PHYTOCHEMICAL, ANTIBACTERIAL AND ANTIFUNGAL STUDY OF *DOMBEYA TORRIDA* (J.F. GMEL) AND *HYDNORA ABYSSINICA* (A. BRAUN)

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This thesis is my original work and has not been presented for a degree in any other University.

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LIST OF SYMBOLS AND ABBREVIATIONS

$CDCl_3$	Deuterated chloroform
$CHCl_3$	Chloroform
d	Doublet
DMSO	Dimethylsulfoxide
DEPT	Distortionless enhancement by polarization transfer
EtOAc	Ethyl acetate
h	Hour
IR	Infra-red
L	Litre
Ltd	Limited
Med	Medium
m/z	Mass to charge ratio
MS	Mass spectrometry
MHz	Megahertz
MeOH	Methanol
µg	Microgram
µl	Microlitre
ml	Millilitre
M^+	Molecular ion
m	Multiplet
NMR	Nuclear magnetic resonance
ppm	Parts per million
R_f	Retention factor
s	Singlet

SDA	Sabouraud dextrose agar
str	Strong
TLC	Thin layer chromatography
TSA	Tryptone soy agar
t	Triplet
UV	Ultraviolet
λ	Wavelength
WHO	World Health Organisation

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I thank the LORD for making me what I am and for giving me the greatest gift of all, life.

DEDICATION

Dedicated to

My parents *Luka Ndwiga* and *Luciana Marigu*,

for their sacrifices as they brought us up, always believing in the fruits of education

And to my sons,

David N. Njagih and *Thomas M. Njagih*,

for making it all worthwhile.

ABSTRACT

Introduction

Traditional medicines play an essential role in healthcare. The World Health Organisation (WHO) estimates that approximately 80 % of world inhabitants rely on traditional medicine for their primary healthcare. About 85 % of the traditional medicines involve use of plant extracts. At least 119 pure chemical substances derived from 90 plant species are important drugs currently in use in one or more countries. Of these 119 drugs, 74 % were discovered as a result of chemical studies directed at the isolation of active substances from plants used in traditional medicine. Thus natural products are the skeletal frameworks of about 60 % of modern drugs that are available today. Despite the investigation of terrestrial flora, it is estimated that only 5-15 % of approximately 250,000 species of higher plants have been systematically investigated, chemically and pharmacologically. The potential of large areas of tropical rain forests remains virtually untapped. Thus the interest in nature as a source of potential chemotherapeutic agents continues. In Kenya, many plants are used in management of various disease conditions but have hardly been studied. These plants include *Dombeya torrida* (J. F. Gmel.) and *Hydnora abyssinica* (A. Braun).

The decoction of *D. torrida* bark is taken by the Maasai to treat indigestion after a large meal of meat. Its roots are used for treatment of chest pains and colds. The pseudo-rhizome of *H. abyssinica* is normally boiled and the infusion drunk as a cure for throat complaints, as an astringent in dysentery, for treatment of stomach trouble and for removing the placenta if it does not come out in time. It is also used in treatment of diarrhoea and amoebic dysentery and to treat typhoid, anthrax, cancer and East Coast Fever. *In vitro* bioassay of *H. abyssinica* aqueous, methanol and chloroform extracts, showed ability to inhibit growth of 6 human pathogenic fungi and 4 types of bacteria. Phytochemical analyses showed preponderance of condensed tannins and

phenols in the *H. abyssinica* extracts. Bioassay-guided phytochemical investigation of *H. abyssinica* to isolate secondary metabolites yielded four compounds; tetra-decanoic acid, catechin, tyrosol and benzoic acid. The isolated compounds were demonstrated to have antioxidant and antiglycation potential.

The present study was aimed at isolating compounds and investigating the antibacterial and antifungal activities of *D. torrida and H. abyssinica*.

Methodology

The *D. torrida* stem-bark and leaves; and *H. abyssinica* were collected from various parts of Kenya and identity established. Voucher specimens were kept at the School of Biological Sciences Herbarium, University of Nairobi. The *D. torrida* stem-bark leaves and flowers; and *H. abyssinica* pseudo rhizomes were air-dried, finely ground and the powder stored at room temperature in a dry place until used. Extraction of the plants was carried out using chloroform, methanol, acetone, ethyl acetate, petroleum ether and water.

The water and methanol extracts were dissolved in distilled water while dimethyl sulfoxide was used to solubilise chloroform extract to a concentration of 100 mg/ml. These were then screened for antibacterial activity against *Staphylococcus aureus, Staphylococcus epididermis, Bacillus pumilus* and *Escherichia coli*; and for antifungal activity against *Saccromyces cerevisiae* and *Candida albicans* using agar diffusion method. Gentamicin and nystatin were used as positive controls.

To isolate compounds, open column chromatography using normal phase silica gel and Sephadex LH20 were employed. Both isocratic and gradient fractionation procedures were carried out. Infra-red, mass spectrometry and NMR analysis were carried out to identify the compounds.

Results

Dombeya. torrida bark decoction had the highest activity against *S. aureus* with an inhibition zone diameter of 16.91 mm followed by *D. torrida* bark macerate with 16.73 mm. *Hydnora abyssinica* macerate had least activity against *S. aureus* with a zone diameter of 8.86 mm. *Dombeya torrida* bark decoction had the highest activity against *S. epididermis* with a diameter of 17.05 mm with *Hydnora abyssinica* macerate having the least activity. Activity against *E. coli* was highest for *D. torrida* bark decoction with zone diameter of 16.56 mm followed by *H. abyssinica* chloroform extract at 15.84 mm. *Dombeya torrida* bark macerate and methanol extracts were equipotent with zone diameters of 15.82 mm. *Hydnora abyssinica* chloroform extract had the highest activity against *B. pumilus* with a zone diameter of 17.04 mm with *H. abyssinica* macerate having the least activity at 8.51 mm.

The highest activity observed against *S. cerevisiae* was with *D. torrida* chloroform extract with a zone diameter of 17.69 mm and *H. abyssinica* chloroform extract (17.38 mm) with *H. abyssinica* macerate having the least activity (7.70 mm). *Dombeya torrida* chloroform extract was the most active extract against *C. albicans* with a zone diameter of 20.09 mm.

The autobioassay results showed *H. abyssinica* extracts to have appreciable antifungal and antibacterial activities. In this assay, dichloromethane: methanol extract of *H. abyssinica* had the highest activity against *B. pumillus* with 5 spots of inhibition. This extract had comparable effects to those of *H. abyssinica* chloroform extract against *S. aureus*. The *D. torrida* chloroform extract and *H. abyssinica* chloroform and dichloromethane: methanol extracts had a marked inhibition effects against *C. albicans* with big bands, an indication of the presence of antifungal constituents in these extracts.

Methods of isolation of compounds from *D. torrida* and *H. abyssinica* using open column using normal phase silica gel and chloroform, dichloromethane, hexane, ethyl acetate and methanol were developed. Using these methods fifteen compounds were isolated from *D. torrida* and five from *H. abyssinica.* Five of these were identified as friedelin, friedelan-3β-ol, β-sitosterol, stigmasterol and taraxerol by use of spectroscopic techniques and also by comparison with spectroscopic data of compounds in literature. This is the first time these compounds were isolated from *D. torrida*. Five compounds were also isolated from *H. abyssinica* but could not be identified. Further spectroscopic work is needed to identify these compounds.

Conclusion

The plants under study, *D. torrida* and *H. abyssinica* were chosen on the basis of folklore. These antibacterial and antifungal results support the folklore that *H. abyssinica* is used as a cure for throat complaints, as an astringent in dysentery, treatment of diarrhoea and amoebic dysentery. *Dombeya torrida* extracts also had appreciable antifungal and antibacterial activities supporting its folklore use to treat chest pains and colds as many of these conditions are usually caused by bacterial infections.

The study provides further knowledge regarding the phytochemistry of *D. torrida* with five compounds: friedelin, friedelan-3β-ol, β-sitosterol, taraxerol and stigmasterol being isolated and identified for the first time from this plant.

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

Traditional medicines play an essential role in healthcare. The World Health Organisation (WHO) estimates that approximately 80 % of world inhabitants rely on traditional medicine for their primary healthcare. Plant products also play an important role in healthcare systems of the remaining 20 % of population who mainly reside in developed countries [Cragg and Newman, 2001]. In Africa, reliance on traditional medicines is partly due to the high cost of conventional medicines and inaccessibility of modern healthcare facilities, and also because traditional medicine is often deemed a more appropriate method of treatment [Marshall, 1998]. According to Musila *et al.*, (2004) more than 90 % of the population in some parts of Kenya use medicinal plants at one time or another as their primary source of healthcare. In rural areas and among the urban poor, herbal medicine is in most cases the only form of healthcare and persons only consult physicians as a last resort. Even in areas where modern medicine is available, the interest on herbal medicines and their utilization have been increasing rapidly in recent years [WHO, 1998]. About 3.5 to 4 billion people in the world rely on plants as source of drugs [Farnsworth *et al*., 1985].

About 85 % of the traditional medicines involve use of plant extracts [Farnsworth *et al*., 1985]. The number of higher plant species on earth is about 250,000. It is estimated that 35,000 to 70,000 species have, at one time or another, been used in some cultures for medicinal purposes (WHO, 1998). In Hong Kong with a population of 5.6 million, there were at least 346 independent herbalists and 1477 herbal shops in 1981. The same year there were 3362 registered physicians and 375 registered pharmacies. Hong Kong imported herbal products worth over 190 million US dollars in 1982 compared to conventional medicines worth 80 million [Farnsworth, 1988]. In a survey of 4, 000 Japanese clinics conducted in 1983, 42.7 % of respondents reported using traditional medicines in their daily practices mostly to treat chronic diseases such as asthma, high blood pressure, colds, diabetes mellitus and gastritis [Terasawa, 1986]. In China, medicinal plants and their products had a 33.1% share of the pharmaceutical market in 1995. In Malaysia, the market for traditional medicine is estimated at about 1 billion Malaysia rinngit (313 million USD) annually (WHO, 1998).

Analysis of prescriptions dispensed from community pharmacies in the United States of America (U.S.A.) from 1959-1980 indicates that about 25 % contained plant extracts or active principles derived from higher plants. At least 119 pure chemical substances derived from 90 plant species are important drugs currently in use. Of these 119 drugs, 74 % were discovered as a result of chemical studies directed at the isolation of active substances from plants used in traditional medicine [Farnsworth *et al*., 1985]. Thus natural products are the lead compounds of about 60 % of modern drugs that are available today. Of these, about 80 % of antibiotics and 90 % of antimalarial drugs are natural derivatives [Wangchuk, 2004]. In the years 1983 to 1994, over 60 % of the approved anticancer and anti-infective agents were developed from knowledge of natural products [Cragg *et al*., 1997].

1.1.1. Drugs isolated from plants and nature

Many well-known drugs listed in modern pharmacopoeia have their origin in nature. The serendipitous discovery of penicillin from the filamentous fungus *Penicillum* by Fleming in 1929 and the observation of its broad therapeutic effects in 1940s ushered in the golden era of antibiotics and promoted intensive investigation of nature as a source of bioactive agents [Scriabine, 1999]. Other micro-organisms have yielded some of the most important products of pharmaceutical industries. These include antibacterial agents such as cephalosporins, aminoglycosides, erythromycins and tetracyclines; immunosuppresive agents such as cyclosporin and rapamycin; cholesterol lowering agents such as mevastatin; and anthelmintics and antiparasitic drugs such as ivermectins [Buss and Waigh, 1995].

French pharmacists Caventou and Pellentier reported isolation of antimalarial drug quinine from *Cinchona species* in 1820 [Buss and Waigh, 1995]. The bark had been used by indigenous groups in Amazon region for treatment of fevers and was introduced in Europe in the 17th Century for treatment of malaria. Quinine formed the basis for synthesis of commonly used antimalarials, namely chloroquine, primaquine, amodiaquine and mefloquine [Cragg and Newman, 2001]. With emergence of resistance to these drugs in many tropical regions, another plant long used in traditional Chinese medicine, *Artemesia annua* (quinhaousu) has yielded the drug artemisinin and its derivatives artemether, artesunate and artether which are effective against resistant strains of *Plasmodium falciparum* [Buss and Waigh, 1995].

Artemesinin-based combination therapy (ACT) is now recommended for the treatment of resistant malaria infections. However in order to prevent the development of resistance to artemesinin it is only recommended in combination with another non-artemesinin based therapy. It produces a very rapid reduction in the parasite biomass with an associated reduction in clinical symptoms and is known to cause a reduction in the transmission of gametocytes thus decreasing the potential for the spread of resistant alleles. Examples of ACTs are dihydroartemisinin and piperaquine, artesunate and sulfadoxine/pyrimethamine, artemether and lumefantrine, artesunate and mefloquine and artesunate and amodiaquine [WHO, 2010].

The analgesic morphine isolated from opium poppy *Papaver somniferum* (Papaveraceae) laid the basis for alkaloid chemistry and development of a range of highly effective analgesic agents such as pethidine, codeine and buprenorphine. *Papaver somniferum* was used as a drug and its seeds were used for baking in ancient Mesopotamia. Ephedrine, from *Ephedra sinica* (Ma huang), a plant long used in traditional Chinese medicine is now used as an ingredient for nasal decongestants. It is also the basis for synthesis of the anti-asthmatic agents (beta agonists) salbutamol and salmetrol [Evans, 1996; Cragg and Newman, 2001].

The anti-neoplastics vincristine and vinblastine were isolated from *Catharanthus roseus* (Madagascar periwinkle) during investigation of the plant as a source of potential oral hypoglycaemic agents. Two clinically active agents, etoposide and taniposide are semisynthetic derivatives of natural product epipodophyllotoxin. Epipodophyllotoxin is an isomer of podophyllotoxin, which was isolated as the active antitumour agent from roots of various species of genus *Podophyllum*. Paclitaxel was initially isolated from the plant *Taxus brevifolia* and is used as an anti-cancer agent [Cragg and Newman, 2001].

Cortex Pruni Africanae consists of the dried bark of the trunk of *Prunus africana* found in Kenya [WHO, 2004]. Its medicinal uses which are supported by clinical data are treatment of urinary tract symptoms of benign prostatic hyperplasia stages I and II, such as nocturia, polyuria and urinary retention as defined by Alken; in cases where diagnosis of prostate cancer is negative [Donkervoort *et al*. 1977; Barth 1981; Bartlet *et al.*, 1990]. The purported active constituents of a lipophilic extract of Cortex Pruni Africanae include docosanol, β-sitosterol, alkanols, fatty acids, sterols and triterpenes [Martinelli *et al.*, 1986; Bombardelli and Morazzoni, 1997]. Owing to over exploitation and other factors, *Prunus africana* has been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora [Cunningham *et al*. 1997].

The drug reserpine is isolated from *Rauwolfia serpentina* and was formerly used in psychiatric treatment and as an antihypertensive. Reserpine is no longer used because of side effects. *Rauwolfia serpentina* is used in Ayurvedic medicine for treatment of insects' skin bites and other ailments [Evans, 1996].

The advent of some incurable diseases especially Human Immunodeficiency Virus Acquired Immunodeficiency Syndrome (HIV/AIDS) has led to increased investigation of natural products in search for their cure. Plant chemicals have been shown to inhibit the HIV *in vitro* at many different points of its replication cycle. These include the terpene glycyrrhizin from licorice, lignanolides from *Ipomea* species and many phenolics, quinones and alkaloids [Fellows, 1992].

1.1.2. Natural compounds for production of pharmaceuticals

Although total synthesis of many medicinal products is employed in pharmaceutical companies, there is great demand for natural products which serve as starting materials for partial synthesis of other drugs like steroids. Many natural derivatives are available in sufficient amounts for synthetic purposes of steroidal pharmaceuticals. These include diosgenin, hecogenin, solasodine, deoxycholic acid, stigmasterol and sitosterol [Evans, 1996; Singh and Kaushal, 2007]

Hecogenin is the starting material for the synthesis of corticosteroids while diosgenin is used for manufacture of oral contraceptives and the sex hormones. Diosgenin can also be used for synthesis of corticosteroids employing microbiological fermentation to introduce oxygen into the 11α-position of pregnane nucleus. Steroidal alkaloids (for example solasodine from *Solanum* species) are also used in the synthesis of corticosteroids [Evans, 1996].

The yield of hecogenin is about 0.01 % from *Agave sisalana* (sisal) leaves and is obtained as an acetate. In Kenya leave waste stripped during removal of fibre gives, a hecogenin containing sisal concentrate. From this concentrate, the ‘’juice’’ is separated by filtration and allowed to ferment for 7 days. The sludge produced contains about 80 % of the hecogenin originally present in the leaves. This crude hecogenin is taken to pharmaceutical companies for further processing and cortisone manufacture [Evans, 1996].

Despite the investigation of terrestrial flora, it is estimated that only 5-15 % of approximately 250,000 species of higher plants have been systematically investigated, chemically and pharmacologically. The potential of large areas of tropical rain forests remains virtually untapped. Thus the interest in nature as a source of potential chemotherapeutic agents continues [Cragg and Newman, 2001]. In Kenya, many plants are used in management of various disease conditions [Gachathi, 1989; Musila *et al*., 2004; Kokwaro, 2009] but have hardly been studied. These plants include *Dombeya torrida* (J. F. Gmel) and *Hydnora abyssinica* (A. Braun).

1.2. Literature review

1.2.1. *Dombeya* species

1.2.1.1. Introduction

Dombeya species belongs to the division Spermatophyta (seed bearing plants), class Angiospermae (flowering plants), Sterculiaceae family (commonly called Cola family) and is dicotyledonous. This family is found throughout the world. It comprises 50 genera and approximately 1,200 species, mainly trees and shrubs found in tropical and subtropical regions. Their leaves are usually deeply cordate at base, often with overlapping basal lobes; 3-7 veined from base; often with mixed stellate and simple hairs. Flowers have reflexed sepals; petals are oblique, asymmetric and persistent. Fruits are round, hairy and quite small, hidden within the persistent flower [Beentje, 1994; Aubrey, 2001]. This genus includes the species *D. torrida* (J.F. Gmel), *D. burgessiae* Gerrard, *D. kirkii* Mast, *D. quinqueseta* (Del.) Exell, *D. rotundifolia* (Hochst) Planch and *D. taylorii* Bak.

1.2.1.2. Traditional uses

Dombeya mupangae is used for the treatment of venereal diseases and insanity [Beentje, 1994]. *Dombeya shupange* is used for the management of malaria, abdominal pains, irregular menses, amenorrhoea, wounds and to induce abortion [Sawhney *et al.*, 1978; Chhabra *et al.*, 1993; Gessler *et al*., 1994; Gessler *et al*., 1995]. *Dombeya acutangula* hot water extract is orally used to treat dysentery in human adult. Dried entire plant is used in Reunion Island as an astringent [Vera *et al.*, 1990]. In Tanzania, *D. amaniensis* dried root hot water extract is orally used for filariasis and lymphatic disorders [Chhabra *et al.*, 1984], while *D. burgessiae* leaf decoction is orally used as an antimalarial, for stomach pains and for leprosy [Chhabra *et al.*, 1984; Comley, 1990]. *Dombeya cincinnata* root decoction is also used in Tanzania in management of vomiting, abdominal pains, cough, chest pains and dysmenorrhoea [Chhabra *et al*., 1993].

In Tanzania, *D. mupangae* is used for management of threatened abortion, wounds, amenorrhoea, stomach pains and constipation [Haerdi, 1964; Hedberg, 1983]. *Dombeya quinqueseta* root bark is used for schistosomiasis and for stomach ache [Chhabra *et al*., 1993]. *Dombeya wallichii* is reported to be used as a stomach medicine in Thailand [Anderson, 1986]. *Dombeya buettneri* dried leaf is used in Nigeria as an antiulcer agent [Okwari *et al*., 2000], while *D. mastersii* aerial parts have been demonstrated in India to have cytotoxic and hypotensive activity [Bhakuni, 1971].

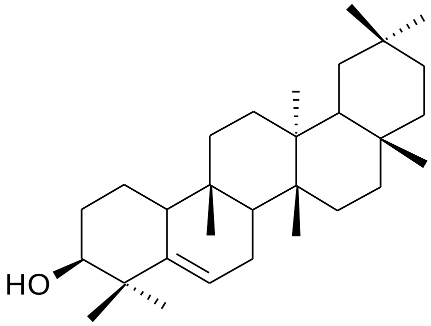
Dombeya rotundifolia roots are pounded, soaked in water and extract given to children with diarrhoea [Kokwaro, 2009]. Its stems and roots are be used to treat syphilis and in rituals [Iwu, 1993]. It is also used to treat heart problems, nausea in pregnant women, headaches, haemorrhoids, dyspepsia, regulate the menses and to hasten the onset of labour [Watt and Breyer-Brandwijk, 1962]. The dried entire plant of *D. rotundifolia* is used in South Africa for diarrhoea and in Tanzania for intestinal upset [Mc Gaw *et al*., 2000]. It is also used to treat abdominal pains, intestinal ulceration, headaches, and haemorrhage, as a tonic and to cause abortion [Reid *et al*., 2001].

1.2.1.3. Scientific work done on *Dombeya* species

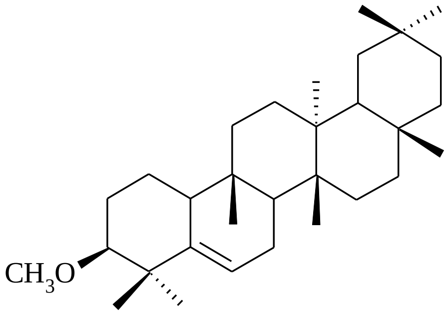
Dombeya buettneri aqueous extract causes significant reduction in basal and histamine-stimulated gastric acid secretion in rats. Pre-treatment with the extract also reduces the extent of gastric mucosal damage induced with oral ethanol (75 % v/v), but has no effect on mucus secretion. It is suggested that the consumption of an extract of the leaves of *D. buettneri* may be beneficial in the prevention and treatment of peptic ulcer disease [Okwari *et al*., 2000].

Dombeya acutangula ssp. *acutangula* contains alkaloids and phenols [Vera *et al*., 1990] while sterols and/or triterpenes have been isolated from the leaf of *D. burgessiae* [Chhabra *et al*., 1993]. *Dombeya multiflora* stem bark contains alkaloids, sterols and flavonoids but lacks cardiac glycosides and quinones [El-Kheir and Salih, 1980]. *Dombeya mukole* has been demonstrated to contain phenolics and flavonoids [Mc Key *et al*. 1978] but no alkaloids [Gartlan *et al*., 1980]. *Dombeya mastersii* lacks tannins on aerial parts [Atal *et al*., 1978], while *D. populnea* contains alkaloids, flavonoids, phenols and tannins in entire plant [Vera *et al*., 1990]. The ethanol extract of dried stem bark of *D. rotundifolia* var. *rotundifolia* exhibits angiotensin-converting enzyme inhibitory activity [Duncan *et al*., 1999].

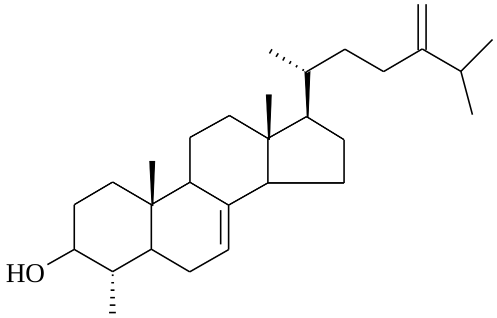
The compound 3β-hydroxyglutin-5-ene has been isolated from *D. rotundifolia, D. torrida, D. taylorii, D. quingueseta* and from *D. burgessiae*. The compounds 3β-methoxyglutin-5-ene and 4α-methyl-5α-ergosta-7,24(28)-dien-3β-ol (24-methylenelophenol) have been isolated from *D. taylorii* [Chepkwony, 2001]. Two other compounds, β-sitosterol and lupeol have been isolated from *D. rotundifolia* [Ndwigah *et al.*, 2005].



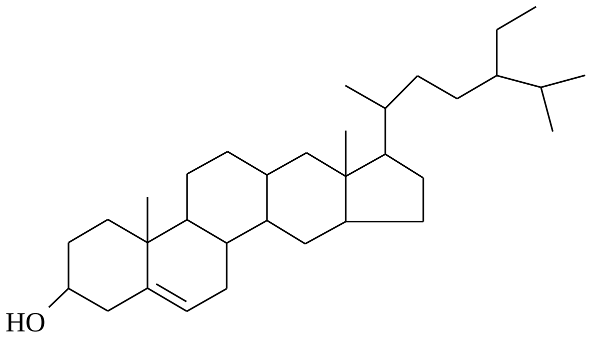
3β-Hydroxyglutin-5-ene



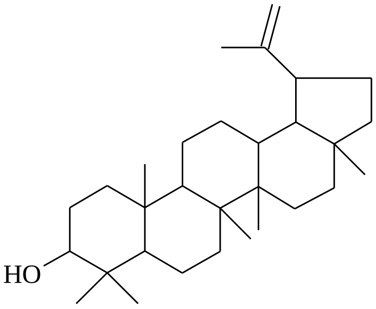
3β-Methoxyglutin-5-ene



4α-Methyl-5α-ergosta-7,24(28)-dien-3β-ol



β-Sitosterol



Lupeol

Figure 1.1. Structures of compounds previously isolated from *Dombeya* species

1.2.2. *Dombeya torrida*

1.2.2.1. Botanical description

Dombeya torrida (J.F. Gmel) was formerly known as *D. goetzenii* (K. Schum.) or *Dombeya faucicola* (K. Schum.). In Kenya it is known in many different names: *Mukeu* (Trade), *Mukeu* (Kikuyu), *Silibwet* (Kipsigis), *Borowa* (Marakwet), *Boroa* (Turkana), *Mukeu* (Meru) and *Ol-Subukiai-Oro* (Maasai) [Beentje, 1994].

