ISOLATION AND CHARACTERIZATION OF ANTIMICROBIAL COMPOUNDS FROM THE PLANTS, *ERYTHRINA ABYSSINICA* DC. AND *CHASMANTHERA DEPENDENS* HOCHST

IRUNGU STANLEY [B.Ed, Sc]
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September, 2012

DECLARATION

DECLARATION BY CANDIDATE

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

Irungu Stanley
Department of Chemistry

Signature ……………..……………………………Date ………………………

DECLARATION BY SUPERVISORS

This thesis has been submitted in partial fulfillment of Masters of Science degree of Kenyatta University with our approval as supervisors.

Dr. Alex K. Machocho
Department of Chemistry
Kenyatta University

Signature ………….……………………….Date ………………………………

Prof. Paul K. Tarus
Department of Chemistry and Biochemistry
Chepkoilel University College (Moi University)

Signature ………….……………………….Date ………………………………

DEDICATION

To my parents, my wife Rachael and children Sharon and Macfarland

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LIST OF ABBREVIATIONS AND ACRONYMS

μg	Microgram
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
CC	Column Chromatography
COSY	Correlation Spectroscopy
d	Doublet
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl Sulfoxide
EtOAc	Ethylacetate
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum Coherence
J	Coupling constant
KEMRI	Kenya Medical Research Institute
m	Multiplet
MBC	Minimum Bacterialcidal Concentration
MDRC	Multiple Drug Resistant Strain
MeOH	Methanol
MFC	Minimum Fungicidal Concentration
MHA	Mueller Hinton Agar
MIC	Minimum Inhibitory Concentration
NA	Nutrient Agar

NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
PDA	Potatoes Dextrose Agar
ppm	parts per million
PTLC	Preparative Thin Layer Chromatography
s	Singlet
SDA	Sabouraud Dextrose Agar
t	Triplet
TLC	Thin Layer Chromatography
UN	United Nations
UV	Ultra Violet
VLC	Vacuum Liquid Chromatography
WHO	World Health Organization
δ	Chemical shift

ABSTRACT

Infectious diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in understanding of microbiology and their pathogenic control, incidences of epidemics due to drug resistant micro-organisms and the emergence of hitherto unknown disease causing microbes, pose enormous public health concerns. Thus, there is need to continue the search for appropriate drugs for anti-microbial treatment without much drawbacks. Historically, plants have provided a good source of anti-infective agents. They are cheap and effective in the treatment of diseases while simultaneously mitigating many of the side effects that are associated with synthetic anti-microbial agents. Phytomedicines derived from plants have shown great promise in the treatment of intractable infectious diseases including opportunistic human immunodeficiency virus (HIV) / acquired immune deficiency (AIDS) infections. The plant species *Erythrina abyssinica* DC. and *Chasmanthera dependens* Hoschst have been reported to exhibit remarkable antimicrobial activities and are used as herbal remedies by traditional medical practioners. In this study, the plant extracts from the two species were screened for their antibacterial activity against selected strains of bacteria, including Gram-negative *Pseudomonas aeruginosa* and Gram-positive *Staphylococcus aureus,* antifungal activities against *Trichophyton mentagrophytes* and yeasts (*Candida albicans*). The isolation and purification of bioactive compounds was done using solvent partition and chromatography: CC, PTLC and VLC. Structure characterization was carried out using standard spectroscopic methods: ^{1}H NMR, ^{13}C NMR, DEPT, COSY, HSQC, HMBC and NOESY. 1,5,4′-trihydroxy-5′-prenylchalcone **(131)**, 2',3′,4′,7-tetrahydroxy-5'-prenylflavanone **(132)** and 2'-methoxy-nor-glycyrrisoflavanone **(134)** were isolated from *Erythrina abyssinica*. 2,3-epoxycolumbin (furano diterpenoid) **(137)** was isolated from *Chasmanthera dependens*. One compound 2'-methoxy-nor-glycyrrisoflavanone **(134)** showed high activity against the gram-positive bacteria *Staphylococcus aureus* with inhibition zone of 15 mm. The DCM crude extract of *E. abyssinica* stem barks had activity against *Staphylococcus aureus* and the fungi *Trichophyton mentagrophytes* with inhibition zones of 10 and 16 mm, respectively. The hexane extract of root tuber of *C. dependens* was highly active against *Staphylococcus aureus* and *Trichophyton mentagrophytes* with inhibition zones of 16 and 23 mm, respectively. The study demonstrated that there is need for further investigation and isolation of more pure compounds from both plants. In addition the crude extracts and the pure compounds be subjected to further bioassay against other disease causing microbes.

