ± 1.92	6± 1.05
Compound **116**	8 ± 1.15	6 ±1.56	6± 1.43
Compound **119**	10 ± 1.07	6 ±1.34	6± 1.05
Compound **120**	9 ± 1.06	6 ±1.43	6± 1.62
Gentamycin ((1000 µg/disk)	19 ± 1.34	15 ±1.63	14± 1.71

Gentamycin gave activities with inhibition zones of 19, 15 and 14 mm against *S. aureus, E. coli* and *P. aeruginosa and* respectively (Table 4.18). Compounds **112** and **113** exhibited a mild activity of 8 mm against *S. aureus* but no activity against *E. coli* and *P. aeruginosa*

(Table 4.18). Compounds **114** and **116** exhibited mild activities with inhibition zones of of 7 and 8 mm, respectively against Gram-positive *S. aureus* but in-active against *E. colli* and *P. aeroginosa.* The compound **115** exhibited no activity against all the test organisms. Compound **119** exhibited a moderate activity with an inhibition zone of of 10 mm against Gram-positive *S. aureus* (table 4.12) but showed no activity on the Gram-negative test organisms; *E. colli* and *P. aeroginosa*. Compound **120** exhibited a mild activity with an inhibition zone of 9 mm against Gram-positive *S. aureus* (Table 4.18) but showed no activity on the Gram-negative test organisms. The antibacterial activities of compounds **119** and **120** indicated their potency. This justified the use of *E. hispidus* and *G. similis* in traditional medicine by different ethinic communities against bacterial diseases.

4.4.9 Antifungal activity of isolated compounds

The isolated compounds were subjected to antifungal assays using the diffusion method assay against *Cryptococcus neoformans.* The anhibition zones were measured after 24 hours in triplicates, 2 mg/disk (diameter 6 mm) (Chhabra and Uiso, 1992).

Table 4.19: Antifungal activities (mm) of isolated compounds

Compound (1000 µg/disk)	*C. neoformans*
Compound **112**	6 ± 0.18
Compound **113**	6 ± 1.07
Compound **114**	6 ± 1.56
Compound **115**	33 ± 2.33
Compound **116**	6 ± 1.13
Compound **119**	33 ± 2.23
Compound **120**	27 ± 2.32
Amphotericin B (1000 mµ/disk)	38 ± 1.78
DMSO	6 ± 0.09

Compounds **119** and **120** exhibited a high antifungal activity of 33 and 27 mm, respectively against *C. neoformans* (Table 4.13). The compounds **114** and **115** showed no activity against *C. neoformans.* The high antifungal activities of compounds **119** and **120** indicated their potency. This justified the use of *E. hispidus* in traditional medicine for the treatment of fungal diseases by different ethinic communities.

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

The results showed that the percentage yields decreased from hexane to DCM and then increased from DCM to EtOAC for both plants (table 4.1). These results suggested that both plants are very rich in non-polar and polar metabolites.

The hexane/DCM extract of *G. similis* showed a very strong antibacterial activity of 15 mm compared to 14 mm that of gentamycin against *S. aureus.* This extract exhibited no antifungal properties against *C. neoformans.* The EtOAc extract of *E. hispidus* showed mild antibacterial activity of 9 mm against *S. aureus* while the DCM and EtOAc extracts of *E. hispidus* exhibited moderate antifungal activities with inhibition zones of 13 and 15 mm against *C. neoformans*, respectively

Phytochemical characterization of the compounds isolated from the hexane/DCM root extract of *G. similis* led to 3β-sitosterol (**112)** and 3β-stigmasterol (**113**)**.** The hexane root extract of *E. hispidus* led to a teraxerane triterpenoid acetyltaraxerol (**114)**, sesquiterpenoid cameroonan-7α-ol (**115**), furofuran lignans membrin-8α-ol (**116)** and membrin-8β-ol (**117**), and a polyacetylene thiophene 4-[5-(penta-1,3-dieynyl) thien-2-yl] but-3-ynyl alcohol (**119**). Compound cameroonan-7α-ol (**115**) had the characteristic colour and odour of the oil of *E. hispidus* hence it is responsible for the fruity smell of the oil. The DCM root extracts of *E.*