Dombeya torrida is a shrub (or tree) and its height range is 6-24 m tall. Its leaves are broadly ovate, base deeply cordate, apex acuminate, margin serrate to entire, 4-25 by 3-15 cm and densely pubescent. Branches are pubescent when young and glabrous when older. Flowers are white, red at the base inside, in umbels with branched stalks. Its petals are 11-21 mm long while its fruits are round and hairy [Blundell, 1992; Beentje, 1994].

The photographs of a young and adult *D. torrida* plant are shown in Figure 1.2 and 1.3. A close up photograph of *D. torrida* flowers is shown in Figure 1.4 while Figure 1.5 is a closer view of the flowers on an adult plant.

1.2.2.2. Distribution

Dombeya torrida grows within an altitude of 1850 to 2700 m [Beentje, 1994]. It is widely distributed from Eritrea and Ethiopia southward through Central and East Africa to southern Malawi; it also occurs in Yemen (Brink, 2007). In Kenya *D. torrida* is found in highlands forests throughout Kenya and is splendid when in flower [Blundell, 1992]. It is found in Aberdare's forest, Ngong Hills, Cherangani, Kericho, Mt. Kenya region, Eldoret, Elburgon, Kajiado and Narok.

Figure 1.2. A photograph of a young *Dombeya torrida* plant

Figure 1.3. A photograph of *Dombeya torrida* adult trees

Figure 1.4. A photograph of *Dombeya torrida* flowers

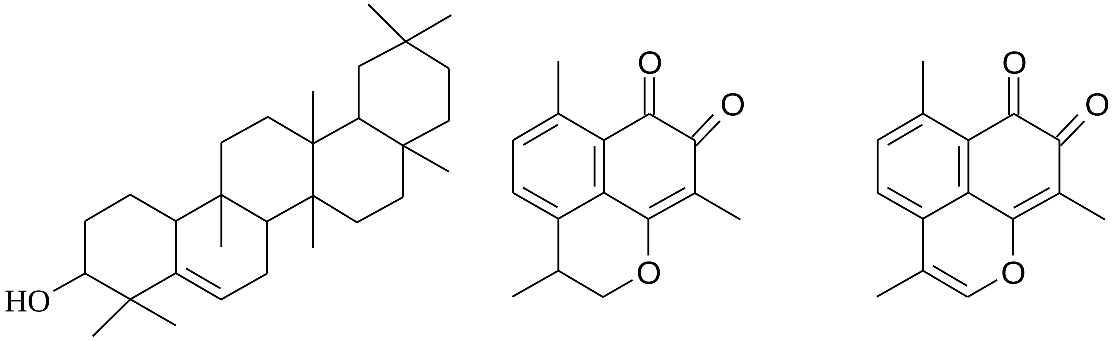
Figure 1.5. A photograph of *Dombeya torrida* leaves

1.2.2.3. Uses of *Dombeya torrida*

In East Africa a decoction of the flowers and bark is taken against indigestion and is taken by the Maasai especially after a large meal of meat [Brink, 2007; Kokwaro, 2009]. Its roots are used for treatment of chest pains and colds [Gachathi, 1989]. The wood of *Dombeya torrida* is suitable for flooring and for building ships, boats, vehicle bodies, furniture, handles, ladders, sporting goods, agricultural implements, yokes, poles, sppons, bows and piles. In Tanzania logs are carved into traditional stools. *Dombeya torrida* is also used as firewood and for making charcoal. Fibre from the bark is made into rope, string and cloth. The flowers produce good nectar for bees. Fallen leaves improve the soil [Brink, 2007].

1.2.2.4. Previous work done on *Dombeya torrida*

Work done on *D. torrida* yielded 3β-hydroxyglutin-5-ene and 2 sesquiterpenoid quinines; mansonone E and mansonone F (Chepkwony, 2001).



3β-Hydroxyglutin-5-ene

Mansonone E

Mansonone F

Figure 1.6. Structures of compounds previously isolated from *Dombeya torrida*

1.2.3. *Hydnoraceae* family

1.2.3.1. Introduction

The Hydnoraceae family has only *Hydnora* and *Prosopanche* genera. Both Hydnora and Prosopanche genera are holoparasites and are totally dependent upon their hosts for their existence. Only the flower emerges from the soil during flowering [Anonymous, 2004]. The Hydnoraceae are the only known angiosperms with no leaves or scales of any sort. *Prosopanche*, with two species, occurs in Central and South America [Nickrent, *et al*., 2002]. *Hydnora*, with five species, is largely African in distribution with some records from the Arabian Peninsula [Maass and Musselman, 2001]. *Hydnora species* include *H. abyssinica*, *H. esculenta*, *H. triceps*, *H. visseri* and *H. africana* [Nickrent, *et al*., 2002]. The vegetative plant body is highly reduced consisting of only roots and flowers. In addition to constituting the only angiosperms lacking leaves and being holoparasitic, species of Hydnoraceae are further distinguished by being found mainly in the semiarid tropics. These subterranean "paleoherbs" emerge only when flowering or fruiting [Maass and Musselman, 2001].

Hydnoraceae are parasites without chlorophyll. They attach to the roots of trees and shrubs by haustoria on a ‘‘pseudo-rhizome’’. The pseudo-rhizomes are warty, simple or branched, rounded or angled and without scale leaves. The flowers are large, solitary and appear above the ground. Their sepals are thick, fleshy, and valvately 3-5 lobed. They lack petals. Stamens are 3-5 in number inserted on the sepal tube and united to form a ring around the stigma. Anthers are numerous and stalkless. The ovary is inferior, 1-chambered with numerous apical or parietal placentas bearing abundant ovules. The stigma is stalkless, often rudimentary. They form berry like fruits underground with numerous very small seeds in a glutinous pulp [Agnew and Agnew, 1994].

1.2.3.2. Work done on *Hydnora* species

The Hydnoraceae is a family with a furtive nature [Maass *et al*., 2004]. Even with their furtive nature, a lot work seems to have been done in the last 5 years. For example a study to assess the intensity and regulation of respiration in *H. africana, H. abyssinica* and *H. esculenta* of African *Hydnora* showed exceptionally low thermogenesis. This in *Hydnora* appears to be associated with scent production and possibly gynoecial development, but has little direct benefit to beetle pollinators [Seymour *et al*., 2009].

A study of the structural attributes of the hypogeous holoparasite *Hydnora triceps* Drège & Meyer (Hydnoraceae) shows that its morphology is highly reduced and its vegetative body difficult to interpret as with many holoparasites,. The vegetative body of *H. triceps* consists of a rhizome with a thickened root-cap-like structure that covers a vegetative shoot apical meristem. Procambial strands originate from the apical meristem and develop into endarch collateral vascular bundles arranged radially around a pith without an inter-fascicular cambium [Tennakoon *et al*. 2007].

1.2.4. *Hydnora abyssinica*

1.2.4.1. Botanical description

Hydnora abyssinica (A. Braun), Hydnoraceae is also known as *H. johannis* (Beccari), *H. abyssinica* var. *quinquefida* (Engler), *H. angolensis* (Decaisne), *H. bogoensis* (Beccari), *H. cornii* (Vaccaneo), *H. gigantea* (Chiovenda), *H. hanningtonii* (Rendle), *H. michaelis* (Peter), *H. ruspolii* (Chiovenda), and *H. solmsiana* (Dinter) [Anonymous, 2004]. It is known locally as *Muthigira* (Kikuyu), *Mũtũmũra Nthĩ* (Embu/Mbere) and *Kimela* (Kamba) [Gachathi, 1989; Musila *et al*., 2004].

Hydnora abyssinica has an evil smelling flower that can grow up to 20 cm from a completely warty pseudo-rhizome. It is fleshy, has 4 thick sepals and has no petals. Its stamens are inserted on the sepal tube and united to form a ring around the stigma. Its inner surface is cream above and bright red and bristly below [Agnew and Agnew, 1994; Blundell, 1992]. It is parasitic and grows underground on acacia roots in dry bushland and is only seen when the tip of the flowers breaks the surface of the ground. The hosts for *H. abyssinica* are species of *Acacia* (Gachathi, 1989; Agnew and Shirley, 1994; Anonymous, 2000]. The local name in Embu/Mbere, Mũtũmũra Nthĩ means ``breaking of the ground''. Figure 1.7 shows photographs of *H. abyssinica* pseudo rhizomes freshly collected from Embu, Kenya.

1.2.4.2 Distribution

Hydnora abyssinica is the most widespread and frequently collected *Hydnora* species. It has wide distribution from western Namibia, northern Botswana, Zimbabwe, Zaire, Tanzania, Kenya, Ethiopia, Somalia, along the Blue Nile to Khartoum in Sudan; and in the Arabian Peninsula in southern Saudi Arabia, northern Yemen and the Dhofar region of Oman [Anonymous, 2004]. It grows at 100-1500 m above sea level [Agnew and Agnew, 1994]. In Kenya it is found in South Nyanza, Mbere and lower dry areas of Meru, of Eastern Province.

1.2.4.3. Uses

The pseudo-rhizome is normally boiled and the infusion drunk as a cure for throat complaints, as an astringent in dysentery, for treatment of stomach trouble and for removing the placenta if it does not come out in time [Kokwaro, 2009]. It is also used in treatment of diarrhoea and amoebic dysentery [Musila, *et al*., 2004] and to treat typhoid, anthrax, cancer and East Coast Fever [Mwangi *et al*., 2001].

a b

c d

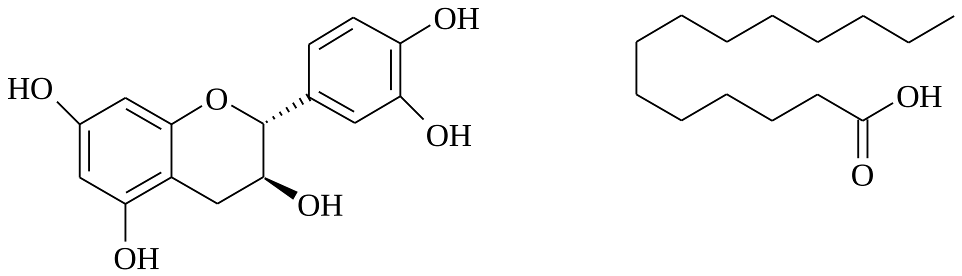
Figure 1.7. Photographs of *Hydnora abyssinica* pseudo rhizomes a: a freshly dug pseudo rhizome, b: a heap of pseudo rhizomes, c: dried pseudo rhizomes and c: size of a freshly dug pseudo rhizome comparing to a human hand.

1.2.4.4. Previous work done on *Hydnora abyssinica*

In vitro bioassay of *H. abyssinica* aqueous, methanol and chloroform extracts, showed ability to inhibit growth of 6 human pathogenic fungi and 4 types of bacteria. The aqueous extract was the most inhibitory followed by methanol extract, while the chloroform extract was the least effective. Growth of pathogens was inhibited by all extracts in a concentration dependent manner

[Saadabi and Ayoub, 2009]. *Aspergillus niger* has been identified as the fungus that associates with the fruits of the *Hydnora abyssinica* and it has been proposed that the fungus helps in digesting the fruits to facilitate easy seed dispersal [Chimwamurombe *et al.,* 2007].

Phytochemical analyses showed preponderance of condensed tannins and phenols in the *H. abyssinica* extracts [Saadabi and Ayoub, 2009]. Bioassay-guided phytochemical investigation of *H. abyssinica* to isolate secondary metabolites yielded four compounds; tetra-decanoic acid, catechin, tyrosol and benzoic acid. The isolated compounds were demonstrated to have antioxidant and antiglycation potential [Waleed *et al.,* 2009].



Catechin Tetra-decanoic acid

Tyrosol Benzoic acid

Figure 1.8. Structures compounds previously isolated from *Hydnora abyssinica*

1.3. Study justification

Throughout human history, natural products have been the foundation for the discovery and development of therapeutics used to treat diseases ranging from cardiovascular disease to cancer. Natural products are from plants, marine organisms, microorganisms and animals [Evans, 1996]. The chemical diversity and complexity of natural products have provided structural scaffolds for small-molecule drugs and have served as inspiration for medicinal drug design [Chang and Keasling, 2006].

Plants have been instrumental in the pharmaceutical drug discovery. Many drugs used in modern medicine, such as vinblastine (Madagascar periwinkle), digitalis (purple foxglove) and codeine (opium poppy), are derived from herbal remedies and are used for diverse purposes such as treatment of cancer, heart conditions and pain [Chang and Keasling, 2006]. It is reported that 61% of the 877 small-molecule drugs introduced in the last 20 years can trace their ancestry to natural products, with 38% comprising natural products and their close derivatives [Newman, *et al*., 2003]

"The proven natural product drug discovery track record, coupled with the continuing threat to biodiversity through the destruction of terrestrial and marine ecosystems, provide a compelling argument in favour of expanded exploration of nature as a source of novel compounds for the development of drugs and other valuable bioactive agents" [Cragg and Newman, 2001]. It is thus apparent that nature can provide novel chemical scaffolds for elaboration by chemical and biochemical approaches leading to agents that can be optimised on the basis of their biological activities.

Literature and folklore give medicinal uses of *D. torrida* and *H. abyssinica*. Some of these medicinal uses such as treatment of anthrax, typhoid, dysentery, expelling placenta and stomach ailments are of great health and economic importance. A lot remains to be done to isolate compounds responsible for these activities. There is a need therefore to carry out systematic studies on these plants to determine their phytochemical composition and isolate compounds responsible for any pharmacological activity.

1.4. Research question

Do *Dombeya torrida* and *Hydnora abyssinica* have isolable chemical principles? And do they have demonstrable antibacterial and antifungal activities *in vitro*? Are the antibacterial and antifungal activities of *Dombeya torrida* and *Hydnora abyssinica* attributable to specific isolable chemical principles?

1.5. Objectives

1.5.1. General objective

The main aim of this work is to carry out phytochemical investigation of *Dombeya torrida* and *Hydnora abyssinica* to isolate compounds and to determine the antibacterial and antifungal activities of both plants.

1.5.2. Specific objectives

1. To determine the antifungal and antibacterial activity of *Dombeya torrida* and *Hydnora abyssinica* extracts.
2. To develop methods of isolation of compounds from *D. torrida* and *H. abyssinica*.
3. To carry out structural elucidation of isolated compounds using spectroscopic methods.

CHAPTER TWO

EXPERIMENTAL

2.1. Materials and reagents

2.1.1. Plant collection, identification and preservation

2.1.1.1. *Dombeya torrida*

The *Dombeya torrida* stem-bark, leaves and flowers were collected on 3rd August 2006 and on 17th August 2007 at Kinale forest in Mukeu Sub-location, Kinale Location, Lari Division, Kiambu West District, Central Province, Kenya. A plant taxonomist, Mr. S. G. Mathenge (University of Nairobi, Department of Botany), was involved in the collection and identification of the plant. A voucher specimen number 2006/002 is deposited at the School of Biological Sciences herbarium, University of Nairobi. The stem-bark, leaves and flowers were air-dried at room temperature, finely ground and the powder stored at room temperature in a dry place until used.

2.1.1.2. *Hydnora abyssinica*

The first batch of *Hydnora abyssinica* was collected in May 2004 at Kimunyi Village, Nguthi Sub-Location, Ivurori Location, Siakago Division, Mbeere District, Kenya. The second batch was collected at Mashiara region at the boundary between Mbeere and Meru districts on 3rd March 2007. Identification was done in Department of Botany, University of Nairobi. A voucher specimen number 2006/001 is kept at School of Biological Sciences herbarium, University of Nairobi. The pseudo rhizomes were air-dried, finely ground and the powder was stored at room temperature in a dry place until used.

2.1.2. Chromatographic and microbiological materials

Normal phase and reversed-phase thin layer chromatography (TLC) pre-coated plates with silica gel $60F_{254}$ and $18F_{254S}$ were from Sigma-Aldrich Chemie (Steinheim, Germany) and Merck (Darmstadt, Germany), respectively. Column chromatography was carried out on silica gel for column chromatography (0.032-0.63 mm) from Sigma-Aldrich Laborchemikalien (Seelze, Germany). Sephadex LH_{20} was also from Sigma-Aldrich Laborchemikalien.

Methanol, dichloromethane, acetone, n-hexane and chloroform were from Kobian Kenya Ltd (Nairobi, Kenya), while petroleum ether 60-80, ethyl acetate and ethanol were from Synerchemie Chemicals (Nairobi, Kenya). All these solvents were of general purpose grade and were distilled in glass apparatus before use. Vanillin was acquired from Laboratory Chemicals, (Nairobi, Kenya). Concentrated sulphuric acid was from Kanha Laboratory Supplies (Nairobi, Kenya).

Sabouraud Dextrose Agar (CM 0041) and Tryptone Soy Agar (CM 0131) both from Oxoid Ltd (Bashing Stoke, Hampshire, England) were used as culture media for growth of fungi and bacteria, respectively.

2.2. Equipment

A Delta Range top loading balance PB 303 (Mettler Toledo, Zurich, Switzerland) was used to weigh samples above 100 g and a Sartorius R200D (Sartorius, Goettingen, Germany) analytical balance was used to weigh samples below 100 g.

Extractions were done using a 2,000 ml Soxhlet apparatus (Quickfit, Birmingham, U.K.) or by percolation using a 1 m long glass column of 80 mm in diameter made in Science Workshop, Department of Chemistry, University of Nairobi. Aqueous extracts were reduced on a rotary evaporator (Heidolph VV2000, Heidelberg, Germany) connected to an Edwards vacuum oil pump Rv 5 (Edwards High Vacuum International, West Sussex, U.K.). Organic extracts were reduced on a rotary evaporator (Heidolph VV2000) connected to a rotary vane pump KNF Laboport (Neuberger, Freiburg Germany). A Heto-Holten A/S FD 1.0-110 freeze drier (Gydevang, Allerod, Denmark) connected to an Edwards vacuum oil pump Rv 5 was used to lyophilize the water extracts. Filtration for qualitative analysis was done using filter papers from Whatman Laboratory division (Kent, U.K.). Fractions were collected using Superfrac fraction collector from Pharmacia LKB Biotechnology (Uppsala, Sweden).

A Min UV/Vis box (Desaga GMBH, Heidelberg, Germany) was used for visualising chromatograms on TLC plates. A Perkin-Elmer Lambda UV/Visible spectrophotometer (Berlin, Germany) was used for ultraviolet (UV) analysis. High resolution mass spectrometric (MS) analysis was done using a GC Mate II Jeol (Tokyo, Japan). Sample inlet was by direct probe and ionisation was by electron impact. A Shimadzu Fourier Transform IR Prestige-21 spectrophotometer (Shimadzu Corporation, Kyoto, Japan) was used for infrared (IR) analysis. Nuclear magnetic resonance (NMR) spectroscopic analysis of isolated compounds was carried out using a Mercury Varian 200 MHz from Varian Inc. (Palo Alto, California, U.S.A.), with a magnet from Oxford Instruments (Oxford, U.K.) and the computer software from Sun Micro-Systems (California, U.S.A.). A Voss of Maldon vibrator Type WFSM/8 from Voss Instruments Ltd. (Maldon, Essex, England) was used during the packing of the chromatographic columns.

2.3. Procedures

2.3.1. Preparation of 1 % vanillin spraying reagent

One gram of vanillin was weighed into a conical flask. About 99 ml of 36 N sulphuric acid was added into the flask and the contents swirled until all the vanillin had dissolved to form a light yellowish solution. This formed 1 % vanillin in concentrated H_2SO_4 which was sprayed onto developed TLC plates and the plates heated at 100-105° C for visualization. It was used as a spray reagent for detection of steroids on the TLC whereby they form coloured spots.

2.3.2. Packing of fractionating columns

Typically, about 235 g of silica gel for column chromatography was weighed with a top loading balance and suspended in 1,000 ml of distilled chloroform. It was packed into a column 1 m in length and 2.5 cm diameter under low pressure using a hand pump. Such column was used in isocratic fractionation of the chloroform and petroleum ether extracts. Another 300 g of the silica gel was similarly weighed and suspended in 1,000 ml of distilled n-hexane. It was packed into a column 1 m in length and 3.5 cm in diameter with continuous vibration using a Voss of Maldon vibrating machine. This kind of column was used in gradient fractionation of extracts of ethyl acetate, dichloromethane/ethyl acetate mixture and dichloromethane/methanol mixture.

About 50 g of Sephadex LH20 was soaked in dichloromethane/methanol (50:50) mixture over 24 h. It was then loaded into a fractionating column 84 cm in length and 2.3 cm diameter and allowed to settle freely. This was used in separating fractions which had more than one spot obtained from the silica columns.

2.3.3. Preparation of *Dombeya torrida* extracts

2.3.3.1. Stem bark chloroform extract

About 1 kg of the stem bark powder was weighed and extracted with chloroform for 48 h in a Soxhlet apparatus. The extract was filtered through filter paper, reduced *in vacuo* to dryness and then stored at 2-8 °C until further processing. This process was repeated 9 times to avail approximately 139 g from 10 kg crude material (1.39 % yield) of extract.

2.3.3.2. Stem bark methanol extract

The stem bark material that was extracted by Soxhlet with chloroform was dried at room temperature and re-extracted with methanol for 48 h. The extract was filtered through filter paper, reduced *in vacuo* to yield 100 g (10 % yield) of methanol extract and then stored at 2-8 °C.

2.3.3.3. Stem bark acetone extract

Another 1 kg stem bark material earlier extracted with chloroform was dried at room temperature and further extracted with acetone for 48 h. The extract was filtered through filter paper, reduced *in vacuo* and then stored until further use. The yield was 131 g (13.1 % yield) of acetone extract from 1kg of material.

2.3.3.4. Stem bark ethyl acetate extract

The stem bark powder (1 kg) was weighed and packed into a 2 L column, 1 m in length and 8 cm in diameter. Distilled ethyl acetate was added on top and let to percolate slowly over 72 h with 12 h breaks when the tap was closed and the material was left to soak in the extracting solvent to allow for maximum extraction. Around 5,000 ml of the ethyl acetate was used for the percolation to avail 35.6 g (3.56 % yield) of a brown shiny gummy extract.

2.3.3.5. Stem bark macerate

About 402 g of stem bark material was weighed into a conical flask and subjected to cold maceration with 1,000 ml of water for 7 h with occasional stirring. The extract was filtered through filter paper and reduced *in vacuo* to about 100 ml, freeze-dried and then stored at 2-8 °C. A yield of 38 g (9.4 %) of *D. torrida* macerate was obtained.

2.3.3.6. Stem bark decoction

About 405 g of stem bark material was added to 2,000 ml distilled water. The mixture was heated to boiling point using a hot plate and allowed to boil for 5 min then allowed to cool, with continuous stirring to yield a decoction. The extract was filtered through filter paper and reduced *in vacuo* to about 100 ml, freeze-dried and stored at 2-8 °C. A yield of 39.6 g (9.8 %) of *D. torrida* decoction was realised.

2.3.3.7. Stem bark petroleum ether extract

About 1 kg of the stem bark powder was weighed and extracted with petroleum ether for 48 h in a 2,000 ml flask using Soxhlet apparatus. The extract was filtered through filter paper and reduced *in vacuo* to dryness to yield 6.4 g (0.64 % yield) of petroleum ether extract. The extract was stored at 2-8 °C.

2.3.3.7. Leaves dichloromethane:methanol extract

Around 2 kg of the leaf powder was weighed and packed into a 2 L column, 1.6 m in length and 80 mm in diameter. A 50:50 mixture of dichloromethane and methanol was added on top and let to percolate slowly over 72 h with 12 h breaks when the tap was closed and the material was left to soak in the extracting solvent to allow for maximum extraction. Five litres of the dichloromethane:methanol mixture was used for the percolation to avail 120 g (6 % yield) of a blue/black gummy extract.

2.3.4. Preparation of *Hydnora abyssinica* extracts

2.3.4.1. Petroleum ether extract

About 1 kg of *Hydnora abyssinica* powder was weighed and extracted with petroleum ether using Soxhlet for 48 h. The extract was filtered through filter paper, reduced to dryness *in vacuo* to yield 7.0 g (0.7 % yield) and then stored at 2-8 °C until processed further.

2.3.4.2. Chloroform extract

About 1 kg of powder was weighed and extracted with chloroform using Soxhlet with for 48 h. The extract was filtered through filter paper, reduced *in vacuo* to dryness to yield 6.5 g (0.6 % yield) and then stored at 2-8 °C until further processed.

2.3.4.3. Methanol extract

The material that was extracted by Soxhlet with chloroform was dried at room temperature and re-extracted by Soxhlet with methanol for 48 h. The extract was filtered through filter paper, reduced *in vacuo* to yield 60.0 g (6 % yield) and then stored at 2-8 °C until processed further.

2.3.4.4. Macerate

About 100 g of powder material was weighed into a conical flask and subjected to cold maceration with 250 ml of water for 7 h with occasional stirring. The extract was filtered through filter paper and reduced *in vacuo* to about 100 ml then freeze-dried. One hundred grams of material yielded 1.85 g of macerate which was then stored at 2-8 °C.

2.3.4.5. Decoction

About 109 g of fresh plant material was added to 400 ml of boiling water. The mixture was heated to boiling using a hot plate with stirring for 5 min then allowed to cool, with continuous stirring. The extract was filtered though filter paper and reduced *in vacuo* to about 100 ml then freeze-dried. The 109 g of material yielded 2.5 g of dried decoction which was stored at 2-8 °C.

2.3.4.6. Ethyl acetate:dichloromethane mixture extract

Around 5 kg of the pseudo rhizome powder was weighed and packed into a 2 L column, 1 m in length and 8 cm in diameter. Distilled ethyl acetate and dichloromethane mixture (50:50) was added on top and let to percolate slowly over 72 h with 12 h breaks when the tap was closed and the material was left to soak in the extracting solvent to allow for maximum extraction. Around 10 l of the mixture was used for the percolation to avail 141.0 g (2.8 % yield) of a brown shiny gummy extract.

2.3.5. Microbiological screening and autobioassay

Dombeya torrida and *Hydnora abyssinica* extracts were screened for antimicrobial activity as described by Hewitt and Vincent (1989) using 2 gram-positive and 2 gram-negative bacteria; and 2 species of fungi. Autobioassay was also carried out for these extracts to determine which components had antimicrobial activity after carrying out TLC of the said extracts.

2.3.5.1. Microorganisms

Gram-positive and gram negative bacteria and fungi were used in the screening for antimicrobial activity. The gram positive were *Staphylococcus aureus* and *Staphylococcus epididermis* with strain code of NC 07447and NC1336, respectively. Gram-negative bacteria were *Bacillus pumilus* (NC 08241) and *Escherichia coli* (ATTC 25922). The fungi were *Saccromyces cerevisiae* and *Candida albicans* and both were normal yeasts. These microorganisms were sourced from Drug Analysis and Research Unit, Department of Pharmaceutical Chemistry, University of Nairobi.

2.3.5.2. Preparation of the media

Trytone Soy Agar (TSA) was used to culture bacteria. Around 4 g of TSA was suspended in 100 ml distilled water for each strain of the bacteria. It was heated to boiling to dissolve completely. It was then sterilised by autoclaving at 121 °C for 15 min. It was allowed to cool to about 50 °C and inoculated with 1 ml of bacterial culture innoculum [approximately 10^6 colony forming units (cfu) per ml]. It was swirled and 20 ml poured per plate (in 4 plates). It was allowed to cool and set for about 20 min at 40 °C. Six wells were made in the plate using a cork borer.

Sabouraud Dextrose Agar (SDA) was used to culture *C. albicans* and *S. cerevisiae*. About 13 g of SDA was suspended in 200 ml distilled water and heated to boiling to dissolve completely. It was sterilised by autoclaving at 121 °C for 15 min. It was allowed to cool to about 50 °C and inoculated with 1 ml of fungal culture inoculum [approximately 10^6 colony forming units (cfu) per ml]. It was swirled and 20 ml poured per plate (in 2 plates). It was allowed to cool and set for about 20 min at 40 °C. Six wells were made in the plate using a cork borer.

2.3.5.3. Preparation of the samples

About 100 mg of plant extracts was dissolved in 1 ml distilled water to avail 100 mg/ml samples. The extracts were first coded as shown in Appendix 1 (a).

2.3.5.4. Screening for antimicrobial activity

Fifty micro litres of samples containing 100 mg/ml were introduced per well. The standard (positive control) and blank (negative control) were included. The plates with the samples were left at room temperature in the laminar flow equipment for about 1 h for the extract to diffuse. The plates were then incubated for 18 h in an incubator at 37 °C for bacteria and 35 °C for fungi after which zones of inhibitions were read. All screenings were done in duplicate.

2.3.5.5. Preparation of TLC plates for autobioassay

Glass plates (20 x 20 cm) were cleaned with distilled water and dried. Silica gel GF_{254} (normal phase) was used to make a slurry in a ratio of 1:2 of silica with distilled water. The glass plates were put in a special applicator and the slurry poured onto them. A spreader was passed over them at a uniform speed to receive a uniform coating of adsorbent layer of 0.75 mm. The slurry was allowed to dry. After 30 min, the plates were activated by heating in an oven at 105 ^{o}C for 1 h.

After cooling down, the plates were spotted with *D. torrida* methanol, chloroform and dichloromethane/methanol extracts; and *H. abyssinica* methanol, chloroform and dichloromethane/methanol extracts 2.5 cm apart. About 100 µl of samples containing 100 mg/ml were spotted. The amounts spotted were high since this was for preparative work. The plates were then developed in a saturated tank using chloroform-methanol (90:10) mixture as the mobile phase. This allowed for separation of the components of each extract depending on its polarity. The developed TLC plate was removed from the developing tank and allowed to dry at room temperature for 30 min. About 50 µl erythromycin and nystatin solutions (containing 0.3 mg/ml) were spotted as standards for antibacterial and for antifungal effects, respectively.

2.3.5.6. Autobioassay

The dried TLC plate was put on a glass plate. Sabouraud Dextrose Agar and TSA already inoculated with individual microbial culture inoculum (as in 2.3.5.2 above) was swirled and 50 ml poured on to the TLC plate to form an even layer covering the developed plate. It was allowed to cool and set for about 20 min. The plates were then incubated in an oven at 37 °C for bacteria and 35 °C for fungi for 18 h after which zones of inhibitions were read. The auto bioassay was done in duplicate.

CHAPTER THREE

ISOLATION OF COMPOUNDS FROM *DOMBEYA TORRIDA* AND *HYDNORA ABYSSINICA*

3.1. Isolation of compounds from *Dombeya torrida*

3.1.1. Stem bark chloroform extract

About 24 g of the extract was introduced into a column packed with 235 g silica, eluted with chloroform and fractions were collected using a fraction collector. The flow rate was set at about 30 drops per min and fractions were collected every 15 min. Fractions were monitored by TLC and those with profiles similar were pooled. Appendix 1(b) gives the TLC profile of fractions from *Dombeya torrida* bark. After fractionation, the column was washed with 400 ml of methanol and the methanol washing designated DTM. The pooled fractions were reduced to dryness *in vacuo* using a rotary evaporator and yields determined. Table 3.1 below shows the fractions pooled together from 8 different columns that gave similar TLC profiles.

Table 3.1. Yields of fractions from *Dombeya torrida* stem bark chloroform extract

Fraction	Weight (mg)	% yield from chloroform extract	Fraction	Weight (mg)	% yield from chloroform extract
D1	400	1.67	D9	300	1.25
D2	500	2.08	D10	400	1.67
D3	200	0.83	D11	460	1.92
D4	700	2.92	D12	1,400	5.83
D5	200	0.83	D13	1,600	6.67
D6	250	1.04	D14	400	1.67
D7	150	0.63	D15	1,080	4.50
D8	350	1.46	DTM	9,500	39.5

The fractions were dissolved in minimal amount of chloroform and left to stand at room temperature. Fractions D1 and D2 gave fatty residues on drying and after dissolving in petroleum ether, they availed an amorphous oily residue on solvent evaporation. No compounds were isolated from these two fractions and no further work was done on them.

The residue of fraction D3 was dissolved in ethyl acetate and left to evaporate slowly at room temperature. White star-like crystals formed at the bottom of the tube. The mother liquor was pipetted out and the crystals washed with cold ethyl acetate. The crystals were designated compound **i** giving a yield of 0.07 % (17 mg of compound **i** was isolated from 24 g of crude chloroform extract). Compound **i** is in chapter 4 designated as compound **1** for purpose of discussion and identification. Similarly, Appendix 1 (c) tabulates all the isolated compounds and gives Arabic codes to those discussed in chapter 4. Compound **i** was also obtained from *D. torrida* petroleum ether extract fractionated in a similar manner to the chloroform extract and thus it is an alternative isolation procedure of this compound.

After D3 fraction formed good crystals (compound **i**) in ethyl acetate, ethyl acetate was chosen as an alternative solvent to chloroform for crystallization. After isolation of compound **i**, the mother liquor was left to evaporate slowly. It formed some creamy amorphous material at the bottom of tube. Cold ethyl acetate was added to the tube and the amorphous material washed by shaking as it did not easily dissolve in the cold ethyl acetate. Filtration availed an amorphous white material with a single spot on TLC which was designated compound **ii**.

As D4 evaporated, a few crystals formed on top of the solution meaning they were less dense than chloroform. The fraction was left until almost all the chloroform had dried to maximize on formation of these crystals. Ethyl acetate was then added into the tube and clear glassy crystals settled at the bottom of the tube. These were recovered by filtration and washed with cold ethyl acetate and designated compound **iii (2)**. Three mg of compound **iii** were isolated from 24 g of crude *Dombeya torrida* chloroform extract. During the subsequent fractionation, the fraction was dissolved directly in ethyl acetate and crystals formed at the bottom of the tube. The yields of compound **iii** were low availing only 23 mg from 100 mg of crude extract.

White crystals formed in D10 and in D11 fractions within 24 h after the fractions were dissolved in chloroform and left to stand at room temperature. The fractions were allowed to stand for a further 48 h during which most of the solvent evaporated. Ethyl acetate was added to the tubes and the crystals settled at the bottom of the tubes. The mother liquors were pipetted out. The crystals were then washed with ethyl acetate and filtered. It was observed that they had very poor solubility in ethyl acetate. On spotting the two crystals from D10 and D11 on TLC, and developing using chloroform as the mobile phase, they had the same profile and were merged and designated compound **iv**.

The mother liquors of compound **iv** were reduced to dryness and then ethyl acetate was added. Some white crystals did not dissolve in ethyl acetate and after recovery by filtration were confirmed to be same as compound **iv**. The filtrates were left to stand at room temperature. Needle like crystals formed in both D10 and D11 and were recovered by pipetting the mother liquor out. These were designated compound **v (3** and **4)**. The mother liquor of compound **v** was re-dissolved in around 10 ml chloroform and 2 ml of methanol added. After 48 h clear glassy crystals formed and were recovered by filtration. Thin layer chromatography showed that the clear glassy crystals were same as compound **v** and these were pooled. Around 30 mg of compound **iv** and 100 mg of compound **v** were isolated from 24 g of *Dombeya torrida* chloroform extract.

The fractions D5, D6, D7, D8, D9, D12, D13, D14 and D15 did not afford any isolates but the later eluting fractions had a similar spot on TLC. These later eluting fractions D12, D13, D14 and D15 were further pooled to give about 5 g of material. This material was fractionated on 60 g of silica, eluted with chloroform. Eight fractions designated LD1 to LD8 were realised. These were reduced to dryness and re-dissolved in chloroform. Fractions LD5 and LD6 formed some amorphous material which was colourless on filtration but turned yellow on drying. This was designated compound **vi**. The later eluting fractions in subsequent column were again pooled to give around 3 g of material. This material was fractionated 1 g at a time using a column packed with 50 g Sephadex LH20 with dichloromethane: methanol (50:50) mobile phase to avail a white amorphous powder designated compound **vii**. Thin layer chromatography showed compound **vi** and compound **vii** to have similar spots but the compounds were different in appearance.

Isolation process is summarised in the figure 3.1 and TLC results in table 3.2. A total of 7 compounds were isolated from *Dombeya torrida* stem bark chloroform extract, 4 crystalline and 3 amorphous.

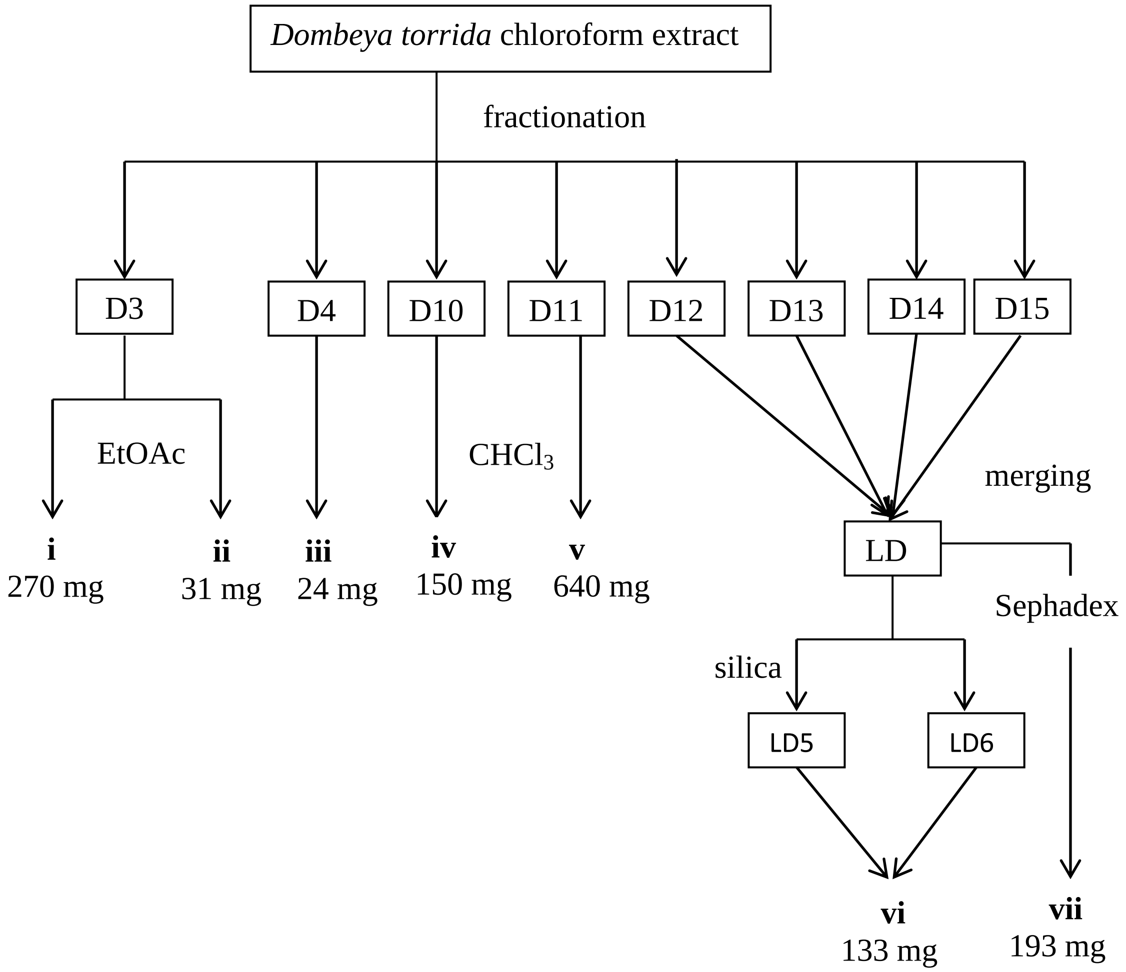


Figure 3.1. A flow chart of isolation of compounds from *Dombeya torrida* stem bark chloroform extract

Thin layer chromatography was carried out on the isolates and those with similar profiles were pooled. Compounds **i** and **ii** formed pink spots after spraying with vanillin but showed no spots in UV or iodine. Compound **iii** absorbed iodine and formed a pink spot after spraying with vanillin but could not be visualized in UV light. Compound **iv** quenched UV_{254} and could not be visualised at UV_{366}, in iodine or with vanillin. Compound **v** could be visualized in iodine and with vanillin but not in UV. Compounds **vi** and **vii** formed similar pink spots after spraying with vanillin but could not be visualised in UV light. The profiles of the isolates visualised using UV 254 nm, UV 366 nm, iodine and vanillin and are summarised in table 3.2 below.

Table 3.2. Characteristics of isolates from *Dombeya torrida* stem bark chloroform extract

	Amount (mg)	Appearance	R_f	UV_{254}	UV_{366}	Iodine	Vanillin
i	270	White, star like crystals	0.66^a	-	-	-	+
ii	31	White amorphous powder	0.59^a	-	-	+	+
iii	23	Colourless glassy crystals	0.48^a	-	-	-	+
iv	150	White sugar like crystals	0.10^a	+	-	-	-
v	640	White needle like crystals	0.22^a	-	-	+	+
vi	133	Yellow amorphous powder	0.62^b	-	-	+	+
vii	133	White amorphous powder	0.62^b	-	-	+	+

a: R_f in chloroform, b: R_f in ethyl acetate, +: visualised, -: not visualised

3.1.2. Stem bark ethyl acetate extract

About 30 g of ethyl acetate extract was dissolved in 200 ml methanol in a 1,000 ml reducing flask. Forty grams of silica for column chromatography was added and the methanol evaporated using a rotary vacuum evaporator to give a particulate residue containing the extract adsorbed onto the silica. The residue was ground to a fine powder, suspended in 200 ml of n-hexane and introduced into the column packed with 360 g of silica gel for column chromatography.

The column was eluted with 1,000 ml each of various solvents of increasing polarity as given in table 3.3. The elution was free-flow under gravity and fractions were collected every 7.5 min (approximately 16 ml). Fractions were monitored by TLC using dichloromethane, ethyl acetate and ethyl acetate:methanol mixture as the developing solvent for early eluting fractions, intermediate and last fractions, respectively. Similar fractions were pooled to yield different fractions as shown in table 3.3.

Table 3.3. Eluting solvents and yields of fractions from *Dombeya torrida* stem bark ethyl acetate extract

Fractionating solvent (1,000 ml)	**Fraction**	**Yield (mg)**	**% yield**
100 % n-hexane	A	520	1.73
10 % DCM in n-hexane	B	110	0.37
20 % DCM in n-hexane	C	100	0.33
40 % DCM in n-hexane	D1	100	0.33
	D2	130	0.43
50 % DCM in n-hexane	E1	30	0.10
	E2	70	0.23
	E3	200	0.67
	E4	160	0.53
75 % DCM in n-hexane	F1	250	0.83
	F2	50	0.17
	F3	190	0.63
100 % dichloromethane	G	90	0.30
10 % EA in dichloromethane	H	590	1.97
20 % EA in dichloromethane	I	50	0.17
40 % EA in dichloromethane	J	500	1.67
75 % EA in dichloromethane	K	2,886	9.6
100 % Ethyl acetate	L	2,000	6.67
10 % Acetone in ethyl acetate	M	2,900	9.67
20 % Acetone in ethyl acetate	N	1,250	4.16
50 % Acetone in ethyl acetate	O	900	3.00
75 % Acetone in ethyl acetate	P	5,300	17.67
100 % Acetone	Q	5,500	18.33

EA: ethyl acetate, DCM: dichloromethane

Each of fractions A to J were dissolved in ethyl acetate while fractions K to P were dissolved in acetone. Fraction A evaporated forming a fatty amorphous residue. Fraction B formed white needle like crystals at the bottom of the pear shaped flask which were recovered by pipetting out the mother liquor and washing the crystals with cold ethyl acetate. These were designated compound **i** after TLC and weighed 17 mg. Fraction D1 formed clear crystals at the bottom of the tube and these were designated compound **iii** (9 mg). Fraction C formed some feathery white crystals which were recovered by filtration and designated CC. When spotted on TLC, CC was found to be a mixture of compound **i** and compound **iii**. Fractions E3 and E4 formed some clear glassy crystals which were found to be the same on carrying out TLC and were same as compound **v** (63 mg). The isolation is summarised as shown in figure 3.2 below.

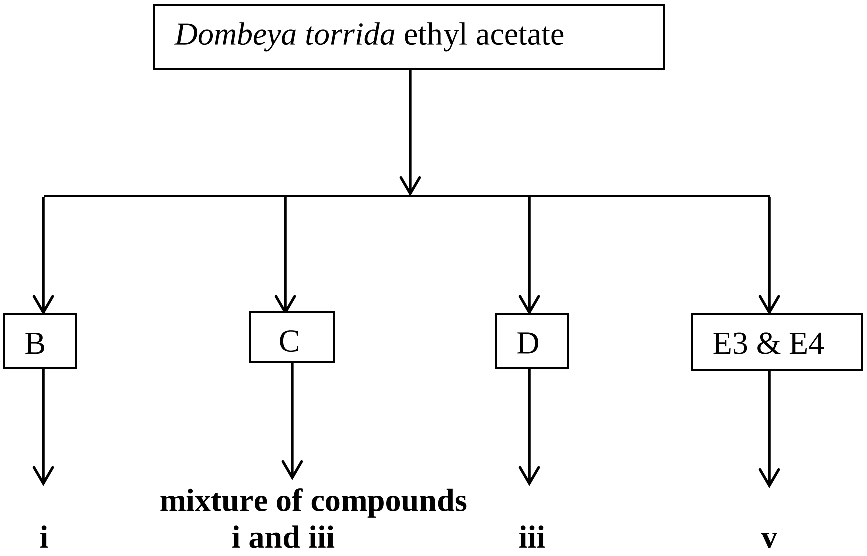


Figure 3.2. A flow chart of the isolation of compounds from ethyl acetate extract of *Dombeya torrida* stem bark

3.1.3. Leaves dichloromethane:methanol extract

About 50 g of dichloromethane: methanol leaves extract was dissolved in 300 ml dichloromethane/methanol mixture in a 1,000 ml reducing flask. Seventy five grams of silica for column chromatography was added and the contents mixed by swirling to allow for adsorption of the extract onto the silica. The dissolving solvent was then evaporated using a rotary vacuum evaporator to give a dark particulate residue containing the extract adsorbed onto the silica. The residue was ground to a fine powder using mortar and pestle, suspended in approximately 300 ml of n-hexane and introduced onto a column packed with 250 g of silica gel for column chromatography.

The extract was eluted with solvents of increasing polarity as given in table 3.4. The elution was free-flow under gravity and fractions were collected every 7.5 min (approximately 16 ml). Each solvent mixture used for elution was 1,500 ml. Fractions were monitored by TLC using dichloromethane, ethyl acetate and ethyl acetate-methanol mixture as the developing solvent for early eluting fractions, intermediate and last fractions, respectively. Appendices 1 d and 1 e gives the TLC profile of fractions from *D. torrida* leaves dichloromethane:methanol extract. Similar fractions were pooled to yield different fractions as shown in table 3.4.

The fractions were reduced *in vacuo* to 15-50 ml volumes (depending with fraction size) and left to stand in glass tubes or in pear shaped reducing flask in the eluting solvents. Fraction DTLA evaporated forming clear glassy crystals designated compound **viii** (20 mg) which settled at the bottom of the tube. Fraction DTLB1 formed white sugar like crystals at the bottom of the pear shaped flask which were recovered by pipetting out the mother liquor. These were designated compound **ix** and weighed 102 mg. Fraction DTLB4 formed sheet like crystals at the sides of the pear shaped flask which changed to needle like crystals when re-crystallised in methanol-dichloromethane (1:1). These weighed 120 mg and were designated compound **x (6).** Fraction DTLD2 formed sugar like crystals which settled at the bottom of the pear shaped flask which were recovered by pipetting out the mother liquor and designated compound **xi (7)** (120 mg).

Table 3.4. Eluting solvents and fractions obtained from *Dombeya torrida* leaves dichloromethane-methanol extract

Fractionating solvent	Fraction
100 % n-hexane	DTLA
10 % dichloromethane in n-hexane	DTLB1, DTLB2, DTLB3 and DTLB4
20 % dichloromethane in n-hexane	DTLC1, DTLC2, DTLC3 and DTLC4
40 % dichloromethane in n-hexane	DTLD1, DTLD2, DTLD3 and DTLD4
80% dichloromethane in n-hexane	DTLE1, DTLE2, DTLE3 and DTLE4
100 % dichloromethane	DTLF1 and DTLF2
20 % ethyl acetate in dichloromethane	DTLG1, DTLG2, DTLG3, DTLG4 and DTLG5
50 % ethyl acetate in dichloromethane	DTLH1, DTLH2, DTLH3, DTLH4, DTLH5 and DTLH6
100 % ethyl acetate	DTLJ1, DTLJ2, DTLJ3, DTLJ4 and DTLJ5
10 % methanol in ethyl acetate	DTLK1, DTLK2, DTLK3, DTLK4 and DTLK5

Fraction DTLD3 formed a white amorphous precipitate and these were designated compound **xii** (60 mg). Fraction DTLD4 formed sugar like crystals which were recovered by filtration and designated compound **xiii (5)** (23 mg). Fractions DTLE3 formed some clear glassy crystals (83 mg) which were the same as compound **v** on TLC.

The other fractions DTLB2, DTLB3 and DTLCs formed fatty amorphous residues as the solvents evaporated while fractions DTLFs contained mainly chlorophyll and no further work was done on them. The fractions DTLG4, DTLH1, DTLH2, DTLH5 and DTLH6 were dark coloured due to chlorophyll but their TLC profiles showed some prominent spots with vanillin. Each of these was thus fractionated further using Sephadex LH20 with dichloromethane/methanol (1:1) as the eluting solvent. Fractions DTLH1 and DTLH2 each availed feathery crystals designated compound **xiv** and compound **xv**, respectively. The isolation of compounds from *D. torrida* dichloromethane/methanol leaves extract is summarised as shown in figure 3.3.