hispidus led to a polyacetylene thiophene 4-[5-(penta-1,3-dieynyl) thien-2-yl] but-3-ynyl diol (**120**)**.** The 3β-sitosterol (**112**) and 3β-stigmasterol (**113**) are being reported from *G. similis* for the first time while the compounds acetyltaraxerol **(114)**, cameroonan-7α-ol (**115**) and 4-[5-(penta-1,3-dieynyl)thien-2-yl]but-3-ynyl alcohol (**119**) are being reported from *E. hispidus* for the first time. The compounds membrin-8α-ol (**116**), membrin-8β-ol (**117**) and 4-[5-(penta-1,3-dieynyl) thien-2-yl]but-3-ynyl diol (**120**) are new compounds.

The isolated compounds **112** and **113** showed mild activities of 10 and 8 mm, respectively, against *S. aureus*. Compound **119** and **120** exhibited potent antifungal activities of 33 and 27 mm against *C. neoformans*, respectively (Table 4.19). Compounds **119** and **120** had high activities and may be potential lead compounds to be further structurally modified leading to novel and effective therapeutics. From the results, *E. hispidus* is a better antifungal agent while *G. similis* is a better antibacterial agent. In conclusion, we have provided the experimental data to support the use of *E. hispidus* Fresen *and G. similis* K. Schum as herbal medicine.

5.2 Recommendations

i. The compounds isolated are not the only compounds present in the two plants extracts as evidenced from the TLC analysis. Further work should therefore be carried out to isolate other compounds which may be more bioactive.

ii. There is need to extend phytochemical and antimicrobial investigation of these plants to the methanol extracts and other fractions which were not analysed in this study.

iii. More research should be carried out on both the crude extracts and the isolated compounds to include antiplasmodial, anticancer, antimalarial, antivenom, antihypertensive and insecticidal activities.

iv. In particular anticancer screening of *E. hispidus*, *G. similis* and compounds **112, 113**, 116, **119** and **120** should be carried out as the two plants and these compounds are reputed in cancer treatment.

v. Derivatives of compounds **112** and **113** with conventional antibiotics should be synthesised and their antimicrobial activities studied as this may produce more potent drugs.

vi. Further testing of **115** should be carried out in the cosmetic and fragrance industry.

vii. *In vivo* antimicrobial efficacy tests should be carried out to validate the *in vitro* results.

viii. Both plants are in high demand owing to their traditional uses with *G. similis* being valued for its edible fruits and twings used as chewing sticks. Measures should therefore put in place to conserve these plants from extinction.

ix. Synergic effects among the crude extracts, the isolated compounds and the conventional antibiotics should be investigated.

x. Dose response anti-bacterial and anti-fungal screening to be done on the compounds isolated in this work.

REFERENCES

Abadi, J., Nachman, S., Kressel, A.B. and Pirofski, L. (1999). Cryptococcosis in children with AIDS. *Clinical Infectious Diseases* **28**, 309.

Agrawal, P.K., Thakur, R.S. and Bansal, M.C. (1989). In studies in organic chemistry 39, Carbon -13 NMR of flavonoids; Agrawal, P.K., ed. Elsevier Science publishing Company Inc. New York, USA pp 123.

Ahmad, V.U., Aliya, R., Perveen, S. and Shameel, M. (1994). *Phytochemistry* **31**, 1429.

Ahmad, V.U. and Rahman, A.U. (1996). Pentacyclic triterpenoids, Handbook of Natural Products Data, Elsevieer, Amsterdam pp 235.

Akerere, O. (1993) Summary of WHO guideline for the assessment of herbal medicines. *Herbalgram*, **28**, 13.

Alam, M.S., Chopra, N., Ali, M. and Niwa, M. (1996). Oleanen and stigmasterol derivatives from *Ambronna Augusta*. *Phytochemistry* **41**, 1197.