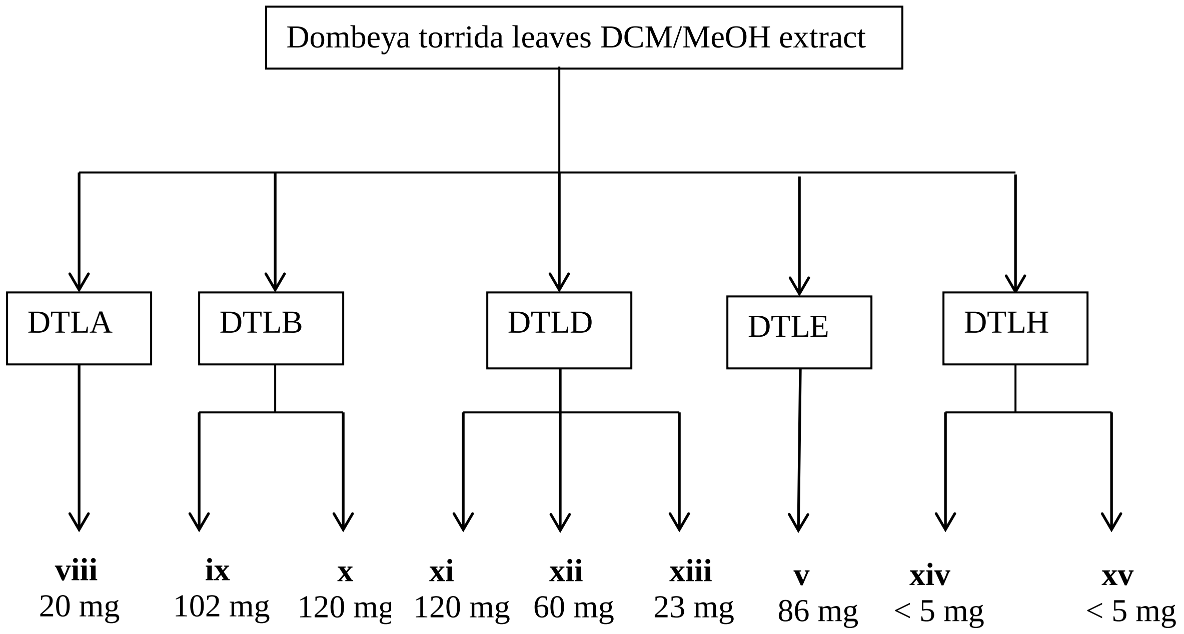


Figure 3.3. A flow chart showing isolation of compounds from the dichloromethane:methanol extract of *Dombeya torrida* leaves

Thin layer chromatography carried on these isolates from leaves show that they are different from compounds earlier isolated from *D. torrida* stem bark with exception of crystals from fraction DTLE which had same spot with compound **v** earlier isolated from chloroform stem bark extract.

3.2. Isolation of compounds from *Hydnora abyssinica* pseudo rhizome

3.2.1. *Hydnora abyssinica* petroleum ether extract

About 19 g of the extract was introduced into a column packed with 160 g of silica for column chromatography and eluted isocratically with chloroform. The flow rate was set at around 25 drops per min and fractions of about 7.5 ml were collected every 15 min. Fractions were monitored on TLC and similar fractions from the column were pooled to give 12 fractions designated P1 to P12. Appendix 1(f) gives the TLC profile of some fractions from *H. abyssinica* petroleum ether extract. The fractions were reduced *in vacuo* to dryness and yields determined. Table 3.5 shows yields for various fractions.

Table 3.5. Yields of fractions from *Hydnora abyssinica* petroleum ether extract

Fraction	Weight (g)	% yield	Fraction	Weight (mg)	% yield
P1	1.22	6.4	P7	2.10	11.1
P2	0.68	3.5	P8	2.40	12.6
P3	0.06	0.3	P9	3.85	20.3
P4	1.06	5.6	P10	1.15	6.1
P5	0.94	4.9	P11	0.55	2.9
P6	2.39	12.6	P12	1.43	7.5

All the fractions P1 to P12 were dissolved in chloroform and left to stand. Fractions P5 and P8 formed a white amorphous material on top of the tubes which was recovered by filtration (compound **xvi** and compound **xvii**, respectively). All the other fractions also formed variable amounts of amorphous white powder. The yields of these powders were high but none of them could be visualised on TLC by use of vanillin, UV or iodine.

3.2.2. *Hydnora abyssinica* ethyl acetate:dichloromethane mixture extract

About 50 g of dichloromethane-ethyl acetate mixture (1:1) extract was dissolved in 300 ml methanol in a 1,000 ml reducing flask. Silica gel for column chromatography (100 g) was added into the reducing flask and the methanol evaporated using a rotary vacuum evaporator to give a particulate residue containing the extract adsorbed onto the silica. The residue was ground to a fine powder, suspended in 200 ml of n-hexane and introduced onto the column packed with 360 g of silica gel for column chromatography.

The extract was eluted using an open column with various solvents of increasing polarity as given in table 3.6. The elution was free-flow under gravity and fractions of around 16 ml were collected every 6 min. Each defined solvent mixture used for elution was 1,200 ml. Fractions were monitored by TLC using dichloromethane, ethyl acetate and ethyl acetate/methanol mixture as the developing solvent for early eluting fractions, intermediate and last fractions, respectively. Similar fractions were pooled to yield different fractions as shown in table 3.6.

Fractions HA to HG were dissolved in dichloromethane, while fractions HH and HJ were dissolved in ethyl acetate. After adding ethyl acetate to HF and heating on a water bath to dissolve, it formed a white crystalline powder on cooling which were filtered and designated compound **xviii**. Other fractions HA to HH formed white amorphous material like the one seen in earlier *H. abyssinica* petroleum ether extract column. Similarly, this white material could also not be visualised with UV, iodine or vanillin. Spots were still seen on TLC profile of fractions and the mother liquor.

Table 3.6. Yields of fractions from *Hydnora abyssinica* dichloromethane:ethyl acetate extract

Fractionating solvent	Fraction	Yield (mg)
100 % n-hexane	HA	50
10 % dichloromethane in n-hexane	HB	50
20 % dichloromethane in n-hexane	HC	170
30 % dichloromethane in n-hexane	HD	1470
60 % dichloromethane in n-hexane	HE	130
80 % dichloromethane in n-hexane	HF	1450
100 % dichloromethane	HG	900
20 % ethyl acetate in dichloromethane	HH	3000
50 % ethyl acetate in dichloromethane	HI	700
100 % ethyl acetate	HJ	1500

These fractions were fractionated further using Sephadex LH_{20} to try isolate compounds responsible for the spots seen on TLC. Fraction HG was dissolved in methanol/dichloromethane mixture (25:75) and eluted with the same solvent mixture using a column packed with 50 g of Sephadex LH_{20}. Twenty fractions each of 12 ml were collected. The fractions were left to stand for 5 days at room temperature. Fractions 1-4 were blank containing only the mobile phase, 5-9 formed white powdery material while 11-13 formed clear glassy crystals. These crystals were recovered by pipetting out the mother liquor and were designated compound **xix**. They formed a pink spot on TLC when sprayed with vanillin and heating at 105 ^{o}C and within 5 min turned blue.

Fraction HJ was dissolved in methanol/dichloromethane mixture (50:50) and eluted with the same solvent mixture using a column packed with 50 g of Sephadex LH_{20}. Thirty five fractions each of 12 ml were collected. The fractions were left to stand for 5 days at room temperature.

Fractions 21-25 formed needle like crystals on sides of the tubes which were recovered by scrapping and designated compound **xx**. They quenched short UV and formed brown/yellowish spots on TLC when exposed to iodine. No compounds were isolated from the other fractions. Isolation from *H. abyssinica* is summarised in Figure 3.4 below.

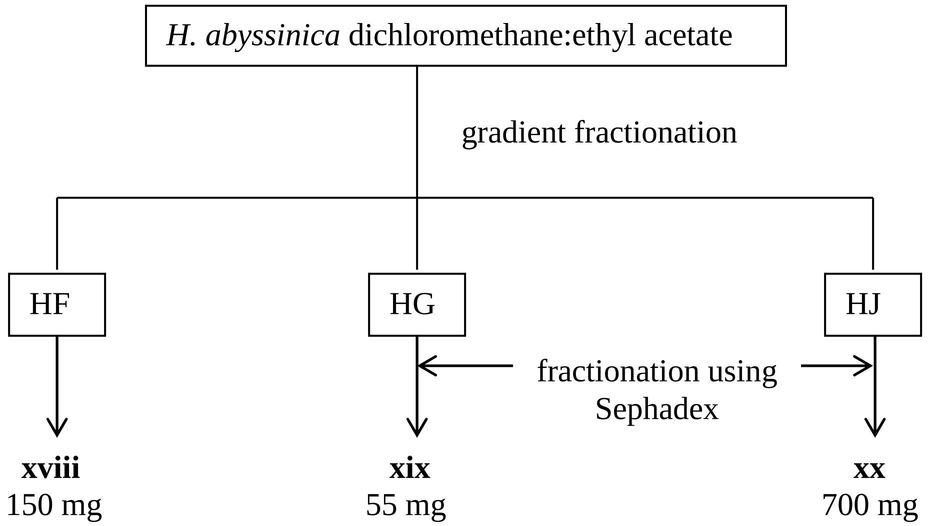


Figure 3.4. A flow chart of isolation of compounds from *Hydnora abyssinica* pseudo rhizome dichloromethane:ethyl acetate extract

CHAPTER FOUR

STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

Identification of compounds involves use of various spectroscopic techniques which include infra-red (IR) spectroscopy, ultraviolet (UV) spectroscopy, nuclear magnetic resonance (NMR) and mass spectrometry (MS). The isolated compounds were subjected to: one dimensional NMR analysis which are proton NMR (^{1}H-NMR), ^{13}carbon NMR (^{13}C-NMR) and distortionless enhancement by polarization transfer (DEPT), MS and IR analysis. Appendix 1 (c) tabulates all the compounds discussed in this chapter giving their Roman codes as they appear in chapter 3.

4.1. Compounds from *Dombeya torrida*

4.1.1. Compound 1 (Friedelin)

Compound **1** was obtained as white star-like crystals in ethyl acetate. This compound could not be visualised under UV meaning it lacked extended conjugation, aromatic rings or any chromophores. It gave a purple spot when the TLC plate was sprayed with vanillin/sulphuric acid reagent and heated to 105 °C. This is a characteristic of steroids. Compound **1** had a melting point of 261-265 °C. Further, compound **1** gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 2962.66- 2868.15 (CH, CH_2 and CH_3 -C-H str), 1710.86 (C=O str), 1460.11 (CH_2 and CH_3 med) [Appendix 2].

MS *m/z* (relative intensity %): 426.47 (M^+, 27), 425.46 (M^+-H, 75), 411.53 (M^+-CH_3, 5), 410.54 (15), 341.58 (3), 302.55 (15), 301.58 (37), 273.57 (34), 272.61 (54), 247.68 (21), 231.61 (34), 230.61 (29), 217.69 (48), 204.70 (56), 178.74 (45), 162.76 (46), 148.77 (29), 136.80 (46), 124.82 (87), 122.81 (85), 108.82 (89), 94.83 (100), 68.86 (96), 66.86 (40), 54.87 (57), 41.17 (29) [Appendices 3a-b].

^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.70, 0.88 0.89, 1.02, 1.07, 1.20, 1.4, 1.6, 2.3 [Appendix 4].

^{13}C-NMR (200 MHz, $CDCl_3$) δ: 22.51 (C-1), 41.51 (C-2), 213.51 (C-3), 58.44 (C-4), 42.37 (C-5), 41.76 (C-6), 18.46 (C-7), 53.32 (C-8), 37.66 (C-9), 59.68 (C-10), 35.56 (C-11), 30.73 (C-12), 39.91 (C-13), 38.51 (C-14), 32.34 (C-15), 36.23 (C-16), 30.22 (C-17), 43.00 (C-18), 35.25 (C-19), 28.40 (C-20), 32.64 (C-21), 39.47 (C-22), 7.06 (C-23), 14.88 (C-24), 18.18 (C-25), 20.49 (C-26), 18.90 (C-27), 32.01 (C-28), 35.84 (C-29), 32.99 (C-30) [Appendices 5 a-b].

It was identified as friedelin (Figure 4.1) based on its spectroscopic data, which were in agreement with those reported in literature [Chandler and Hooper, 1978; Boonyaratavej and Petsom, 1991; Akihisa *et al*., 1992; El Deeb *et al*., 2003; Phan *et al*., 2004 and Manoharan *et al*., 2005].

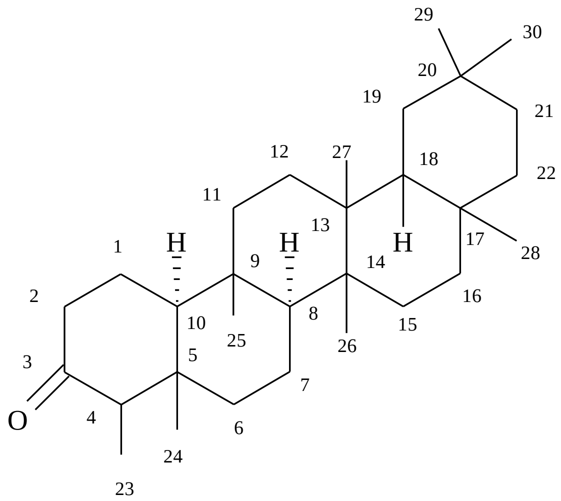


Figure 4.1. Structure of friedelin

IR spectrum data showed the presence of sp^3 C-H groups and aliphatic system at 2962.66 to 2868.15 cm^{-1} corresponding to C-H vibration of CH, CH_2 and CH_3 groups. A strong absorption at 1710.86 cm^{-1} indicated a ketone. A ketone typically absorbs in the region of 1750 - 1705 cm^{-1} [Silverstein *et al*., 1991].

The MS data was in agreement with fragmentation of pentacyclic triterpenoids and steroidal systems as described by Shiojima *et al*., (1992) and Wulfson *et al*., (1964), respectively. The MS spectrum showed a molecular ion at *m/z* 426.47. Friedelin has molecular weight of 426.47 and corresponds to molecular formula $C_{30}H_{50}O$. The fragment ion at *m/z* 425.46 corresponds to the loss of one proton from the molecular ion while *m/z* 411.53 corresponds to the loss of one methyl group from the molecular ion. Figure 4.2 gives a proposed fragmentation of friedelin which is in agreement with literature. Other significant ions at *m/z* 341.58, 273.57, 205.70 and 123.79 can be attributed to fragmentation of rings A, B, C and D, respectively. These assignments suggest the compound is friedelin.

In the ^{1}H-NMR spectrum the singlet peak at δ 0.70 could be attributed to the C-24 methyl protons while a singlet at δ 0.88 were due to C-30 protons. Peaks at δ 0.89, 1.02, 1.07 and 1.20 could be attributed to methyl protons at C-29, C-26, C-27 and C-28, respectively. The peak around δ 7.3 (Appendix 4) was caused by isotopic impurity of $CDCl_3$.

The ^{13}C NMR spectrum showed a total of 30 peaks accounting for 30 carbons. The triplet at around δ 77 was due to the ^{13}C NMR of the solvent $CDCl_3$. The distortionless enhancement by polarization transfer (DEPT) spectrum (Appendices 6 a-b) showed 8 methyl carbons, 11 methylene carbons and 4 methine carbons, which was supported by IR data and was in conformity with friedelin structure. The chemical shifts of δ 213.51 could be attributed to C-3. Ketone carbons resonate low field at 205-220 ppm. The chemical shifts of δ 7.06, 14.88, 18.18, 20.49, 18.90, 32.01, 35.84 and 32.99 could be attributed to methyl carbons C_{23}, C_{24}, C_{25}, C_{26}, C_{27}, C_{28}, C_{29} and C_{30}, respectively. The other carbon atoms were assigned chemical shifts by comparing with the figures given in literature by Boonyaratavej and Petsom, 1991; Akihisa *et al*., 1992; Mahato and Kundu, 1994; El Deeb *et al*., 2003; Phan *et al*., 2004 and Manoharan *et al*., 2005. Table 4.1 shows ^{13}C-NMR data of compound **1** and reference data for friedelin.

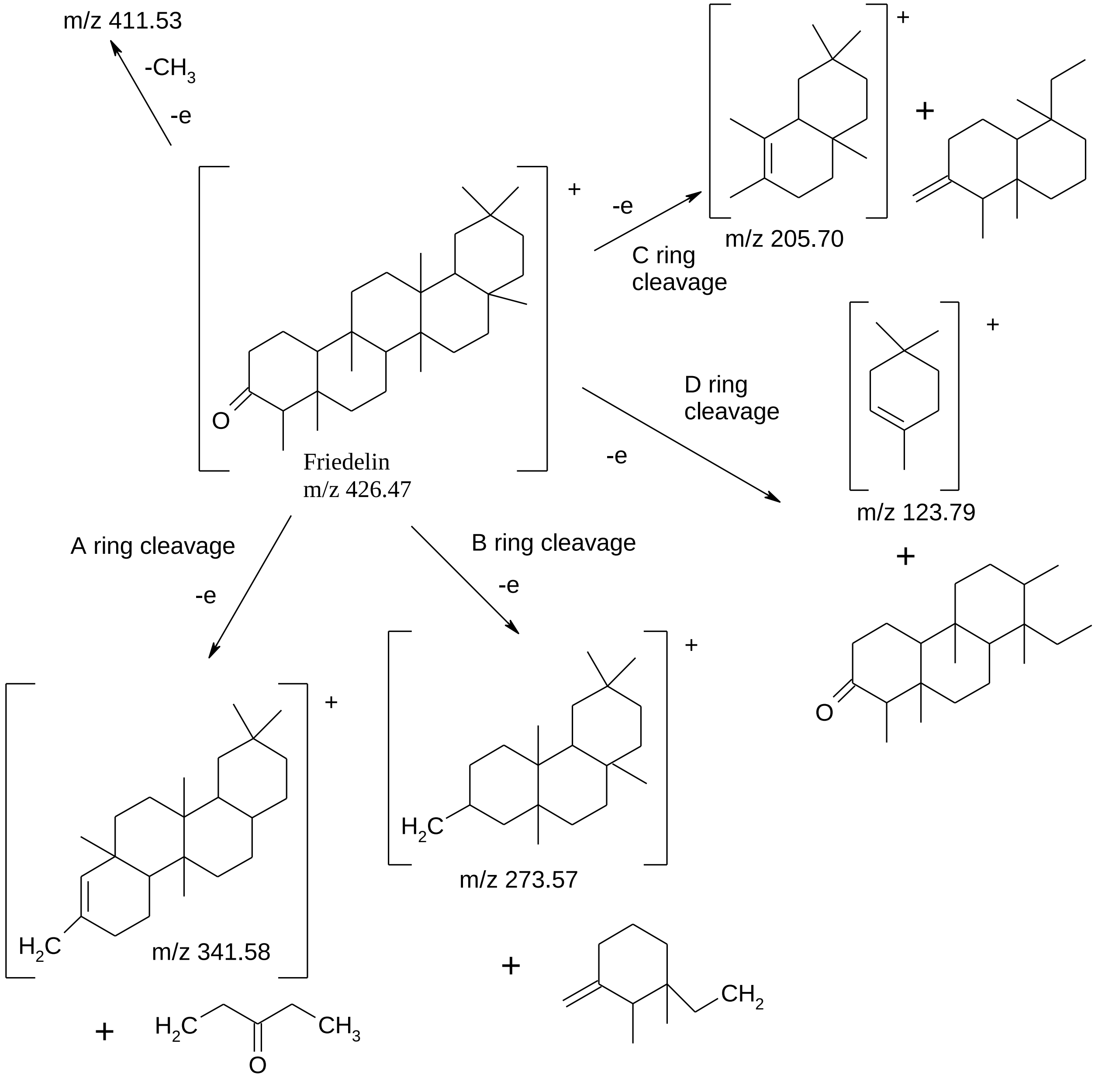


Figure 4.2. Proposed fragmentation of friedelin

Table 4.1. Comparison of the chemical shifts for carbon atoms of compound 1 with literature values of friedelin

	Compound **1** δ values	Friedelin δ values from literature*		Compound **1** δ values	Friedelin δ values from literature*
C-1	22.51	22.3	C-16	36.23	36.0
C-2	41.51	41.5	C-17	30.22	30.0
C-3	213.51	213.2	C-18	43.00	42.8
C-4	58.44	58.2	C-19	35.25	35.3
C-5	42.37	42.1	C-20	28.40	28.1
C-6	41.76	41.3	C-21	32.64	32.7
C-7	18.46	18.2	C-22	39.47	39.2
C-8	53.32	53.1	C-23	7.06	6.8
C-9	37.66	37.4	C-24	14.88	14.6
C-10	59.68	59.4	C-25	18.18	17.9
C-11	35.56	35.6	C-26	20.49	20.2
C-12	30.73	30.5	C-27	18.90	18.6
C-13	39.91	39.7	C-28	32.01	32.1
C-14	38.51	38.3	C-29	35.84	35.0
C-15	32.34	32.4	C-30	32.99	31.8

*(Akihisa *et al.*, 1992)

4.1.2. Compound 2 (Friedelan-3β-ol)

Compound **2** was obtained as white star-like crystals in ethyl acetate. This compound could not be visualised under UV meaning it lacked UV active chromophores. It gave a purple spot when the TLC plate was sprayed with vanillin/sulphuric acid reagent and heated to 105 °C. This is a characteristic of steroids. Compound **2** gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 3624.25, 3479.46 (O-H str), 2937.59-2866.22 (CH, CH_2 and CH_{3} -C-H str), 1462.04 (CH_2 and CH3 med), 1382.96 (OH med) [Appendix 7].

MS *m/z* (relative intensity %): 428.39 (M^+, 82), 426.38 (13), 424.37 (4), 413.38 (M^+-CH_3, 54), 395.39 (9), 346.33 (5), 341.32 (4), 276.25 (24), 275.23 (69), 273.24 (8), 275.23 (69), 261.26 (15), 259.24 (18), 257.23 (16), 248.22 (23), 234.20 (34), 233.19 (38), 231.20 (42), 220.18 (41), 205.20 (46), 177.16 (48), 165.13 (100), 149.12 (28), 137.13 (35), 125.13 (64), 123.12 (57), 121.10 (51), 96.10 (91), 95.09 (91), 81.07 (53), 69.07 (66) [Appendices 8 a-b].

^{1}H-NMR (400 MHz, $CDCl_3$ + Acetone-D_6) δ: 0.16 0.19, 0.23, 0.27, 0.29, 0.31, 0.46, 0.63, 0.66, 0.81, 0.84, 0.87, 0.99, 1.06, 1.14, 1.19, 1.36, 1.38, 1.39, 2.10, 3.00, 7.00 [Appendices 9 a-b].

^{13}C-NMR (200 MHz, $CDCl_3$ + Acetone-D_6) δ: 16.05 (C-1), 35.01 (C-2), 71.46 (C-3), 49.16 (C-4), 38.12 (C-5), 41.63 (C-6), 17.33 (C-7), 52.96 (C-8), 36.86 (C-9), 61.28 (C-10), 35.32 (C-11), 30.54 (C-12), 37.69 (C-13), 39.39 (C-14), 32.02 (C-15), 35.79 (C-16), 30.40 (C-17), 42.58 (C-18), 35.32 (C-19), 27.82 (C-20), 32.51 (C-21), 38.96 (C-22), 11.37 (C-23), 15.67 (C-24), 17.92 (C-25), 19.75 (C-26), 18.29 (C-27), 31.68 (C-28), 34.56 (C-29), 31.38 (C-30) [Appendices 10 a-b].

Compound **2** was identified as friedelan-3β-ol (Figure 4.3) by comparison of its ^{13}C-NMR data with those reported in literature by Boonyaratavej and Petsom, 1991; Mahato and Kundu, 1994; Salazar, *et al.*, 2000.

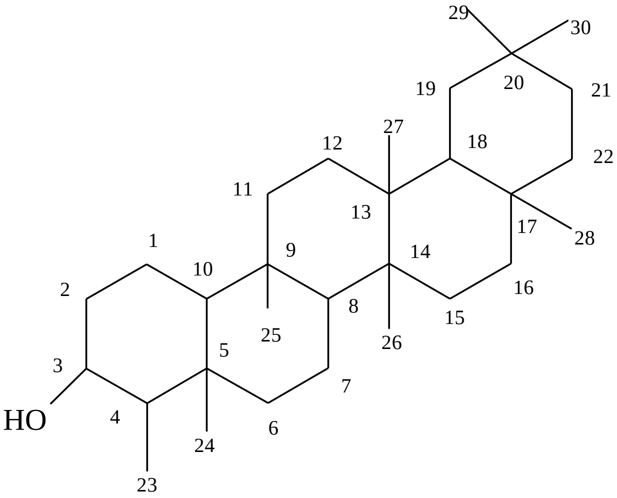


Figure 4.3. Structure of friedelan-3β-ol

Infra-red spectrum peaks at 3479.46 cm^{-1} and at 1382.96 cm^{-1} showed O-H stretching and bending vibrations, respectively, while bands at 2937.59-2866.22 cm^{-1} were attributed to C-H stretching vibrations of aliphatic CH, CH_2 and CH_3. The peak at 1462.04 cm^{-1} was caused by CH_2 and CH_3 deformation.

The MS spectrum [Appendices 8 a-b] gives a molecular ion at 428.40 (82 % relative intensity). Four peaks above this with highest at 448.11 were due to impurities in the compound and are in small quantities. The fragmentation pattern is in agreement with literature fragments for fridelanes as described by Shiojima *et al*., (1992). Figure 4.4 gives fragmentation pattern of friedelan-3β-ol.

Two solvents: acetone-D_6 and $CDCl_3$ were used to dissolve compound **2** during NMR analysis. The peaks at δ 206.04 and a multiplet centred around δ 29 could be attributed to acetone-D_6 while peaks at δ 77 were attributed to deuterated chloroform. The other peaks in the spectrum were 29 (one of them 35.32 ppm integrating for 2 carbons) accounting for 30 carbons. The chemical shift of δ 71.45 could be attributed to C_3 due to the hydroxyl group. The other carbon atoms were assigned chemical shifts by comparing with the figures given in literature by Boonyaratavej & Petsom, 1991, Mahato and Kundu, 1994 and Salazar, *et al*., 2000. Table 4.2. shows ^{13}C-NMR data for compound **2** and literature data of friedelan-3β-ol.

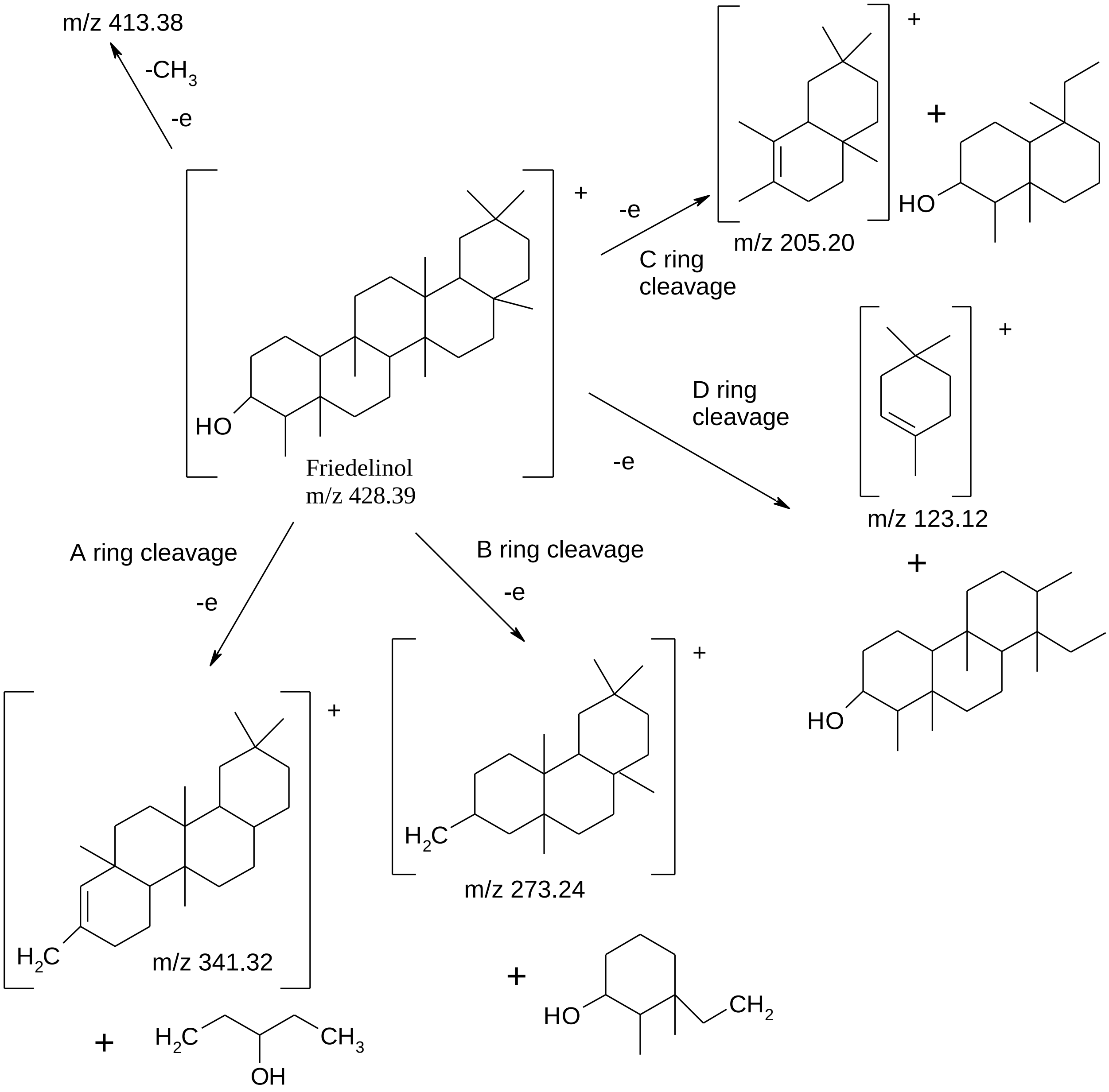


Figure 4.4 Proposed fragmentation of friedelin-3β-ol

Table 4.2. Comparison of the chemical shifts for carbon atoms of compound 2 with literature values of friedelan-3β-ol

	Compound **2** δ values	Friedelan-3β-ol δ values from literature*		Compound **2** δ values	Friedelan-3β-ol δ values from literature*
C-1	16.05	16.16	C-16	35.79	35.90
C-2	35.01	36.14	C-17	30.40	30.02
C-3	71.46	71.59	C-18	42.58	42.88
C-4	49.16	49.62	C-19	35.32	35.36
C-5	38.12	38.09	C-20	27.82	28.17
C-6	41.63	41.99	C-21	32.51	32.88
C-7	17.33	17.69	C-22	38.96	39.28
C-8	52.96	53.27	C-23	11.37	12.09
C-9	36.86	37.18	C-24	15.67	16.58
C-10	61.28	61.65	C-25	17.92	18.35
C-11	35.32	35.66	C-26	19.75	20.13
C-12	30.54	30.69	C-27	18.29	18.69
C-13	37.69	38.38	C-28	31.68	32.12
C-14	39.39	39.69	C-29	34.56	35.02
C-15	32.02	32.34	C-30	31.38	31.85

*(Salazar, *et al.*, 2000)

4.1.3. Compounds 3 (β-Sitosterol) and 4 (Stigmasterol)

Compounds **3** and **4** were isolated together as colourless needle-like crystals in ethyl acetate. The mixture could not be visualized under UV, but gave one yellow spot when the TLC plate was put in a saturated iodine tank and a purple colour when the plate was sprayed with vanillin/sulphuric acid reagent. The mixture had a melting point of 136-145 °C. Spectroscopic analysis showed that the mixture contained two compounds with molecular weights of 414 and 412 corresponding to molecular formulas $C_{29}H_{50}O$ and $C_{29}H_{48}O$, respectively. The molecular weights and the fragmentation pattern indicate that it is a mixture of β-sitosterol and stigmasterol. Other ion peaks are m/z 413 (100, M^{+}-1) for sitosterol and 411 (25, M^{+}-1) for stigmasterol. The DEPT spectrum [Appendices 15 a-b] showed 6 methyl carbons, 11 methylene carbons and 11 methine carbons, which was in conformity with structures of sitosterol and stigmasterol. The only difference between the two compounds is the presence of $C_{22}=C_{23}$ double bond in stigmasterol as compared to C_{22}-C_{23} single bond in β-sitosterol and hence, the lack of practical difference in their R_f value despite the use of several solvent systems in an attempt to separate them. Literature has many examples of these compounds isolated together [Boonyaratavej & Petsom, 1991; Maima *et al*., 2008; Pateh *et al*., 2008; Kamboj and Saluja, 2011].

4.1.3.1: Compound 3 (β-Sitosterol)

Below are the spectroscopic data given by β-sitosterol:

IR ν max (KBr) cm^{-1}: 3423.65 (O-H str), 2970.38 (C-H str), 2860.43 (=C-H str), 1653 (C=C absorption peak), 1462.04 (CH_2), 1375.25, 1055.06, 962.48 (=C-H bend) [Appendix 11].

MS *m/z* (rel. int.): 414 (M^{+}, 36), 413 (100), 399 (9), 396 (28.5), 381 (9), 328 (22), 302 (30), 274 (14), 272 (22), 254 (38), 231(6), 212 (30), 160 (28), 158 (35), 146 (39), 144 (48), 134 (24), 118

(28), 110 (13), 104 (43), 96 (22), 94 (45), 93 (10), 92 (33), 90 (28), 84 (21), 80 (54), 78 (25), 70 (24), 68 (38), 66 (24), 56 (41), 54 (53), 43 (64), 43 (28) [Appendices 12 a-b].

^{1} H NMR ($CDCl_3$, 200 MHz) δ: 0.69 (3H, s, CH_3-18), 0.85 (3H, m, CH_3-29), 0.83 (3H, d, CH_3-26), 0.81 (3H, d, CH_3-27), 1.00 (3H, d, CH3-21), 1.00 (3H, s, CH_3-19), 3.52 (1H, m, H-3), 5.34 (1 H, d, H-6) [Appendices 13 a-c)].

^{13}C-NMR (200 MHz, $CDCl_3$) δ: 37.48 (C-1), 31.86 (C-2), 72.03 (C-3), 42.51 (C-4), 140.97 (C-5), 121.95 (C-6), 32.13 (C-7), 32.13 (C-8), 50.35 (C-9), 36.73 (C-10), 21.31 (C-11), 39.99 (C-12), 42.51 (C-13), 56.99 (C-14), 24.53 (C-15), 28.48 (C-16), 56.17 (C-17), 12.09 (C-18), 19.63 (C-19), 36.38 (C-20), 19.01 (C-21), 34.16 (C-22), 26.27 (C-23), 46.04 (C-24), 29.36 (C-25), 20.06 (C-26), 19.26 (C-27), 23.28 (C-28), 12.21 (C-29) [Appendices 14 a-b].

Based on spectroscopic data, which were in agreement with those reported in literature [Reich *et al*., 1969; Holland *et al*., 1978; Nes *et al*. 1992; Bano *et al*., 2002 and Ndwigah *et al*., 2005], it was concluded that the mixture contained compound **3** (β-sitosterol) (Figure 4.5).

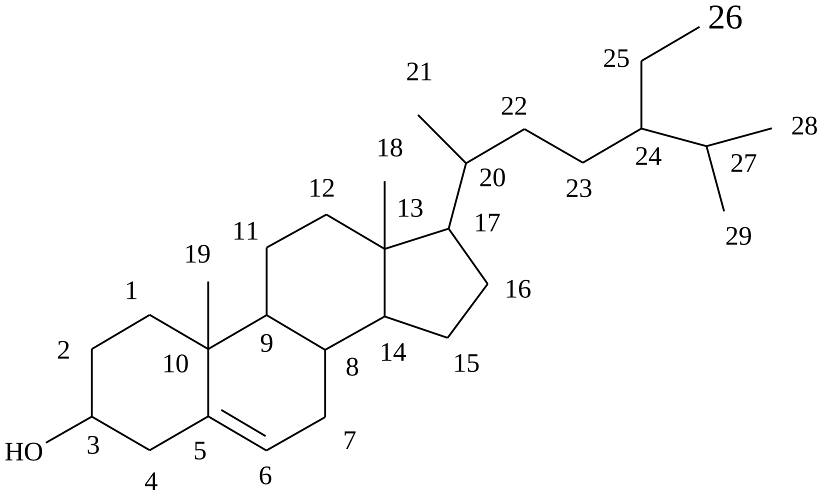


Figure 4.5. Structure of β-sitosterol

The IR spectrum showed the presence of a hydroxyl group at 3423.65 and an aliphatic system 2970.38 (C-H str), 2860.43 (=C-H str), 1653 (C=C absorption peak), 1462.04 (CH_2), 1375.25, 1055.06, 962.48 (=C-H bend). The peak at 1653 cm^{-1} was due to the double bond between C_5 and C_6 [Silverstein *et al*., 1991].

The MS spectrum showed a molecular ion at m/z 414, corresponding to molecular formula $C_{29}H_{50}O$. The fragment ion at m/z 413 corresponds to the loss of one proton from the molecular ion while m/z 396 corresponds to the loss of one water molecule from the molecular ion. The fragment ion at m/z 329 could be due to fragmentation between C_{23} and C_{24} (Figure 4.6), which is characteristic of phytosterols [Wulfson *et al*., 1964].

According to ^1H NMR, there is a doublet at δ 5.34 attributed to vinylic proton at C_6. A multiplet at δ 3.52 was attributed to methine proton at C_3. Doublets at δ 0.83 and δ 0.81 and a multiplet at δ 0.85 integrated for 9 protons representing C_{26}, C_{27} and C_{29} methyl protons, respectively. Two singlets at δ 0.69 and δ 1.00 accounting for 3 protons each were due to methyl protons at C_{18} and C_{19}, respectively. A doublet at δ 1.00 was assigned to C_{21} secondary methyl protons. The peak around δ 7.26 was caused by isotopic impurity of $CDCl_3$ [Pateh *et al*., 2008; Maima *et al*., 2008; Boonyaratavej & Petsom, 1991].

The ^{13}C NMR spectrum showed a total of 27 peaks accounting for 29 carbons (two of these peaks at δ 32.13 and δ 42.51 represented two carbons each). The triplet at around δ 77 was due to the ^{13}C NMR of the solvent $CDCl_3$ [Boonyaratavej & Petsom, 1991; Maima *et al*., 2008; Pateh *et al*., 2008].

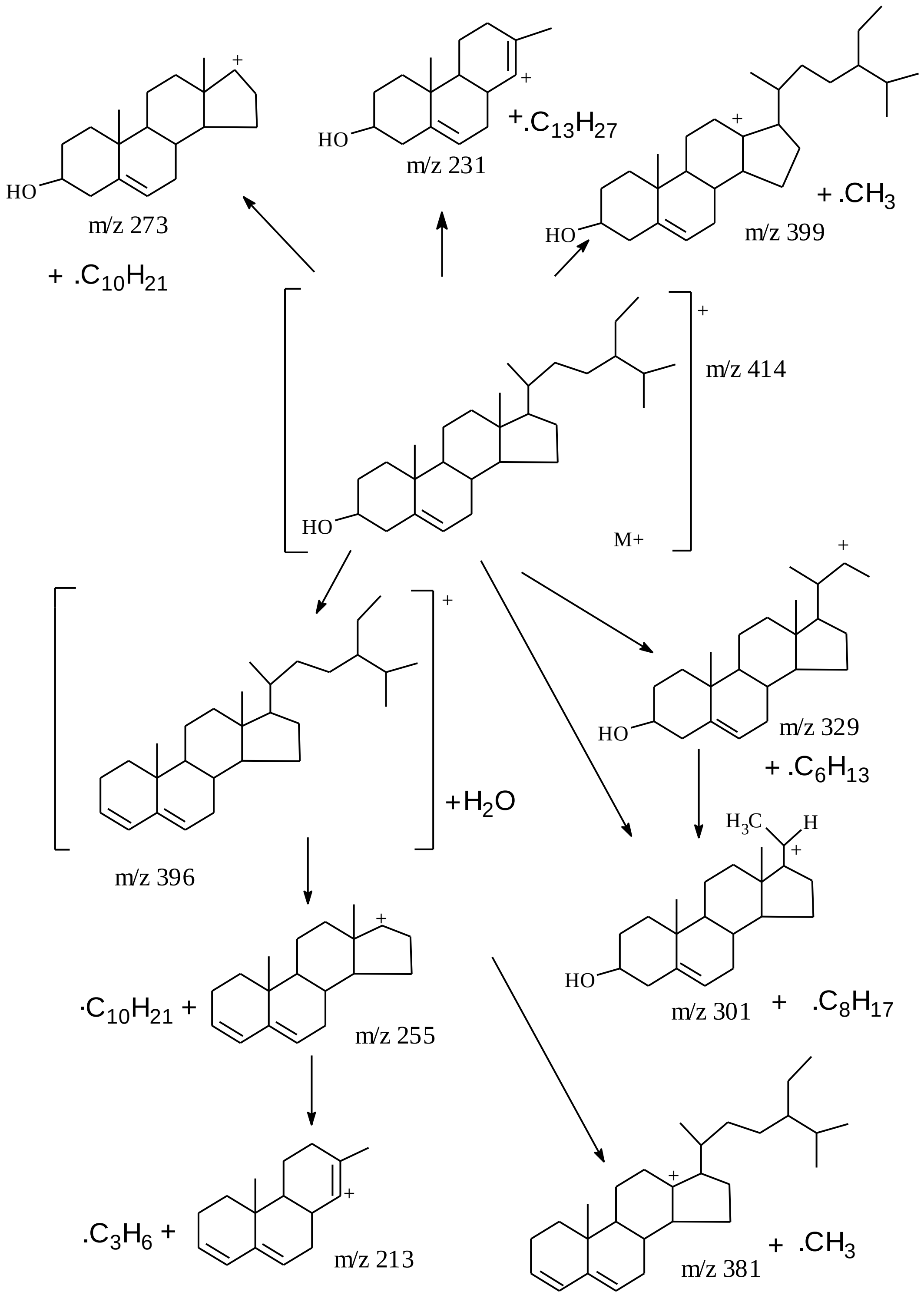


Figure 4.6. Proposed fragmentation pattern of β-sitosterol

The DEPT spectrum (Appendices 15 a-b) showed 6 methyl carbons, 11 methylene carbons and 9 methine carbons, which was in conformity with β-sitosterol structure. The chemical shifts of δ 72.03, δ 140.97 and δ 121.95 could be attributed to C_3, C_5 and C_6 respectively. The other carbon atoms were assigned chemical shifts by comparing with the figures given in literature [Reich *et al*., 1969; Holland *et al*, 1978; Boonyaratavej & Petsom, 1991; Nes *et al*. 1992; Bano *et al*., 2002; Ndwigah *et al*., 2005; Pateh *et al*., 2008; Maima *et al*., 2008].

4.1.3.2: Compound 4 (Stigmasterol)

IR ν max (KBr) cm^{-1}: 3423.65 (O-H str), 2970.38 (C-H str), 2860.43 (C-H str), 1653 (C=C absorption peak), 1462.04 (CH_2), 1375.25, 1055.06, 962.48 (=C-H bend) [Appendices 11 a-b].

MS m/z (rel. int.): 412 (M^+, 4), 411 (25), 398 (21), 395 (74), 380 (21), 302 (30), 274 (14), 272 (22), 254 (38), 231(6), 212 (30), 160 (28), 158 (35), 146 (39), 144 (48), 134 (24), 118 (28), 110 (13), 104 (43), 96 (22), 94 (45), 93 (10), 92 (33), 90 (28), 84 (21), 80 (54), 78 (25), 70 (24), 68 (38), 66 (24), 56 (41), 54 (53), 43 (64), 43 (28) [Appendices 12 a-c].

^{1} H NMR ($CDCl_3$, 200 MHz) δ: 0.69 (3H, s, CH_3-18), 0.81 (3H, m, CH_3-29), 0.85 (3H, d, CH_3-26), 0.80 (3H, d, CH_3-27), 1.03 (3H, d, CH3-21), 1.00 (3H, s, CH_3-19), 3.51 (1H, m, H-3), 5.34 (1 H, d, H-6) [Appendices 13 a-c)].

^{13}C-NMR (200 MHz, $CDCl_3$) δ: 37.48 (C-1), 31.86 (C-2), 72.03 (C-3), 42.51 (C-4), 140.97 (C-5), 121.95 (C-6), 32.13 (C-7), 32.13 (C-8), 50.35 (C-9), 36.73 (C-10), 21.31 (C-11), 39.91 (C-12), 42.51 (C-13), 57.09 (C-14), 24.53 (C-15), 29.15 (C-16), 56.17 (C-17), 12.09 (C-18), 19.26 (C-19), 40.74 (C-20), 21.45 (C-21), 138.56 (C-22), 129.49 (C-23), 51.47 (C-24), 32.13 (C-25), 19.63 (C-26), 20.06 (C-27), 25.64 (C-28), 12.49 (C-29) [Appendices 14 a-b].

Based on its spectroscopic data, which were in agreement with those reported in literature [Holland *et al*., 1978; Forgo and Kövér, 2004; Jamal *et al*., 2008; Jain and Bari, 2010], it was found that the isolate contained compound **4** (stigmasterol) (Figure 4.7).

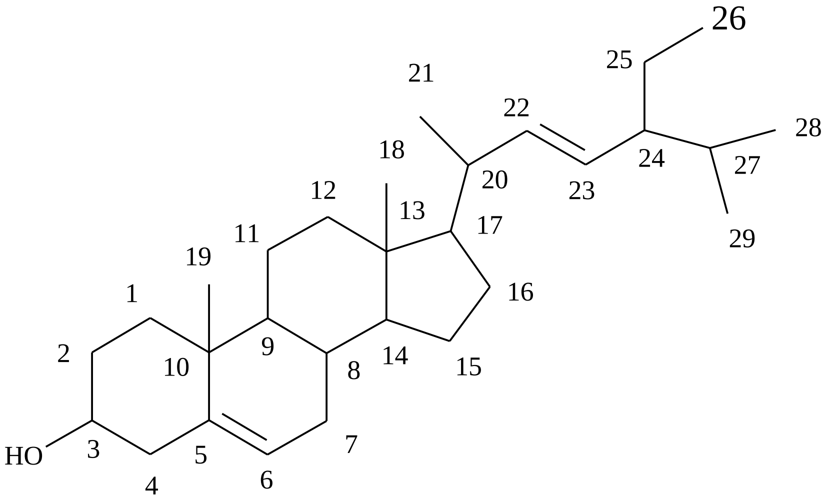


Figure 4.7. Structure of stigmasterol

IR spectrum showed the presence of hydroxyl group at 3423.7 cm^{-1} and an aliphatic system 2930.1 (CH_3 C-H str), 1465.1, 1381.0, 959.0 and 838.2 cm^{-1} (C-H bend). The peak at 1653 cm^{-1} was due to the double bonds between C_5=C_6 and C_{22}=C_{23} [Silverstein *et al.*, 1991].

The mass spectrum indicated fragmentation that was in agreement with fragmentation process of steroidal systems described by Wulfson *et al*. (1964). The MS spectrum showed a molecular ion at m/z 412, corresponding to molecular formula $C_{29}H_{48}O$. Fragmentation of stigmasterol is similar to that of β-sitosterol (Figure 4.6).

According to ^{1}H NMR, there was a doublet at δ 5.34 attributed to vinylic proton at C_6. A multiplet at δ 3.51 is attributed to methine proton at C_3 that is next to a hydroxyl group. Doublets at δ 0.80, a multiplet at δ 0.81 and δ 0.85 and integrated for 9 protons representing C_{26}, C_{27} and C_{29} methyl protons, respectively. Two singlets at δ 0.69 and δ 1.00 accounting for 3 protons each were due to methyl protons at C_{18} and C_{19}, respectively. A doublet at δ 1.03 was assigned to C_{21} secondary methyl protons. The peak around δ 7.26 was caused by isotopic impurity of $CDCl_3$.

The ^{13}C NMR spectrum showed a total of 27 peaks accounting for 29 carbons (two of these peaks at δ 32.13 and δ 42.51 represented two carbons each). The triplet at around δ 77 was due to the ^{13}C NMR of the solvent $CDCl_3$. The DEPT spectrum (Appendices 15 a-b) showed 6 methyl carbons, 9 methylene carbons and 11 methine carbons, which was in conformity with stigmasterol structure. The chemical shifts of δ 72.03, δ 140.97 and δ 121.95 could be attributed to C_3, C_5 and C_6 respectively. Alkene carbons (sp^2 hybridized) such as C_5 and C_6 have chemical shifts between δ 80 and δ 180 while alkyl (sp^3 hybridized) ones resonate more upfield between δ 0- 80. Oxygenated carbons such as C_3 resonate between δ 65 and 82 [Schirmer, 1991; Silverstein *et al.*, 1991].

The other carbon atoms were assigned chemical shifts in comparison with the figures given in literature by Holland *et al*, 1978; Forgo P. and Kövér, 2004; Jamal *et al*, 2008; and Jain and Bari, 2010.