Albuquerque da Costa D., Silva, D.A., Cavalcanti, A.C., Alves de Medeiros, M.A., Tolentino de Lima, J., Cavalcante, J.M.S., Araujo da Silva, B., Maria de Fatima A. and Vanderlei de Souza, M.F.(2007). Chemical constituents from *Bakeridesia pickelii* Monteiro (Malvaceae) and the relaxant activity of kaempferol-3-O-β-D-(6"-E-p -coumaroyl) glucopyranoside on guinea-pig ileum. *Quimica Nova* **30**, 4.

Aregbeyen, J.B.O. (1983). Free health care delivery program in Bendel state of Nigeria. Problems and Prospects. PhD Disertation Washington, D.C, Howaed University pp 156.

Badami, S., Vijayan, P., Mathew, N., Chandrashekhar, R., Godavarthi. A., Dhanaraj, S.A. and Suresh, B. (2003). In vitro cytotoxic properties of *Grewia tiliafolia* bark and lupeol. *Indian Journal of Pharmacology* **35**, 250.

Baldry, P. (1976). The battle against bacteria. A fresh look. Cambridge university press, Hampshire pp 32.

Ball. P., Mandell, L., Niki, Y. and Tillotson, G. (1999). Comparative tolerability of the newer fluoroquinolone antibacterials. *Drug Safety* **21**, 407.

Barnes, P. (2000). Management of drug-resistant Gram-positive pathogens. *Journal of Hospital Infections* **57,** 258.

Beentje, H.J. (1994). Kenya trees , shrubs and lianans **1**. National museums of Kenya, Nairobi. pp 196.

Bijal, M. and Shantu, A. (1990). Synthesis and mutagenicity of trans-dihydrodiol metabolites of benzo[β]naphtha[2,1-α]thiophene. *Chemical Research Toxicology* **3**, 93.

Bodeker, G. (1994). Traditional health knowledge and public policy. *Nature and Resources* **30**, 5.

Calixto, J.B. (2000). Efficacy, safety, quality control, marketing and regulatory guidelines for herbal medicines (Phytotherapeutic agents). *Brazilian Journal of Medical and Biological Research* **33**, 179.

Callejas, A., Ordonez, M.N., Rodriguez, C. and Castaneda, E. (1998). First isolation of *Cryptococcus neoformans* var. gattii, serotype C, from the environment in Colombia. *Medicinal Mycology* **36**, 341.

Casadevall, A., Rosas, A.L. and Nosanchuk, J.D. (2000). Melanin and virulence in *Cryptococcus neoformans*. *Current Microbiology* **3**, 354.

Chaudhuri, P.K. (1992). 7-Hydroxyechinozolinone, a new alkaloid from *Echinops echinatus*. *Journal of Natural Products* **55**, 249.

Chhabra, S.C. and Uiso, F.C. (1992). Antibacterial activity of some Tanzanian plants used in traditional medicine. *Fitoterapia* **62,** 499.

Chien, N.Q., Hung, N.V., and Sung, T.V. (2004). Nghien cuuthanh hoa hoc cay *Kydia glabrescens*. *Tap Chi Hoa Hoc* **42**, 71.

Choi, S.Z., Yang, M.C., Choi, S.U and Lee, K.R. (2006). Cytotoxic terpenes and lignans from the roots *Ainsliaeae acarifolia*. *Archives of Pharmaceutical Research* **29**, 203.

Cornelis P. (2008). *Pseudomonas,* Genomics and Molecular Biology, 1st ed, Caister Academic Press pp 201.

Dey, P.M. and Harborne, J.B. (1991). Methods in plant biochemistry. Terpenoids. Aczdemic press, New York, London **7**, 370.

Dopke, W. and Fritsch, G. (1969). Echinine, a dihydroquinoline from *Echinops ritro* seeds. *Pharmazia* **24**, 782.

Ellis, D. (1999). Household hyphomycetes and other indoor fungi. *Journal of Mededical Veterinary Mycology* **32**, 27.

Elujoba, A.A, Odeleye, O.M. and Ogunyemi C.M. (2005). Traditional Medical Development for medical and dental primary Health care Delivery System in Africa. *African Journal of Traditional, Complementary and Alternative Medicine*, **2**, 46.

Estrada-Reyes, R., Alveraz, A.L.C., Lopez-Rubalcavas, C., Rocha, L., Heinze, G., Moreno, J. and Martinez-Vazquez, M. (2002). Lignans from *Rollina mucosa*. *Z. Naturforsch* **57**, 29.

Farnsworth, N.R, Soejarto, D.D., Guo, Z., Bingel, A.S. and Akerere, O. (1985). Medicinal plants in therapy. *Bulletin of the WHO* **63**, 965.

Fauci, A. (1998). New and emerging diseases, New perspective on international collaboration. *Journal of Natural Products* **16**, 565.

Fokialakis, N., Osbrink, W.L.A., Mamonov, L.K., Gemejieva, N.G., Mims, A.B., Stkaltsounis, A.L, Lax, A.R., and Cantrrell C.L. (2005). Antifeedant and toxicity effects of thiophenes from four *Echinops* species against the *Formosan subterranean* termite, *Coptotermes formosanus*. *Pest Management Science* **62**, 832.

Foye, K., Fujii, I. and Ebizuka, Y. (1989). Anthracycline biosynthesis in *Streptomyces. Galilaeus Chemotherapy* **97**, 2511.

Gomes, A., Saha, A., Chatterjee, I. and Chakravarty, A.K. (2007). Viper and cobra venom neutralization by beta-sitosterol and stigmasterol isolated from the root extract of *Pluchea indica* less. (Asteraceae). *Phytomedicine* **14**, 637.

Gomez, M.A., Garcı´a, M.D. and Saenz, M.T. (2001). Cytostatic activity of *Achillea ageratum* L**.** *Phytotherapy Research* **15**, 633.

Groll, A. and Piscitelli, S. (1998). Clinical pharmacology of systematic and fungal events. A comprehensive review of agents in clinical use. *Current Investigational Compounds and Putative Targets for Antifungal Drug Development* **44,** 35.

Harborne, F.B. (1998). Phytochemical methods 3rd ed. Chapman J.R., Blackburn H.S. (1973). Introductory microbiology, John Wlley and SONS Innc. New York pp. 535.

Heywood, V.H. (1977). *The Biology and Chemistry of Compositae* **2**, 141.

http://www.mobot.mobot.org/.

Hudault, B. (2001). *Escherichia coli* strains colonizing the gastrointestinal tract protect germfree mice against *Salmonella typhimurium* infection. *Gut* **49**, 47.

Hymete, A., Rohloff, J., Kjθsen, H., Iverson, T. (2005). Acetylenic thiophenes from the roots of *Echinops ellenbeckii* from Ethiopia. *Natural Product Research* **19**, 8-14.

Iwu, M.W., Duncan, A.R. and Okunji, C.O. (1999**).** *New antimicrobials of plant origin. Perspectives in new crops and new uses,* ASHS Press, V.A.J. Janick ed. Alexandria pp 457.
Jacobson, E.S. (2000). Pathogenic roles for fungal melanins. *Clinical Microbiology Review* **13**, 708.
Jaspers, M.W., Bashir, A.K., Zwaving, J.H. and Malingre, T.M. (1986). Ivestigation of Grewia bicolar . *Journal of Ethnopharmacology* **17**, 205.

Jayasinghe, U.L.B., Balasooriya, B.A.S., Bandara, A.G.D. and Fujimoto, Y. (2004). *Taylor and Francis Helth Sciences* **18**, 499.

Kamkaen, N., Matsuki, Y., Ichino, C., Yohara, H. and Yamada, H. (2006). Isolation of of the anti-*Heliobacter pyroli* compounds in the seeds of Arctium lappa. *Thai Pharmaceutical and Health Science Journal* **1**, 12.

Knowles, D. (1997). New strategies for anti-bacterial drug design. *Tred in Microbiology* **5**, 379-383.

Kokwaro, J.O. (1996). *An Ethnobotanical study of East African medicinal plants and traditional medicine. Science Symbosium*. Baltimore, Maryland pp.23.

Korfel, A., Menssen, H.D., Schwartz, S. and Thiel, E. (1998). Cryptococcosis in Hodgkin's disease, description of two cases and review of the literature. *Analytical Hematology* **76**, 283.