4.1.4: Compound 5 (Taraxerol)

The compound **5** was obtained as colourless sugar like crystals in dichloromethane. This compound could not be visualised under UV, but gave a yellow spot in iodine and a purple spot when TLC plate was sprayed with vanillin and heated to 105 °C. This is characteristic of triterpenoids. Compound **5** had a melting point of 281-284 °C. Further, compound **5** gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 3487.30 (OH str), 2964.59- 2860.43 (CH, CH_2 and CH_3 - C-H str), 1641.42, 1467.83, 1454.33 (CH_2 and CH_3 med), 1375.25, 1031.92 and 999.13 (Appendix 16)

MS m/z (rel. int. %): 428.31 (M^++2, 3), 426.30 (M^+, 48), 411.27 (17), 303.20 (14), 302.20 (57), 287.17 (30), 269.17 (13), 231.16 (7), 218.15 (26), 205.15 (29), 204.14 (100), 191.14 (10), 189.12 (16), 135.08 (26), 81.05 (11), 69.05 (15) [Appendices 17 a-b].

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.80 (s), 0.82 (s), 0.90 (s), 0.92, 0.94, 0.97, 1.08, 1.25, 1.32, 1.58, 1.96 (m, 2H), 3.18 (m , 1H), 5.53 (dd, 1H) [Appendices 18 a-b].

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 37.93 (C-1), 27.36 (C-2), 79.29 (C-3), 39.18 (C-4), 55.73 (C-5), 19.01 (C-6), 35.32 (C-7), 38.98 (C-8), 48.93 (C-9), 37.77 (C-10), 17.72 (C-11), 36.01 (C-12), 37.93 (C-13), 158.29 (C-14), 117.09 (C-15), 36.89 (C-16), 38.21 (C-17), 49.49 (C-18), 41.52 (C-19), 29.02 (C-20), 33.90 (C-21), 33.30 (C-22), 28.21 (C-23), 15.67 (C-24), 15.67 (C-25), 30.04 (C-26), 26.13 (C-27), 30.15 (C-28), 33.57 (C-29), 21.54 (C-30) [Appendices 19 a-b].

It was identified as taraxerol (Figure 4.8) based on its spectroscopic data, which were in agreement with those reported in literature (Nobuko *et al*., 1987; Mahato and Kundu, 1994; Hernández-Chávez *et al*., 2012).

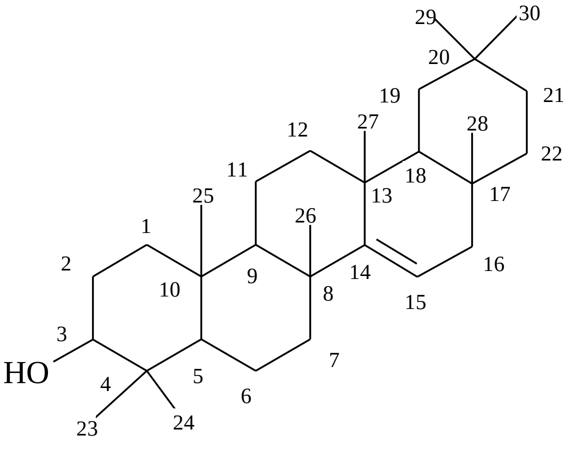


Figure 4.8. Structure of taraxerol

The IR spectrum showed the presence of an OH group at 3487.30, an aliphatic system at 2964.59- 2860.43 (CH, CH_2 and CH_3 -C-H str.), 1467.83, 1454.33 (CH_2 and CH_3 med), and skeletal frequencies at 1375.25, 1031.92 and 999.13. A peak at 1641.42 cm^{-1} indicates a non-conjugated C=C bond on the structure.

The MS data is in agreement with fragmentation of pentacyclic triterpenoids as described by Kutney & Eigendorf, (1969) and Shiojima *et al*., (1992). Figure 4.9 below shows fragmentation pattern of taraxerol. The MS spectrum gives a molecular ion at *m/z* 426.30. An important cleavage process is the retro Diels-Alder collapse of ring D to yield the diene fragment **A**. A subsequent elimination of the C-3 hydroxyl group gives rise to a daughter ion **B** at *m/z* 284 and further loss of a methyl group yields ion **C** *m/z* 269. A second cleavage occurs in ring C involving rupture of the 8, 14 and 11, 12 bonds. The molecular ion generates the ion species, **D**, which in turn leads to **E**. Species E (*m/z* 204.14) is the base peak in the spectra of taraxerol and taraxerone and gives rise to an abundant satellite ion, F (*m/z* 189), by elimination of the methyl group at the C-17 position [Kutney & Eigendorf, 1969].

In the ^{1}H-NMR spectrum, there is a double doublet at δ 5.53 attributed to vinylic proton at C_{15} and a multiplet centred at δ 3.18 attributed to methine proton at C_3. Singlets at 1.08, 0.98, 0.94, 0.92, 0.82 and 0.80 integrating for 3 protons each are attributed to H-26, H-23, H-27, H-25, H-28 and H-24 methyl groups, respectively. Another singlet integrating for 6 protons at δ 0.90 is due to methyl protons at C_{29} and C_{30}.

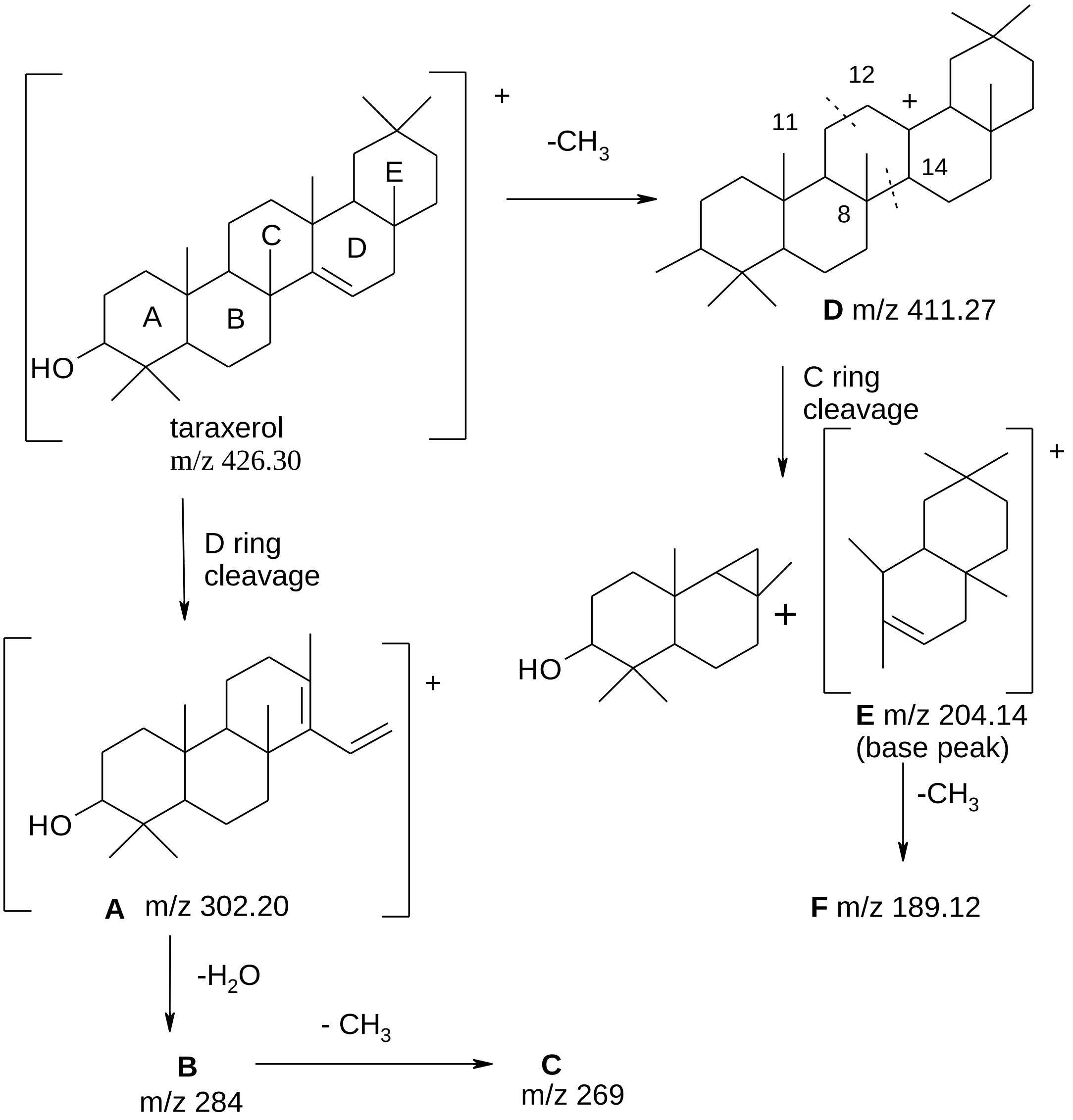


Figure 4.9. Fragmentation pattern of taraxerol

The ^{13}C NMR spectrum showed a total of 28 peaks with peaks at δ 15.67 and at δ 37.93 ppm integrating for 2 carbons each, thus accounting for 30 carbons. The DEPT spectrum (Appendices 20 a-b) showed 8 methyl carbons, 10 methylene carbons and 5 methine carbons, which was supported by IR data and was in conformity with taraxerol structure. The chemical shifts of δ 158.29 and 117.09 could be attributed to C_{14} and C_{15} carbons, respectively. The chemical shift at δ 79.29 is attributed to the C_{13} (sp^3 hybridized) attached to the OH group which resonate between 60 to 85 ppm.The other carbon atoms were assigned chemical shifts by comparing with the figures given in literature by Nobuko *et al.*, 1987; Hernández-Chávez *et al.*, 2012; and Mahato & Kundu, 1994. Table 4.3 lists the chemical shifts for compound **5** with those of literature taraxerol.

Table 4.3. Comparison of the chemical shifts for carbon atoms of compound 5 with literature values of taraxerol

	Compound **5** δ values	Taraxerol δ values from literature*		Compound **5** δ values	Taraxerol δ values from literature*
C-1	37.93	38.1	C-16	36.89	36.9
C-2	27.36	27.3	C-17	38.21	38.1
C-3	79.29	79.2	C-18	49.49	49.4
C-4	39.18	39.1	C-19	41.52	41.4
C-5	55.73	55.7	C-20	29.02	29.0
C-6	19.01	19.0	C-21	33.90	33.9
C-7	35.32	35.3	C-22	33.30	33.2
C-8	38.98	38.9	C-23	28.21	28.1
C-9	48.93	48.9	C-24	15.67	15.6
C-10	37.77	37.9	C-25	15.67	15.6
C-11	17.72	17.7	C-26	30.04	30.1
C-12	36.01	35.9	C-27	26.13	26.0
C-13	37.93	37.9	C-28	30.15	30.1
C-14	158.29	158.1	C-29	33.57	33.5
C-15	117.09	117.0	C-30	21.54	21.5

*(Nobuko *et al.*, 1987)

4.1.5. Compound 6

Compound **6** was isolated as colourless sheet like crystals in dichloromethane which changed to needle like crystals when re-crystallised in methanol/dichloromethane mixture. This compound could not be visualised under UV, but gave a yellow spot in iodine and a purple spot when TLC plate was sprayed with vanillin and heated to 105 °C. Further, compound **6** gave the following spectroscopic data:

MS m/z (rel. int. %): 290.21 (M^+, 2), 276.21 (23), 275.21 (100), 258.21 (18), 257.20 (83), 192.17 (36), 191.16 (25), 177.14 (26), 149.11 (10), 137.11 (24), 123.10 (15), 109.08 (17), 95.07 (22), 81.06 (29), 69.05 (17), 67.20 (15) (Appendices 21 and 22).

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.72 (s, 3H), 0.77 (s, 3H), 0.84 (s, 3H), 0.91 (d), 0.96 (d), 1.12 (s, 4H), 1.21 (s, 4 H), 1.32, 1.45 (m), 1.59 (m), 1.64, 2.20 (m, 1H), 4.92 (m, 2H), 6.00 (m, 1H) (Appendices 23 a-c).

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 147.92, 109.72, 76.30, 73.50, 58.69, 56.67, 43.30, 42.39, 39.56, 37.05, 35.03, 33.56, 32.95, 24.15, 21.47, 20.13, 18.86, 16.08 (Appendix 24).

The ^{13}C NMR shows a 20-carbon compound and gives an indication that compound **6** could be a diterpene with 2 hydroxyl groups similar to labd-14-en-8,13-diol. The DEPT spectrum (Appendices 25 a-b) showed 5 methyl carbons, 7 methylene carbons and 3 methine carbons. The ^{13}C-NMR shows two sp^2 hybridized carbons resonating at δ 147.92 and 109.72 ppm showing a compound with a double bond. Two other carbons resonating at δ 76.30 and 73.50 ppm suggests a dihydroxy molecule. A labdane diterpene labd-14-en-8,13-diol (Figure 4.10 below) has similar chemical shifts as compound **6**. Data suggest compound **6** to be a labdane diterpene. Table 4.4 below shows comparison of compound 10 chemical shifts with literature figures for labd-14-en-8, 13-diol from Pacheco *et al*., 2009.

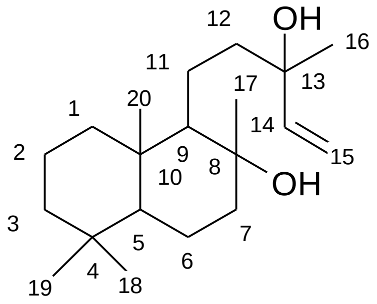


Figure 4.10. Structure of labd-14-en-8,13-diol

Table 4.4. Comparison of the chemical shifts for carbon atoms of compound 6 with literature values of labd-14-en-8,13-diol

	Compound **6** δ values	Labden-8,13-diol δ values from literature*		Compound **6** δ values	Labden-8,13-diol δ values from literature*
C-1	37.05	40.4	C-11	18.86	20.0
C-2	16.08	19.0	C-12	43.30	46.2
C-3	39.56	42.7	C-13	73.50	73.3
C-4	33.56	33.7	C-14	147.92	147.5
C-5	56.67	56.9	C-15	109.72	110.7
C-6	20.13	21.1	C-16	32.95	27.8
C-7	42.39	45.1	C-17	24.15	24.5
C-8	76.30	73.9	C-18	33.56	33.7
C-9	58.69	62.3	C-19	21.47	21.8
C-10	35.03	39.8	C-20	16.08	15.8

*(Pacheco *et al*., 2009)

However a number of things stand out in relation to the chemical shifts. Comparing the ^{13}C-NMR and DEPT spectra (Appendices 25 a-b) of compound **6** and literature values of labden-8, 13-diol, there are many similarities. Both compounds have 2 sp^2 hybridized carbons, 2 hydroxyl groups, 5 methyl carbons, 7 methylene carbons and 3 methine carbons. However, the DEPT spectrum shows of the 2 sp^2 hybridized carbons of compound **6**, one is a methine carbon and the second is not protonated unlike for labden-8,13-diol in which one of the 2 sp^2 hybridized carbons is a methylene and the other a methine carbon. Further work using X-ray crystallography will fully elucidate the structure of compound **6**. Diterpenes are organic compounds of low molecular weight with a skeleton of 20 carbon atoms. They are of significant chemical and commercial interest because of their use as lead compounds in the search for new pharmaceuticals.

4.1.6. Compound 7

Compound **7** was obtained as sugar like crystals in dichloromethane. This compound could not be visualised under UV, but gave a yellow spot in iodine and a purple spot when the TLC plate was sprayed with vanillin and heated to 105 °C. This is a characteristic of steroids. Further, compound **7** gave the following spectroscopic data:

IR ν max (KBr) cm^{-1}: 3437.15 (OH weak), 2924.09, 2858.51(CH, CH_2 and CH_3 -C-H str), 1462.04, 1377.17 and 966.34 [Appendix 26].

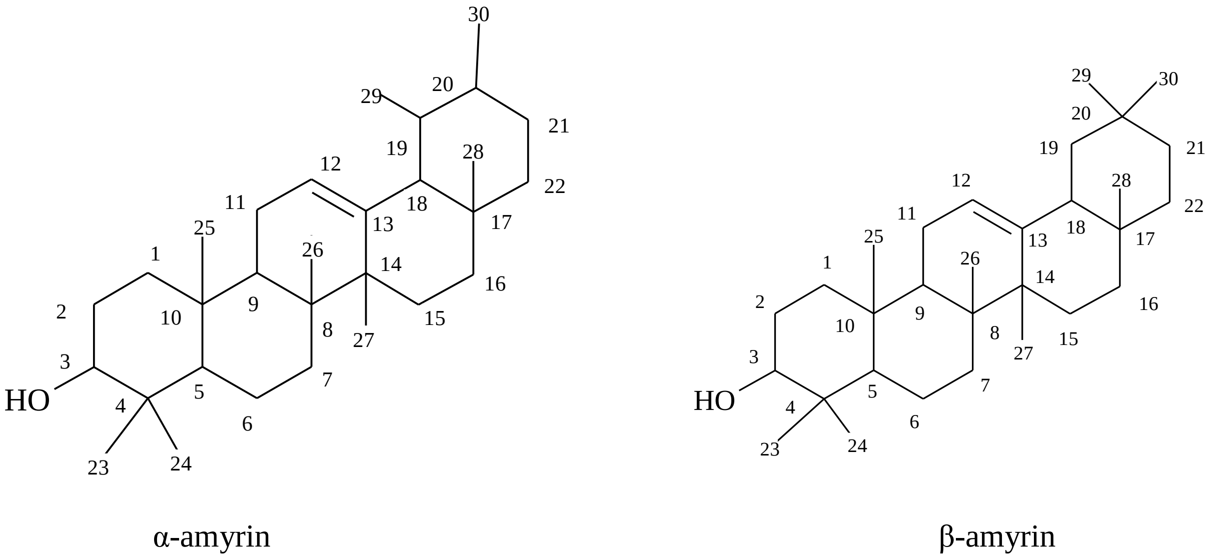
^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.77 (s), 0.81, 0.83, 0.86, 0.89, 0.90, 0.92,1.00, 1.04, 1.13, 1.25, 1.47, 1.59, 3.45 (m , 1H), 5.61 (m, 1H) [Appendices 27 a-c].

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 142.19, 122.22, 60.24, 51.95, 50.45, 44.46, 43.01, 41.05, 39.53, 38.83, 35.63, 35.03, 34.36, 31.01, 29.93, 29.29, 29.21, 28.54, 27.99, 25.70, 24.27, 23.15, 22.18, 20.13, 18.28, 18.09, 16.30, 15.97, 15.23 [Appendices 28 a-b].

The IR spectrum shows presence of a hydroxyl group at 3437 cm^{-1}; an aliphatic system at 2924.09 and 2858.51cm^{-1} (CH, CH_2 and CH_3, C-H str); and skeletal frequencies at 1462.04, 1377.17 and 966.34. This suggests a compound with only 1 hydroxyl group and no other substitutions like carbonyl groups or nitro groups.

The ^{1}H-NMR spectrum shows 8 singlets each integrating for 3 H and indicates presence of 8 methyl groups which are not bordered by any other H hence the singlets. These 7 singlets are centered at δ 0.77, 0.81, 0.84, 0.89, 0.92, 1.00, 1.04 and δ 1.13. The DEPT spectrum (Appendices 29 a-b) confirmed presence of the 8 methyl groups.

The ^{13}C NMR spectra gave 29 peaks with one at δ 29.29 integrating for 2 peaks giving a total of 30 carbons. The chemical shifts at δ 142.19 and 122.22 indicate presence of 2 unsaturated carbons, sp^2 hybridized, in form of a C=C double bond. These normally resonate at δ 100-145 ppm. The δ 60.24 is usually due to a saturated carbon, sp3 hybridized bonded to an oxygen usually inform of an alcohol. Chemical shifts δ 23-58, δ 17-47, and δ 5-25 are due to methine, methylene and methyl carbons, respectively (Silverstein *et al.*, 1991). The DEPT spectrum (Appendices 29 a-b) showed 8 methyl carbons, 9 methylene carbons and 7 methine carbons. Comparison of the chemical shifts for carbon atoms of compound **7** with those of literature gives an indication that it could be a triterpenoid. The ^{13}C NMR and the DEPT spectra show a correlation with the triterpenoids α- and β-amyrin but there are clear differences in the δ values. Figure 4.11 below give the structures of α- and β-amyrin.



α-amyrin β-amyrin

Figure 4.11. Structures of α- and β-amyrin

Below is table 4.5 which shows the chemical shifts of compound **7** compared with the ones of α- and β- amyrin in descending order. Due to the significant differences in the chemical shifts, the compound could not be fully identified. But the spectral data strongly suggests compound **7** is a triterpenoid. The chemical shifts of compound **7** correlates closely with those of α and β amyrin. Further work, possibly X-ray crystallography will help fully elucidate its structure.

Table 4.5. Comparison of the chemical shifts (δ values) for carbon atoms of compound 7 with literature values of α-amyrin and β-amyrin

	Compound **7**	Literature			Compound **7**	Literature*	
		α-amyrin*	β-amyrin**			α-amyrin*	β-myrin**
C-1	142.19	139.3	145.1	C-16	29.29	32.9	32.8
C-2	122.22	124.3	121.8	C-17	29.29	31.2	32.5
C-3	60.24	78.3	79.0	C-18	29.21	28.7	31.1
C-4	51.95	58.9	55.3	C-19	28.54	28.1	28.4
C-5	50.45	55.2	47.7	C-20	27.99	28.1	28.2
C-6	44.46	47.7	47.4	C-21	25.70	27.2	27.3
C-7	43.01	42.0	46.9	C-22	24.27	26.6	27.0
C-8	41.05	41.5	41.8	C-23	23.15	23.3	26.2
C-9	39.53	40.0	38.8	C-24	22.18	23.3	26.0
C-10	38.83	39.6	38.8	C-25	20.13	21.3	23.7
C-11	35.63	39.6	38.7	C-26	18.28	18.3	23.6
C-12	35.03	38.7	37.6	C-27	18.09	17.4	18.5
C-13	34.36	38.7	37.2	C-28	16.30	16.8	16.9
C-14	31.01	36.9	34.8	C-29	15.97	15.6	15.6
C-15	29.93	33.7	33.3	C-30	15.23	15.6	15.5

*Knight S. A., 1974; ** Shujiro *et al*., 1981

4.1.7. Other isolated compounds

The structures of other compounds isolated from *Dombeya torrida* could not be elucidated. This was because some were too small for spectroscopic analysis while others were impure. For example two feathery crystals designated compound **xiv** and compound **xv**, were isolated in small amounts (< 5 mg each). These were not adequate amounts for spectroscopic analysis and were not analysed further. For others, spectroscopic analysis was done but structures could not be determined. The data acquired on them is shown below with preliminary discussion of a probable class of compounds they could belong.

4.1.7.1. Compound 8

Compound **8** was isolated as white sugar like crystals in dichloromethane. This compound could not be visualised under UV, but gave a yellow spot in iodine and a purple spot when TLC plate was sprayed with vanillin and heated to 105° C. Further, compound **8** gave the following spectroscopic data:

IR ν max (KBr) cm^{-1}: 3313.71, 3286.70, 2929.87, 2848.86, 2659.84, 2335.80, 1465.90, 1373.32, 1062.78 (Appendices 30).

MS m/z (rel. int. %): 656.79 (M^+, 1), 632.88 (16), 608.16 (33), 607.28 (74), 582.50 (19), 409.39 (7), 364.39 (19), 312.29 (3), 285.25 (16), 257.32 (100), 256.23 (26), 229.21 (7), 153.15 (4), 111.11 (17), 97.10 (25), 85.10 (19), 83.08 (24), 71.08 (27), 69.07 (20) [Appendices 31 a-b].

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.79 (s), 0.84 (s), 0.86, 0.87, 0.91, 1.15, 1.25 (s, 79 H), 1.35, 1.57, 1.61, 1.64, 2.28 (m, 2H), 4.05 (m, 2H) [Appendices 32 a-b].

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 174.26, 64.62, 34.65, 32.16, 29.94, 29.77, 29.71, 29.60, 29.50, 29.39, 28.87, 26.17, 25.26, 22.93, 14.35 [Appendix 33].

The IR data gives peaks at 3313.71 and 3286.70 (OH stretch); 2929.87 and 2848.86 (aliphatic C-H); 2659.84 and 2335.80 due to C≡C; 1465.90 (CH_2 bend); 1373.32 (CH_3 bend) and 1062.78 due to C-O stretch. (Appendices 30 a-b).

The DEPT spectrum (Appendix 34) shows 3 methyl carbons one at δ 14.35 and two at δ 29.94 ppm. It also shows one methine carbon at δ 29.94 ppm. The 3 methyl groups and methine carbon suggests a branched molecule. Further, the DEPT spectrum (Appendix 34) showed methylene carbons at δ 22.93, 25.26, 26.17, 28.87, 29.39, 29.50, 29.60, 29.71, 29.77, 29.94 (methylene envelope), 32.16 and 34.65 ppm. At δ 29.94, the methylene envelope suggests a long chain of (CH_2) groups which are magnetically equivalent hence resonate at one δ value even in literature (Gunstone, 1993). The 13 C-NMR shows 2 other carbons at δ 174.26 and 64.62 ppm. This NMR data is characteristic of a long chain alkyl molecule with a carbonyl carbon which resonates at 174.26 ppm. However still with the spectroscopic data for compound 9, its structure could not be elucidated.

4.1.7.2. Compound 9

Compound **9** was obtained as white amorphous precipitate in dichloromethane. This compound could not be visualised under UV or in iodine. It made a translucent oily spot on TLC plate suggesting it was a fixed oil. It was colourless when TLC plate was sprayed with vanillin and heated to 105° C showing it was not steroidal in structure. Compound **9** gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 2922.16, 2850.79, 2646.34, 2333.87, 1735.93, 1465.90, 1170.79, 725.23 (Appendices 35).

MS m/z (rel. int. %): 420.41 (M^+, 3), 392.37 (14), 365.35 (12), 364.35 (43), 336.32 (10), 181.16 (9), 167.15 (12), 153..14 (15), 139.12 (22), 125.11 (35), 112.08 (15), 111.10 (57), 110.09 (12), 98.09 (20), 97.08 (94), 96.08 (29), 96.08 (29), 85.09 (39), 84.08 (26), 83.07 (100), 82.07 (51), 71.07 (56), 70.06 (32), 69.06 (33), 67.20 (12) [Appendices 36 a-b].

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.99 (m, 3H), 1.36 (m, 58H), 1.67 (m , 5H), 3.75 (m, 2H) (Appendices 37 a-b)

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 77.43, 63.33, 33.04, 32.15, 29.92, 29.66, 29.59, 25.96, 22.92, 14.35 (Appendices 38)

The spectroscopic data gives an indication of a long chain hydrocarbon. The ^{13} C-NMR shows a the methylene envelope at δ 29.92 suggesting a long chain of $-CH_2-$ groups which are magnetically equivalent hence resonate at one δ value. The MS shows fragmentation with loss of 14 atomic mass units (a.m.u.) consistent with a chain of $-CH_2-$ groups. Further work is needed to fully elucidate the structure for compound **9**.

4.2. Compounds from *Hydnora abyssinica*

4.2.1. Compound 10

Compound **10** was obtained as a white amorphous powder in chloroform. This compound could not be visualised under UV, iodine or with vanillin spray reagent. Compound **10** gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 2763.99, 2318.44, 2200.78, 2046.47, 1880.60, 1693.50, 1587.42 (Appendices 39 a).

MS m/z (rel. int.): 411.65 (M^+, 2), 367.70 (25), 340.85(13), 339.84 (48), 311.77 (27), 283.87 (47), 255.82 (29), 128.86 (53), 94.89 (30), 84.92 (40), 83.91 (27), 82.91 (60), 72.87 (92), 70.92 (57), 59.88 (65), 54.91 (100), 43.03 (98) (Appendices 39 a-b)

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.88 (t, 3H), 1.25 (m, 37 H), 1.62 (t, 3H), 2.34 (t, 2H), 4.15 (m, 2H) [Appendix 40].

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 174.18, 68.62, 65.26, 34.34, 32.16, 29.94, 29.69, 29.60, 29.49, 29.35, 25.12, 22.92, 14.35 [Appendices 41].

The spectroscopic data suggests a fatty acid. The DEPT spectrum [Appendix 42] shows at least 3 methyl carbons one at δ 14.35 and two at δ 29.94 ppm. It also shows one methine carbon at δ 68.62 ppm. The methyl groups and methine carbon suggests a branched molecule. The carbon at δ 174.17 suggests a carboxylic acid group which resonates between 165 to 185 ppm. Further, the DEPT spectrum shows methylene carbons are at δ 22.92, 25.12, 29.39, 29.35, 29.49, 29.60, 29.70, 29.94 (methylene envelope), 32.16 and 34.34 ppm. At δ 29.94, the methylene envelope suggests a long chain of -CH_2- groups which are magnetically equivalent hence resonate at one δ value. This ^{13}C-NMR data is characteristic of a fatty acid which the carbonyl carbon resonates at 174.18 ppm and the hydroxyl carbons resonating at 65.26 and 68.62 ppm. However the of structure of compound **10** could not be elucidated and further work is needed to help identify this compound.

4.2.2: Compound 11

The compound **11** was obtained as white crystalline powder from ethyl acetate and could not be visualised with UV, iodine or vanillin. Further, the compound gave the following spectroscopic data.

IR ν max (KBr) cm^{-1}: 2920.23, 2850.79, 2663.69, 2561.47, 1705.07 (C=O) (Appendix 43)

^{1}H-NMR (200 MHz, $CDCl_3$) δ: 0.88 (t, 2H), 1.25 (m, 36 H), 1.63 (t, 2H), 2.35 (t, 2H) (Appendix 44)

^{13}C-NMR (50 MHz, $CDCl_3$) δ: 179.57, 77.44, 34.15, 32.16, 29.93 (methylene envelope), 29.67, 29.60, 29.47, 29.29, 24.91, 22.93, 14.35 (Appendix 45).

The spectroscopic data suggests a fatty acid derivative. The DEPT spectrum [Appendix 46] shows methyl and methylene carbons but no methine carbon. The 3 methyl groups suggest a branched molecule. The methylene envelope suggests a long chain of -CH_2- groups which are magnetically equivalent hence resonate at one δ value. The 13 C-NMR also shows 1 carbon at δ 179.57 ppm a characteristic of a carbonyl group. This NMR data suggests a fatty acid in which the carbonyl carbon resonates at 179.57 ppm. Further work is needed to fully elucidate the structure for compound **11**.

CHAPTER FIVE

ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY

Microbial screening is done *in vitro* using fungi and bacteria to find out if a plant has antimicrobial compounds. This is because bacteria and fungi are omnipresent in nature and may grow anywhere where there is water, suitable temperature and nutrition source.

5.1. Antibacterial activity

Dombeya torrida and *Hydnora abyssinica* extracts were screened for antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epididermis, Escherichia coli* and *Bacillus pumilus*. Clear inhibitions zones were seen with all extracts although they were less than in the positive control but bigger than those caused by distilled water (blank). The inhibition zones are as shown in table 5.1 below and are also included in appendices 47-50.

Dombeya torrida bark decoction had the highest activity against *S. aureus* with an inhibition zone diameter of 16.91 mm followed by *D. torrida* bark macerate with 16.73 mm. The *D. torrida* leave macerate and decoctions had markedly less activity compared to the bark with zone diameters of 9.81 mm and 9.15 mm, respectively. This means that the constituents with anti-staphylococcus may be concentrated in the bark more than the leaves. *Hydnora abyssinica* macerate had least activity against *S. aureus* with a zone diameter of 8.86 mm.

Dombeya torrida bark decoction had the highest activity against *S. epididermis* with a diameter of 17.05 mm followed by *D. torrida* bark macerate with a zone diameter of 16.29 mm. *Hydnora abyssinica* macerate had least activity against *S. epididermis* with a zone diameter of 8.66 mm.

Table 5.1. Antibacterial activity (zones of inhibition) of various *Dombeya torrida* and *Hydnora abyssinica* extracts

	Zones of inhibition (mm)			
Sample	*Staphylococcus aureus*	*Staphylococcus epididermis*	*Escherichia coli*	*Bacillus pumilus*
Dombeya torrida bark macerate	16.73	16.29	15.82	12.31
Dombeya torrida bark decoction	16.91	17.05	16.56	12.71
Dombeya torrida leaf decoction	9.15	8.92	8.62	9.08
Dombeya torrida leave macerate	9.81	9.60	9.20	9.54
Dombeya torrida bark methanol	16.00	15.69	15.82	13.66
Dombeya torrida $CHCl_3$*	12.07	11.30	11.89	12.07
Hydnora abyssinica decoction	10.48	12.52	12.91	8.58
Hydnora abyssinica macerate	8.86	8.66	9.28	8.51
Hydnora abyssinica $CHCl_3$*	15.83	15.75	15.84	17.04
Hydnora abyssinica methanol	11.87	11.37	12.21	11.66
Water blank	7.84	7.87	7.30	7.92
DMSO blank	8.02	8.02	8.02	8.02
Gentamicin sulphate (0.32mg/ml)	24.20	22.70	23.73	23.66

* Chloroform extracts were solubilized using DMSO

Activity against *E. coli* was highest for *D. torrida* bark decoction with zone diameter of 16.56 mm followed by *H. abyssinica* chloroform extract at 15.84 mm. *Dombeya torrida* bark macerate and methanol extracts were equipotent with equal zone diameters of 15.82 mm. *Dombeya torrida* leaf decoction had least activity at 9.20 mm zone diameter. *Hydnora abyssinica* chloroform extract had the highest activity against *B. pumilus* with a zone diameter of 17.04 mm followed by

D. torrida bark methanol extract 13.66 mm. *Hydnora abyssinica* macerate had least activity against *B. pumilus* at 8.51 mm.

5.2. Antifungal activity

Dombeya torrida and *H. abyssinica* extracts were screened for antifungal activity using *S. cerevisiae* and *C. albicans*. None of the negative controls exhibited antifungal activity while the positive control (nystatin) showed inhibition against both fungi showing that the nutrient media conditions supported fungal growth and inhibition in test extracts was due to the presence of inhibitory constituents in the plant extracts. The inhibition zones are as shown in table 5.2 and are also included in appendix 51.

Table 5.2. Antifungal activity (zones of inhibition) of various *Dombeya torrida* and *Hydnora abyssinica* extracts

	Zones of inhibition (mm)	
Sample	*Saccromyces cerevisiae*	*Candida albicans*
Dombeya torrida bark macerate	7.70	10.55
Dombeya torrida bark decoction	7.70	11.31
Dombeya torrida bark methanol	16.75	11.04
Dombeya torrida $CHCl_3$*	17.69	20.09
Hydnora abyssinica decoction	11.13	11.71
Hydnora abyssinica macerate	7.70	-
Hydnora abyssinica $CHCl_3$*	17.38	-
Hydnora abyssinica methanol	15.88	9.41
Water blank	7.70	7.68
DMSO blank	7.70	7.70
Nystatin (0.3 mg/ml)	15.95	18.15

* Chloroform extracts were solubilized using DMSO

(-) indicate zones for *C. albicans* were not very clear

The highest activity observed against *S. cerevisiae* was with *D. torrida* chloroform extract with a zone diameter of 17.69 mm followed by *H. abyssinica* chloroform extract 17.38 mm with *H. abyssinica* macerate having the least activity. The activity of the chloroform extracts against *S. cerevisiae* was higher than for standard. *Dombeya torrida* chloroform extract was the most active extract against *Candida albicans* with a zone diameter of 20.09 mm. This is in agreement with *in vitro* bioassay of *H. abyssinica* aqueous, methanol and chloroform extracts by Saadabi and Ayoub (2009) which showed ability to inhibit growth of 6 human pathogenic fungi: *Epidermophyton floccosum, Microsporum audouinii, Trichophyton rubrum*, *Trichophyton concentricum*, *Trichophyton tonsurans* and *C. albicans*.

5.3. Autobioassay

Bioassay refers to the determination of the strength or biological activity of a substance, such as a drug, by comparing its effects with those of a standard preparation on a test organism. Bioassays may be qualitative or quantitative and are essential in the development of new drugs. In autobioassay, a crude extract is first separated into its components by thin layer chromatography (TLC). Thereafter a culture media inoculated with a microorganism is poured onto the developed TLC plate after which it is incubated and the plate examined for microbial growth. Around the spots with antimicrobial compounds growth is inhibited, while growth progresses unhindered on the other parts of the TLC plate.

The autobioassay was carried out using *B. pumilus, C. albicans* and *S. aureus.* The extracts showed variable activity. Table 5.3 below give a summary of the results giving the number of active spots and their R_f values. Some extracts had a band of inhibition represented as a R_f range.

Table 5.3. Results for autobioassay of *Dombeya torrida* and *Hydnora abyssinica* extracts

Microorganism	*Bacillus pumilus*		*Staphylococcus aureus*		*Candida albicans*	
Extract	Active spots	R_f values of spots	Active spots	R_f values of spots	Active spots	R_f values of spots
Dombeya torrida bark methanol	1	0	1	0	1	0
Dombeya torrida bark chloroform	2	0.3, 0.7	2	0.4, 0.6	band	0.7 to 0.9
Dombeya torrida leaves dichloromethane: methanol	1	0	2	0, 0.3	1	0
Hydnora abyssinica methanol	1	0	1	0	1	0 to 0.2
Hydnora abyssinica chloroform	2	0.1, 0.6	3	0, 0.2, 0.8	band	0.7 to 0.9
Hydnora abyssinica dichloromethane: methanol	5	0, 0.1, 0.2, 0.3, 0.8	3	0, 0.1, 0.3	band	0 to 0.3
Erythromycin (Standard)	a	a	a	a	-	-
Nystatin (standard)	-	-	-		b	b

a: Clear inhibition zones 3 cm in diameter. b: Clear inhibition zones 1.2 cm in diameter

The *D. torrida* bark methanol and leaves dichloromethane/methanol extracts together with *H. abyssinica* methanol extract showed only one spot of inhibition against the 3 microorganisms at the extract application point on the TLC plate. The *Hydnora abyssinica* dichloromethane/methanol extract had most activity against *B. pumillus* with 5 spots of inhibition. This extract had comparable effects to the ones of *H. abyssinica* chloroform against *S. aureus*. The *D. torrida* chloroform extract and *H. abyssinica* chloroform and dichloromethane/methanol extracts had a marked inhibition effects against *C. albicans* with big bands showing presence of antifungal constituents in these extracts.

These results show *H. abyssinica* extracts had appreciable antifungal and antibacterial activities. These results supports the folklore that *H. abyssinica* is use as a cure for throat complaints, as an astringent in dysentery, treatment of diarrhoea, amoebic dysentery and to treat typhoid, anthrax, cancer and East Coast Fever [Kokwaro, 2009; Musila, *et al*., 2004; Mwangi *et al*., 2001]. This is also in agreements with *in vitro* bioassay of *H. abyssinica* aqueous, methanol and chloroform extracts by Saadabi and Ayoub (2009) which showed ability to inhibit growth of 6 human pathogenic fungi and 4 bacteria. *Dombeya torrida* extracts also had appreciable antifungal and antibacterial activities supporting its folklore use to treat indigestion, chest pains and colds [Gachathi, 1989; Kokwaro, 2009].

CHAPTER SIX

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

6.1. Discussion and conclusion

Since the dawn of civilization, natural products have had an essential role in the treatment of human health and disease [Newman *et al.*, 2000]. They have been the foundation for the discovery and development of therapeutics used to treat many diseases. Their chemical diversity and complexity have provided structural scaffolds for small-molecule drugs and have consistently served as inspiration for medicinal design [Chang and Keasling, 2006]. Many drugs still used in modern medicine are derived from herbal remedies [Koehn & Carter, 2005]. It is reported by Newman *et al*., (2003) that 61% of the 877 small-molecule drugs introduced in the last 20 years can trace their origin to natural products, with 38 % comprising natural products and their close derivatives. The extraction of such molecules is often low-yielding and inefficient, and requires substantial expenditure of resources. For example, it would take approximately six 100-year-old Pacific yew trees to provide enough taxol (paclitaxel) to treat one cancer patient [Horwitz, 1994]. Thus more work requires to be done to isolate molecules from plants, identify them and demonstrate their activities. Further work can lead to derivatization and thus afford same molecules at considerable cheaper costs and also make phytochemicals available to bigger populations for management of various medical conditions.

The plants under study, *D. torrida* and *H. abyssinica,* were chosen on the basis of their folklore uses. The study provides knowledge regarding the phytochemistry of *D. torrida* and *H. abyssinica*. The work describes extraction procedures and outlines clear ways in which compounds can be isolated from these plants. Both isocratic and gradient fractionation procedures using open column chromatography are described as well as way to purify isolated compounds.

The plants were worked on with the view of isolating pure compounds, identifying them and evaluating them for antimicrobial activity.

Fifteen compounds were isolated from *D. torrida.* Five of these were identified as friedelin, friedelan-3β-ol, β-sitosterol, stigmasterol and taraxerol (Figure 6.1) by use of infra-red, mass spectrometry and NMR and also by comparison with spectroscopic data of compounds in literature. This is the first time these compounds were isolated from *D. torrida*. Five compounds were also isolated from *H. abyssinica* but could not be identified. Further spectroscopic work like X-ray diffraction crystallography is needed to identify these other compounds.

Dombeya torrida and *H. abyssinica* extracts were found to have appreciable antimicrobial activity against *S. aureus, S. epididermis, E. coli, B. pumilus, S. cerevisiae* and *C. albicans*. These results support their folklore uses. The *D. torrida* decoction of the bark is taken by the Maasai to treat indigestion after a large meal of meat [Kokwaro, 2009] with its roots being used for treatment of chest pains and colds [Gachathi, 1989]. These conditions are linked to bacterial and fungal infections as both bacteria and fungi lead to respiratory tract infections. Fungal infections with *Aspergillus Histoplasmosis* and *Coccidiomycosis* affect the lungs causing chest pain and chills. Bacteria include *S. aureus*, and *Streptococcus pneumonia*. Stomach infections can also lead to indigestion. Thus the antimicrobial effects of *D. torrida* can give relieve in these conditions.

Hydnora abyssinica is in folklore used for treatment of diarrhoea, amoebic dysentery, typhoid, anthrax, cancer and East Coast Fever [Mwangi *et al*., 2001; Musila, *et al*., 2004]. There are many causes of diarrhea, which include viruses, bacteria and parasites. Amoebic dysentery is caused by the *Entamoeba histolytica*, typhoid is caused by *Salmonella typhi* while anthrax is caused by

Bacillus anthracis in cattle. East Coast fever (theileriosis) is a disease of cattle, sheep and goats caused by the protozoan parasite *Theileria parva*. The antimicrobial activity demonstrated with the plants under study gives an indication of probable ability of *D. torrida* and *H. abyssinica* to manage the conditions claimed.

6.2. Recommendations

Interest in natural-products research has resurged with the growing realization that the structural complexity and diversity that is key to the biological activity of natural products is not easily reproduced in *de novo* synthetic libraries [Koehn & Carter, 2005].

It is recognized that polar components may have a significant contribution towards the antimicrobial activity of these plants. But there are difficulties inherent in screening, isolating and identifying low-abundance secondary metabolites [Koehn & Carter, 2005]. Actually isolation of natural products from their natural sources is typically low yielding [Chang and Keasling, 2006]. Considering that traditionally it is the decoction and the macerates that are used medicinally, the polar fractions should be investigated further using resins and reversed phase silica for column chromatography which can be used to fractionate the polar extracts and get polar compounds.

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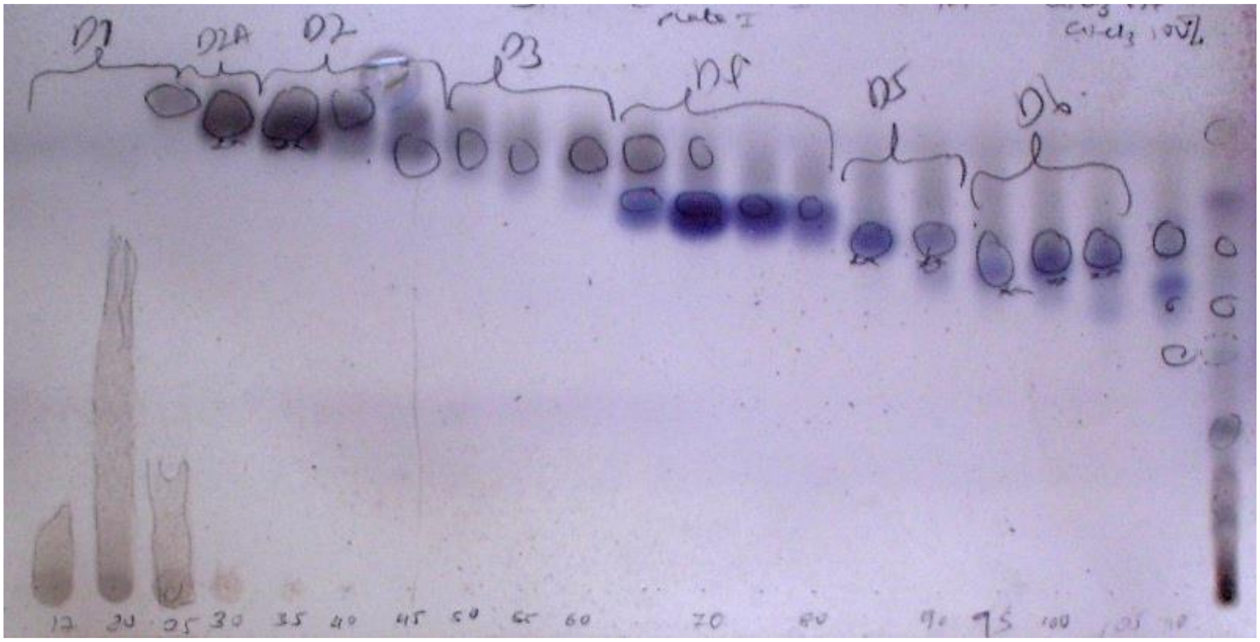
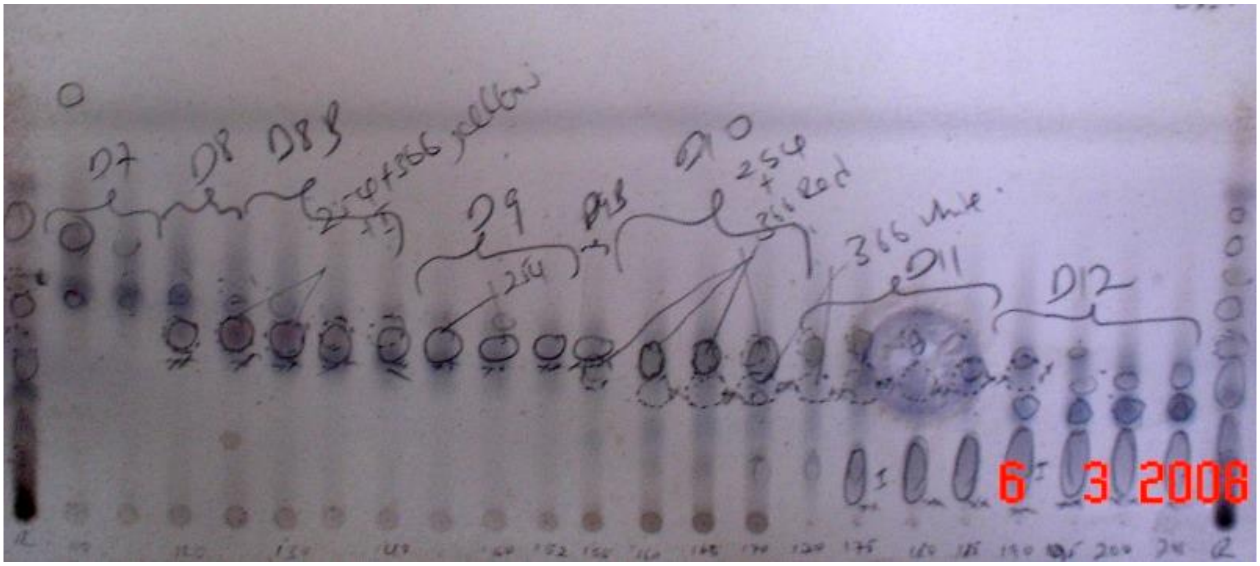
APPENDICES

Appendix 1 (a): Codes of different extracts used for microbiological screening

Extract code	Extract	Weights taken (mg)
1	*Dombeya torrida* bark macerate	103.2
2	*Dombeya torrida* decoction	100.2
3	*Hydnora abyssinica* decoction	104.2
4	*Hydnora abyssinica* macerate	105.4
5	*Dombeya torrida* leaf decoction	108.4
6	*Dombeya torrida* leaves macerate	104.5
7	*Hydnora abyssinica* $CHCl_3$	108.7
8	*Dombeya torrida* bark methanol	107.8
9	*Hydnora abyssinica* methanol	105.3
10	*Dombeya torrida* $CHCl_3$	107.6

Appendix 1 (b): The TLC profile of fractions 1-200 from *Dombeya torrida* bark chloroform extract