Kubitschek, H.E. (1990). Cell volume increase in *Escherichia coli* after shifts to richer media. *Journal of Bacteriology* **172**, 4

Levitz, S.M. (1991). The ecology of *Cryptococcus neoformans* and the epidemiology of cryptococcosis. *Review of Infectious Diseases* **13**, 1163.

Li-Hua X., Teruaki A., Kenjiro H., Takeshi D. and Masao H. (2003). Biotransformation of pinoresinol diglucoside to mammalian lignans by human intestinal microflora, and Isolation of *Enterococcus faecalis* Strain PDG-1 responsible for the transformation of (+)-pinoresinol to (+)-lariciresinol. *Chemical Pharmaceutical Bulletin* **51,** 508.

Ma, C., Zhang, H.J., Tan, G.T. Hung, N.V., Cuong, N.M., Soejerto, D.D., and Fong, H.H. (2006). Antimalarial compounds from *Grewia bilamellata. Journal of Natural Products* **69**, 346.

Mahato, S.B. and Kundu A. (1994). ^{13}C-NMR spectra of pentacyclic triterpenoids, a compilation and some salient features. *Phytochemistry* **37**, 1517.

Mahmoud, Y.A.G. (1999). First environmental isolation of *Cryptococcus neoformans* var. neoformans and var. gatti from the Gharbia Governorate, Egypt. *Mycopathologia* **148**, 83.

Maundu, P., Berger, D.J., Saitabu, C., Nasieku, J., Kipelian, M., Berger, P., Mathenge, S., Marimoto, Y. and Hoft, R. (2001). Ethnobotany of the Loita Maasai; towards community management of the forest of the lost child; experiences from Loita ethnobotany project. *People and Plants Working Paper* **8**, 22.

Makhubu, L. (2003). Traditional medicine, African Journal of Traditional, Complementary and Alternative Medicine. Swaziland pp 131.

Margl, L., Tei, A., Gyurjan, I. and Michael, W.M. (2001a). GLC and GLC-MS Analysis of Thiophene Derivatives in Plants and in *in vitro* Cultures of *Tagetes patula* L. (*Asteraceae*). *Z. Naturforsch* 57, 63.

Margl, L., Eiusenreiich, W.I., Adam, P., Bacher, A. and Zenk, M.H. (2001b). Biosynthesis of thiophenes in *Tagetes patula*. *Phytochemistry* 58, 875.

McChesney, J.D., Clark, A. and Silveira, E. (1991). Antibacterial diterpenes of *Croton sonderianus*, hardwickie and 3, 4-secotrachylobanic acids. *Journal of Natural Products* **54**, 1625.

Min, B., Cui, A.H., Lee H. and Kim, M. R. (2005). Anew furofuran lignan with anti-oxidant and antiseizure activities from the leaves of *Petasites japonicus*. *Archives of Parmaceutical Research* **28**, 1023.

Mulholland, D.A., Sewram, V., Raynor, M., Thornell, K. and Raidoo, D.M. (2002). Coupling SFE to uterotonic bioassay: an online investigation of the uterotonic activity of compounds from *Grewia occidentalis* (Tiliaceae). *South African Journal of Botany* **68**, 68.

Okigbo, R.N., Mbajiuka, C.S. and Njoku, C.O. (2005). Antimicrobial potentials of (Uda) *Xylopia aethiopica* and *Ocimum gratissimum* L. on some pathogens of Man. *International Journal of Molecular Medicine and Advanced Science* **1**, 392.

Owens, R.C. and Ambrose, P.G. (2005). Antimicrobial safety: focus on fluoroquinolones. *Clinical Infectious Diseases* **41**, 144.

Paterson, D.L. and Bonomo, R.A (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Review* **18**, 657.

Peters, W. (1987). Chemotherapy and drug resistance in Malaria. Academic press Ltd, London pp 49.

Rahman, A. A. and Surk-sik, M. (2007). Antimicrobial phenolic derivatives from Dendranthema Zawadskii var. latilobum Kitamura (Asteraceae). *Archives of Pharmaceutical Resesearch* **30**, 1374.

Reid, R.D. (2001). Can bacterial interference prevent infection? *Trends in Microbiology* **9**, 424.

Reynold, W., McLean, S., Poplawski, J., Enjriquez, R., Escobar, L. and Lewon, I. (1999). Flavanoids of *Rhus japonica*. *Tetrahendron* **42**, 3418.

Roshan, B.B., Naren, M.G. and Yvonne, M.F. (2005). Antibiotic activity of marine microorganisms: the antibacterial spectrum. *Helgoland Marine Research* **13**, 188.

Schoffski, P., Freund, M., Wunder, R., Petersen, D., Kohne, C.H., Hecker, H., Schubert, U. and Ganser, A. (1998). Safety and toxicity of amphotericin B in glucose 5% or intralipid 20% in neutropenic patients with pneumonia or fever of unknown origin: randomised study. *British Medical Journal* **8**, 379.

Schroder, P. and Luckner, M. (1968). Structure and synthesis of echinorine, an alkaloid from *Echinops ritro* and *Echinops sphaerocephalu*s (Aesteraceae). *Arch Pharm Ber Dtsch Pharm Ges* **301**, 39.

Shuyun, B., Yi Ding, Zhiwei, D., Proksch, P. and Lin, W. (2007). Rhyncosides A—F, phenolic constituents from the Chinese mangrove plant *Bruguiera sexangula* var. *rhynchopetala*. *Chemical and Pharmaceutical Bulletin* **55**, 1175.

Slatore, C.G. and Tilles, S.A. (2004). Sulphonamides hypersensitivity. *Immunology and Allergy Clinics of America* **24**, 477.

Solio, J., Lutura, M., Lutuluo, R., Kunguru, K., Wood, N. and Mathenge, S. (2006). Plant use of the Maasai of Sekarani valley, Maasai Mara, Kenya. Journal of *Ethnobiology and Ethinomedicine* **2**, 22.

Swartz G.V. (2006). Phytochemical studies of *Helichrysum patulum.* Thesis submitted in partial fulfilment of the requirements for the degree of Masters of Science in the Department of Chemistry, University of the Western Cape pp 20.

Takasaki, M., Konoshima, T., Tokuda, H., Masuda, K., Arai, Y., Shiojima, K. and Ageta, H. (1999). Anti-carcinogenic activity of *Taraxacum* plant. *Biological and Pharmaceutical Bulletin* **22**, 606.

Tanaka, J.C.A., Vidotti, G.J. and Cleuza, C. (2003). A New Tormentic acid derivative from *Luehea divaricata* Mart. (Tiliaceae). *Journal of Brazazilian Chemical Society* **14**, 475.

Tene, M., Tana, P., Sondengam, B.L. and Connolly, J.D. (2004). Lignans from *Echinops giganteus*. *Phytochemistry* **65**, 2101.

Tilles, S.A. (2001). Practical issues in management of hypersensitivity reactions: Sulphonamides. *Southern Medical Journal*. **94**, 817.

Toker, G., Merve, M., Erdem Y., and Mustafa, A. (2004). Main flavonoids of *Tilia argentea* DES. leaves. *Turkish Journal of Chemistry* **28**, 745.

UK Office for National Statistics Online (2007). MRSA deaths continue to rise in 2005 pp 134.

Weyerstahl, P., Marschall, H., Seelmann, I. and Jakupovic, J. (1998). Cameroonane, prenopsane and nopsane, three new tricyclic sesquiterpene skeletons. *European Journal of Organi Chemistry* **1998**, 1205.

WHO (1996). Traditional medicine. *WHO Fact Sheet* **134**, 3.

WHO (1978). Alma Ata Declaration: primary health care. *Health for all series* **1**, 231.

WHO (2000). A publication of united against malaria. *The Magazine of WHO* **3**, 547.

WHO (2001). Legal status of Traditional Medicines and complementary/ Alternative Medicine. A world wide review. WHO publishing **1**, 103.

WHO (2003). Traditional medicine. *WHO Fact Sheet* **34**, 97.

Yadav, A.K. (1999). Phalsa; A potential new small fruit for Georgia. J. Janick ed. pp 348.

Zareh, M.M. (2005). Synopsis of the family Asteraceae in Egypt. *International Journal of Agriculture and Biology* **7**, 832.