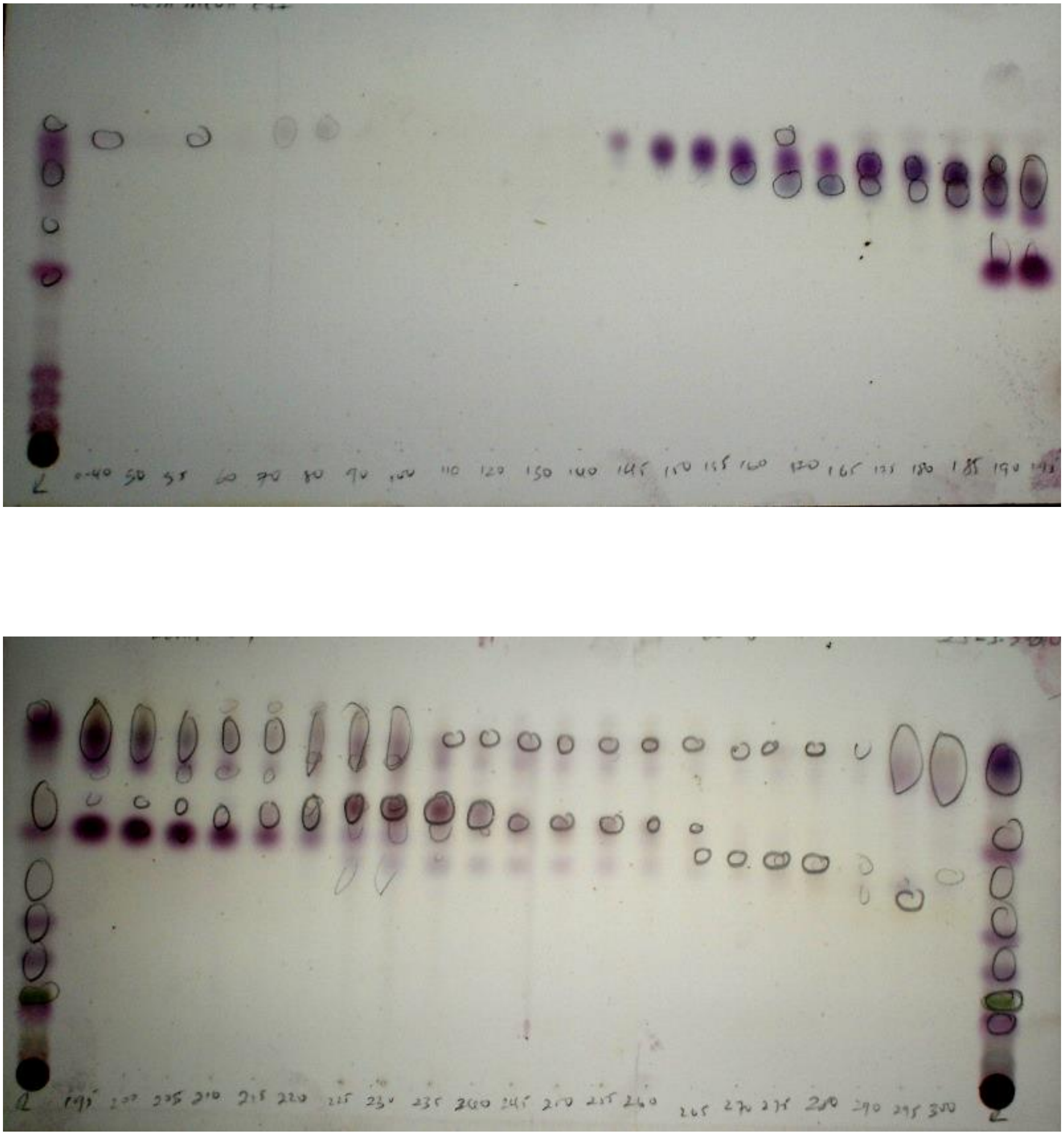




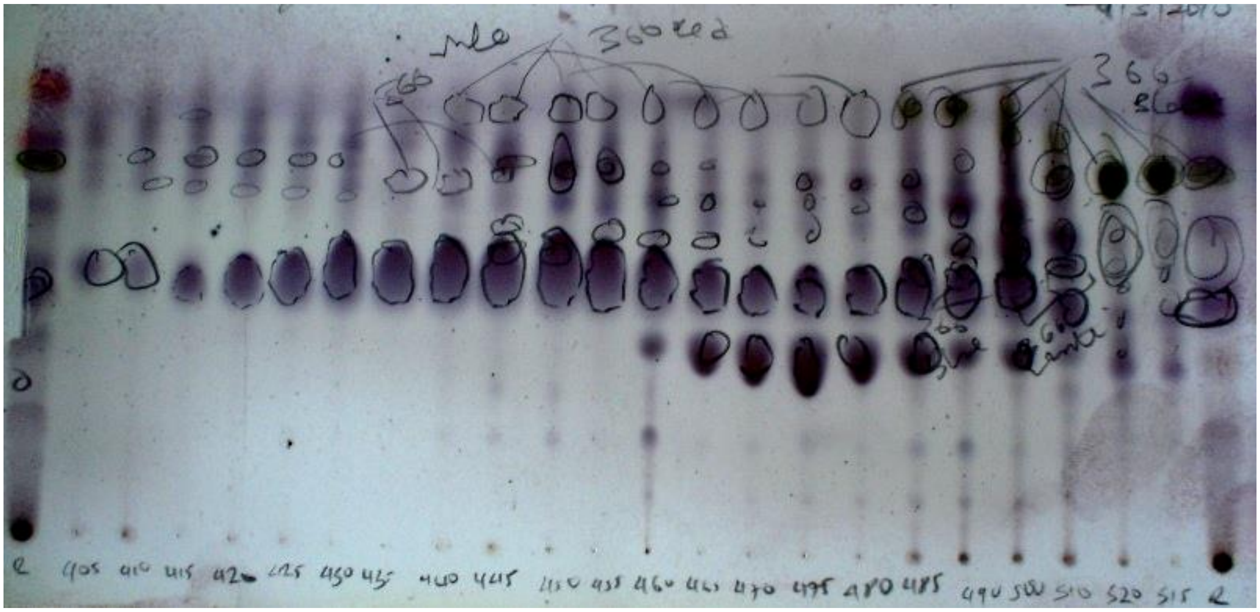
Appendix 1 (c): Isolated compounds and their codes

Compound in Roman numeral	Compound in Arabic numeral	Name
i	**1**	**Friedelin**
ii	**-**	-
iii	**2**	**Friedelan-3β-ol**
iv	**-**	**-**
v	**3**	**B-sitosterol**
	4	**Stigmasterol**
vi	**-**	-
vii	**-**	-
viii	**-**	-
ix	**8**	A long chain alkyl molecule
x	**6**	Labd-14-en-8,13-diol analogue
xi	**7**	Amyrin analogue
xii	**9**	A long chain hydrocarbon
xiii	**5**	**Taraxerol**
xiv	**-**	-
xv	**-**	-
xvi	**10**	A fatty acid
xvii	**11**	A fatty acid derivative
xviii	**-**	-
xix	**-**	-
xx	**-**	-
xxi	**-**	-

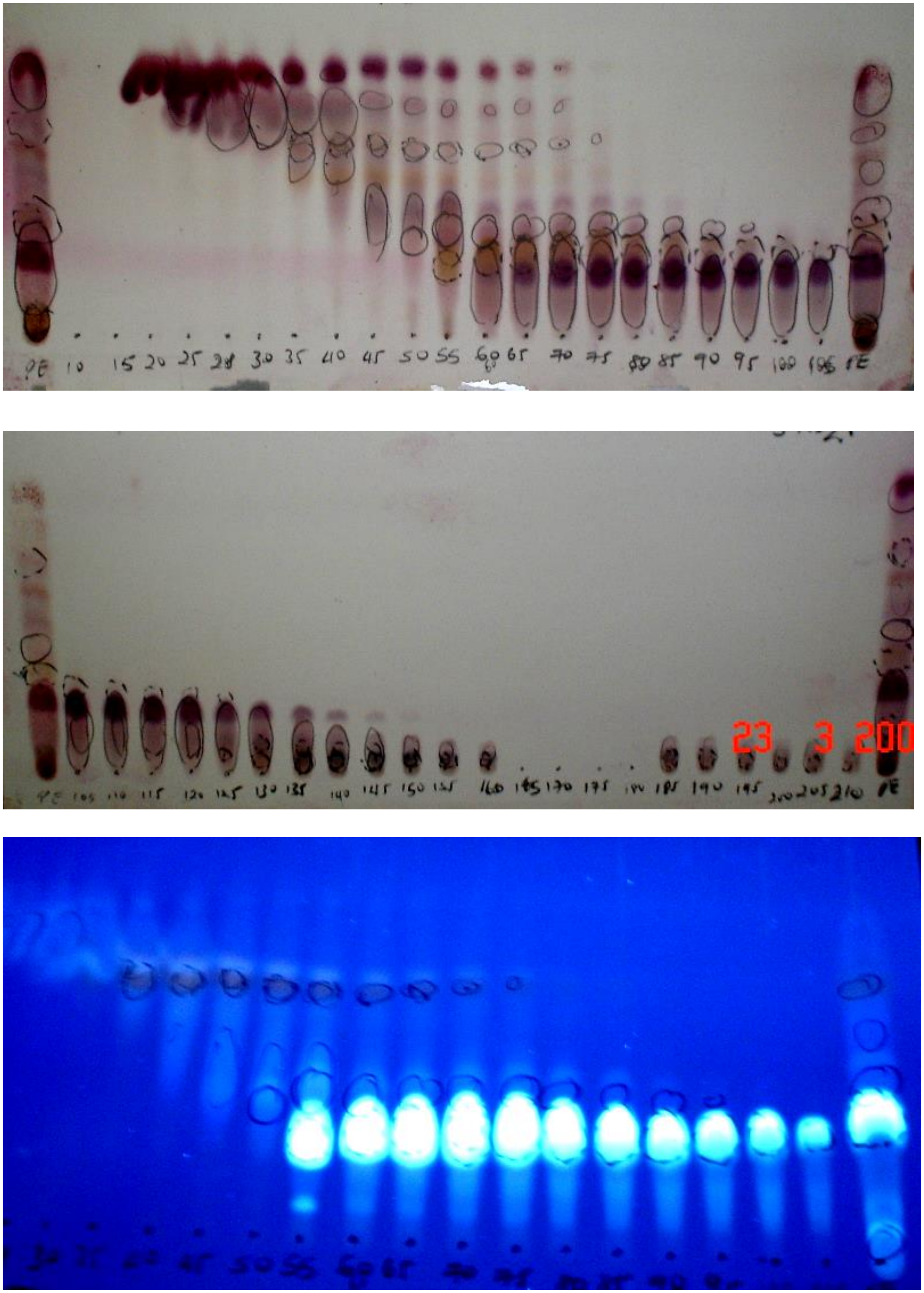
Appendix 1 (d): The TLC profile of fractions 1-300 from *Dombeya torrida* leaves dichloromethane:methanol extract



Appendix 1 (e): The TLC profile of fractions 260-525 from *Dombeya torrida* leaves dichloromethane:methanol extract



Appendix 1 (f): The TLC profile of some fractions from *Hydnora abyssinica* petroleum ether extract



Appendix 2: The infra-red spectrum of compound 1

Appendix 3 (a): The mass spectrum of compound 1

Appendix 3 (b): The mass spectrum data of compound 1

Appendix 3 (b): The mass spectrum data of compound 1

Appendix 4: The ^{1}H-NMR spectrum of compound 1

Appendix 5 (a): The ^{13}C-NMR spectrum of compound 1

Appendix 5 (b): The ^{13}C-NMR spectrum of compound 1

Appendix 6 (a): The DEPT spectrum of compound 1

Appendix 6 (b): The DEPT spectrum of compound 1

Appendix 7: The infra-red spectrum of compound 2

Appendix 8 (a): The mass spectrum of compound 2

Appendix 8 (b): The mass spectrum data of compound 2

Appendix 8 (b): The mass spectrum data of compound 2

Appendix 9 (a): The ^{1}H-NMR of compound 2

Appendix 9 (b): The ^{1}H-NMR of compound 2

Appendix 10 (a): The ^{13}C-NMR of compound 2

Appendix 10 (b): The ^{13}C-NMR of compound 2

Appendix 11: The infra-red spectrum of compounds 3 & 4

Appendix 12 (a): The mass spectrum of compounds 3 & 4

Appendix 12 (b): The mass spectrum data of compounds 3 & 4

Appendix 12 (b): The mass spectrum data of compounds 3 & 4

Appendix 13 (a): The ^{1}H-NMR of compounds 3 & 4

Appendix 13 (b): The ^{1}H-NMR of compounds 3 & 4

Appendix 13 (c): The ^{1}H-NMR compounds 3 & 4

Appendix 14 (a): The ^{13}C-NMR of compounds 3 & 4

Appendix 14 (b): The ^{13}C-NMR of compounds 3 & 4

Appendix 15 (a): The DEPT spectrum of compounds 3 & 4

Appendix 15 (b): The DEPT spectrum of compounds 3 & 4

Appendix 16: The infra-red spectrum of compound 5

Appendix 17 (a): The mass spectrum of compound 5

Appendix 17 (b): The mass spectrum data of compound 5

Appendix 18 (a): The ^{1}H-NMR of compound 5

Appendix 18 (b): The ^{1}H-NMR of compound 5

Appendix 19 (a): The ^{13}C-NMR of compound 5

Appendix 19 (b): The ^{13}C-NMR of compound 5

Appendix 20 (a): The DEPT spectrum of compound 5

Appendix 20 (b): The DEPT spectrum of compound 5

Appendix 21: The mass spectrum of compound 6

Appendix 22: The mass spectrum data of compound 6

Appendix 23 (a): The ^{1}H-NMR spectrum of compound 6

Appendix 23 (b): The ^{1}H-NMR spectrum of compound 6

Appendix 23 (c): The ^{1}H-NMR spectrum of compound 6

Appendix 24 (a): The ^{13}C-NMR spectrum of compound 6

Appendix 24 (b): The ^{13}C-NMR spectrum of compound 6

Appendix 24 (c): The ^{13}C-NMR spectrum of compound 6

Appendix 25 (a): The DEPT spectrum of compound 6

Appendix 25 (b): The DEPT spectrum of compound 6

Appendix 26: The infra-red spectrum of compound 7

Appendix 27 (a): The ^{1}H-NMR of compound 7

Appendix 27 (b): The ^{1}H-NMR of compound 7

Appendix 27 (c): The ^{1}H-NMR of compound 7

Appendix 28 (a): The ^{13}C-NMR of compound 7

Appendix 28 (b): The ^{13}C-NMR of compound 7

Appendix 29 (a): The DEPT spectrum of compound 7

Appendix 29 (b): The DEPT spectrum of compound 7

Appendix 30: The infra-red spectrum of compound 8

Appendix 31(a): The mass spectrum of compound 8

Appendix 31 (b): The mass spectrum data of compound 8

Appendix 32 (a): The ^{1}H-NMR spectrum of compound 8

Appendix 32 (b): The ^{1}H-NMR spectrum of compound 8

Appendix 33 (a): The ^{13}C-NMR spectrum of compound 8

Appendix 33 (b): The ^{13}C-NMR spectrum of compound 8

Appendix 34: The DEPT spectrum of compound 8

Appendix 35: The infra-red spectrum of compound 9

Appendix 36 (a): The mass spectrum of compound 9

Appendix 36 (b): The mass spectrum data of compound 9

Appendix 37 (a): The ^{1}H-NMR of compound 9

Appendix 37 (b): The ^{1}H-NMR of compound 9

Appendix 38 (a): The ^{13}C-NMR of compound 9

Appendix 38 (b): The ^{13}C-NMR of compound 9

Appendix 39 (a): The mass spectrum of compound 10

Appendix 39 (b): The mass spectrum data of compound 10

Appendix 39 (b): The mass spectrum data of compound 10

Appendix 40: The ^{1}H-NMR of compound 10

Appendix 41 (a): The ^{13}C-NMR of compound 10

Appendix 41 (b): The ^{13}C-NMR of compound 10

Appendix 42: The DEPT spectrum of compound 10

Appendix 43: The infra-red spectrum of compound 11

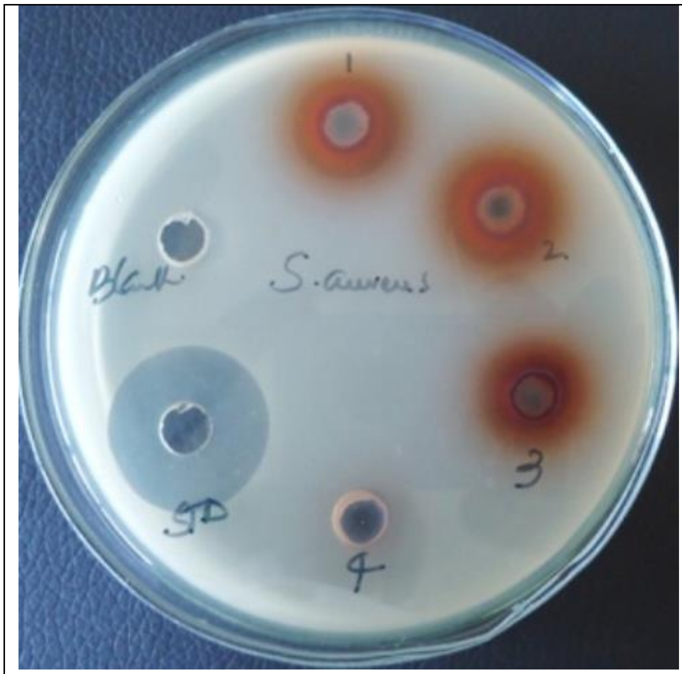
Appendix44: The ^{1}H-NMR of compound 11

Appendix 45 (a): The ^{13}C-NMR of compound 11

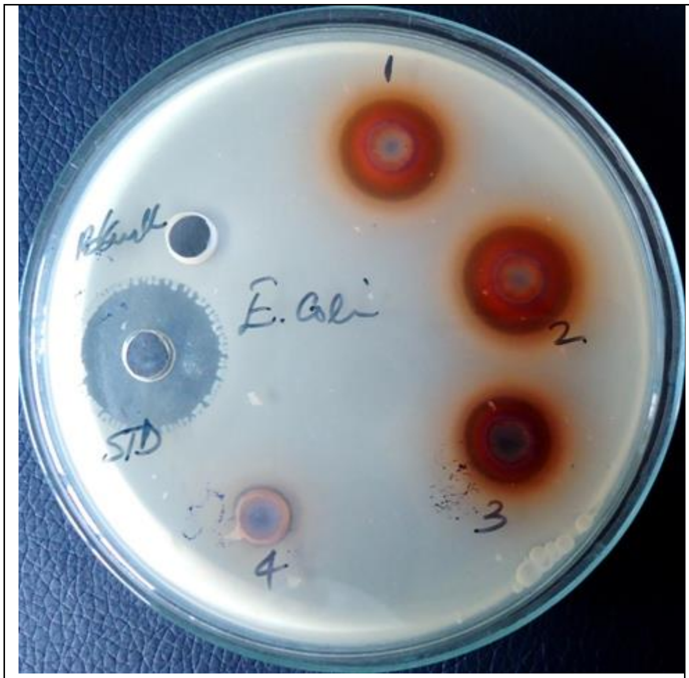
Appendix 45 (b): The ^{13}C-NMR of compound 11

Appendix 46: The DEPT spectrum of compound 11

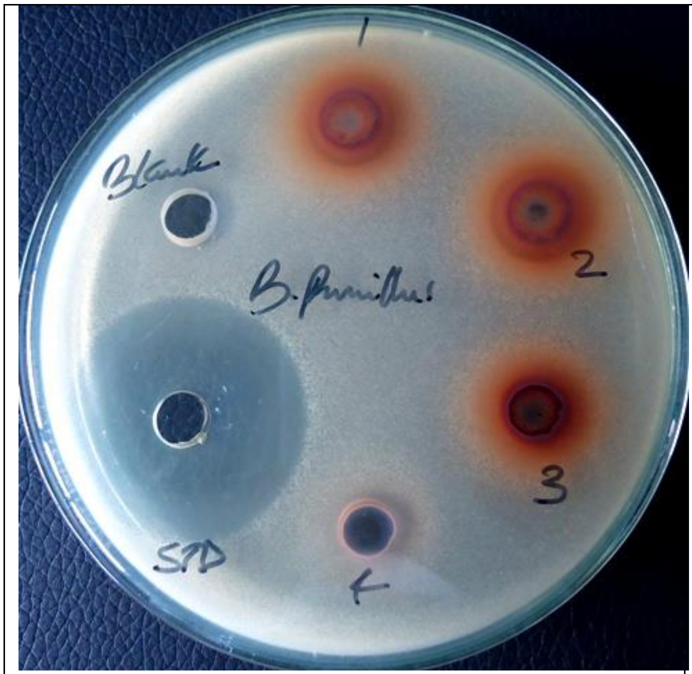
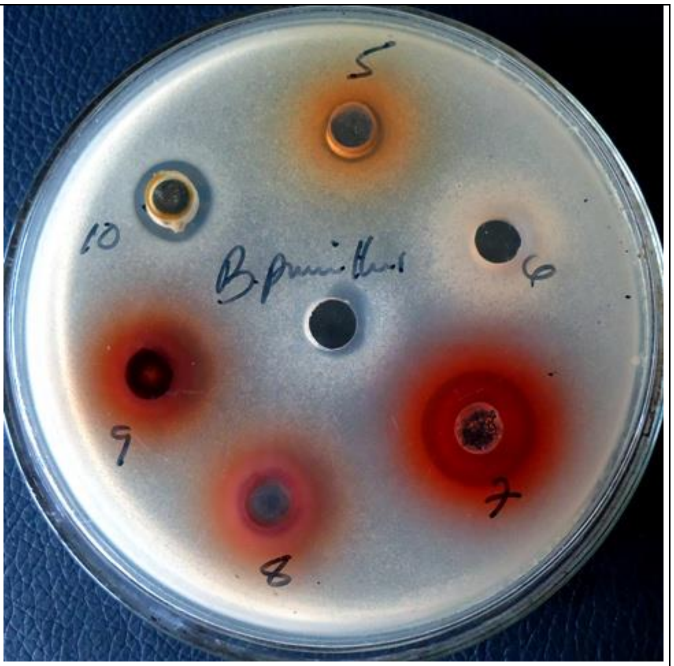
Appendix 47: Inhibition zones of *Staphylococcus aureus*

	
Extract code	**Extract**
1	*Dombeya torrida* bark macerate
2	*Dombeya torrida* decoction
3	*Hydnora abyssinica* decoction
4	*Hydnora abyssinica* macerate
5	*Dombeya torrida* leaf decoction
6	*Dombeya torrida* leave macerate
7	*Hydnora abyssinica* $CHCl_3$
8	*Dombeya torrida* bark methanol
9	*Hydnora abyssinica* methanol
10	*Dombeya torrida* $CHCl_3$

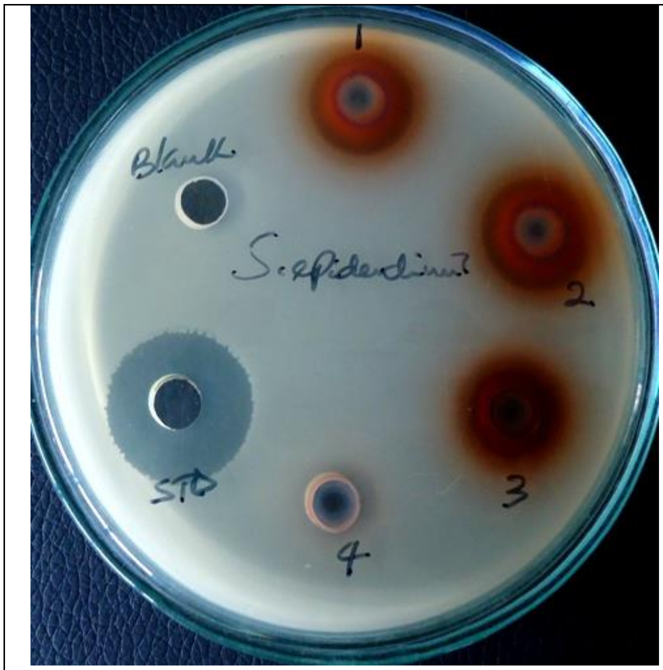
Appendix 48: Inhibition zones of *Escherichia coli*

	
Extract code	**Extract**
1	*Dombeya torrida* bark macerate
2	*Dombeya torrida* decoction
3	*Hydnora abyssinica* decoction
4	*Hydnora abyssinica* macerate
5	*Dombeya torrida* leaf decoction
6	*Dombeya torrida* leave macerate
7	*Hydnora abyssinica* $CHCl_3$
8	*Dombeya torrida* bark methanol
9	*Hydnora abyssinica* methanol
10	*Dombeya torrida* $CHCl_3$

Appendix 49: Inhibition zones of *Bacillus pumilus*

	
Extract code	**Extract**
1	*Dombeya torrida* bark macerate
2	*Dombeya torrida* decoction
3	*Hydnora abyssinica* decoction
4	*Hydnora abyssinica* macerate
5	*Dombeya torrida* leaf decoction
6	*Dombeya torrida* leave macerate
7	*Hydnora abyssinica* $CHCl_3$
8	*Dombeya torrida* bark methanol
9	*Hydnora abyssinica* methanol
10	*Dombeya torrida* $CHCl_3$

Appendix 50: Inhibition zones of *Staphylococcus epididermis*

	
Extract code	**Extract**
1	*Dombeya torrida* bark macerate
2	*Dombeya torrida* decoction
3	*Hydnora abyssinica* decoction
4	*Hydnora abyssinica* macerate
5	*Dombeya torrida* leaf decoction
6	*Dombeya torrida* leave macerate
7	*Hydnora abyssinica* $CHCl_3$
8	*Dombeya torrida* bark methanol
9	*Hydnora abyssinica* methanol
10	*Dombeya torrida* $CHCl_3$

Appendix 51: Inhibition zones of *Saccromyces cerevisiae*

	
Extract code	**Extract**
1	*Dombeya torrida* bark macerate
2	*Dombeya torrida* decoction
3	*Hydnora abyssinica* decoction
4	*Hydnora abyssinica* macerate
5	*Dombeya torrida* leaf decoction
6	*Dombeya torrida* leave macerate
7	*Hydnora abyssinica* $CHCl_3$
8	*Dombeya torrida* bark methanol
9	*Hydnora abyssinica* methanol
10	*Dombeya torrida* $CHCl_3$