CHAPTER ONE

INTRODUCTION

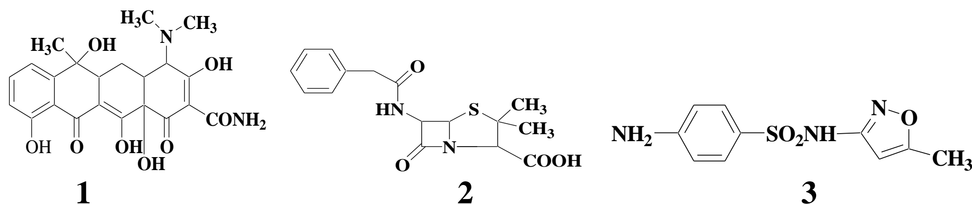
1.1 Background

Infectious diseases are number one cause of death world wide accounting approximately one-half of all deaths in tropical countries. Perhaps it is not surprising to see these statistics in developing nations, but what may be remarkable is that infectious disease mortality rates are actually increasing in developed countries such as the United States of America. Death from infectious disease in U.S.A ranked 5th in 1981 and a 3rd leading cause of death in 1992 with an increase of 58% (Pinner *et al.,* 1996). At the same time man's population is subject to natural atrocities of famine, wars, and infections. Infectious diseases in man are caused by parasitic animals or plants which are derived directly from human or more rarely animal sources. The main form of interspecies competition in which our species is a victim is caused by bacteria, viruses, fungi and protozoa generally referred to as microbes (Grist *et al*., 1987).

This is alarming given that it was once believed that man would eliminate infectious disease by the end of the millennium. The increases are attributed to increases in respiratory tract infections and HIV/AIDS. Other contributing factors are an increase in antibiotic resistance in nosocomial and community acquired infections (Pinner *et al*., 1996). These negative health trends call for a renewed interest in infectious diseases in the medical and public health communities and renewed strategies on treatment and prevention (such as vaccination), improved monitoring and the development of new treatments. It is this last solution that would encompass the development of new antimicrobials (Fauci, 1998). It is certainly feasible that man has almost eaten or chewed berries, bark, root and herbs in order

to try alleviate or cure sickness. By trial and error (in what were the first crude chemical trials) he discovered that certain plants were effective against a number of diseases. The earliest written records of Chinese, Indian, South American and Mediterranean cultures described plant preparations and their therapeutic use (Taylor, 1981). An enormous amount of information about medicinal properties of various plants and animals exist in various ethnic cultures and can be used to provide sources of new medicines (Pandey, 1998). Ethnobotany and ethnomedical studies are today recognized as the most viable methods of identifying new medicinal plants for bioactive constituents (Farnsworth, 1996).

The area of herbal medicine is receiving much and continuous attention. This is as a result of drugs in use which have brought so much complications arising from resistance as in the case of tetracycline **(1)** and penicillin G **(2)** or side effects by sulphonamide **(3)** causing headaches, mental depression and vomiting (Catlin and Reyn, 1982).



Many medicinal plants of Africa have been investigated for their chemical components and some of the isolated compounds have been shown to possess interesting biological activity. Some of these plants include *Creptolepis sanguinolenta* Lindl. (Periplocaceae) which is a shrub that grows in the rain forest and the deciduous belt forest found in the west coast of Africa. It has a medicinal use for the treatment of fever, urinary tract infections especially candida, malaria, hypertension, microbial infection, stomach aches and colic (Iwu, 1993). Active principals identified are indoquinoline alkaloids. Studies on the extracts showed

inhibition against Gram-negative bacteria and yeast (Silva *et al*., 1996). *In vitro* study showed activity against enteric pathogens most notably *Escherichia coli* and some activity against *Candida* (Sawer *et al*., 1995). It has shown histamine antagonism, hypotensive and vasodilatory activities as well as antihyperglycemic properties (Brierer *et al*., 1998).

Xylopia aethiopica Rich (Abbibacceae) is an evergreen, aromatic tree growing up with peppery fruit. It is native to the lowland rainforest in the savanna zones of Africa, in west, central and southern Africa. Medicinal uses of the plant are as carminative, a cough remedy, and a post partum tonic and lactation aid. Other uses are stomachache, bronchitis, biliousness and dysentery (Smith *et al*., 1996). It contains diterpenic and xylopic acid. The fruit extract has been shown to be active as an antimicrobial against Gram positive and negative bacteria. Xylopic acid has also demonstrated activity against *Candida albicans* (Boakye-Yiadom *et al*., 1977).

Garcinia kola Heckel (Guttiferae) is found in most forests, cultivated and distributed throughout west and central Africa. Medicinal uses include purgative, antiparasitic and antimicrobial. The seeds are used in treatment of bronchitis and throat infections, to prevent and relieve colic, cure head or chest cold, relieve cough, treatment of liver disorders and as a chewing stick. The constituents are biflavonoids, xanthones and benzophenones (Iwu, 1993).

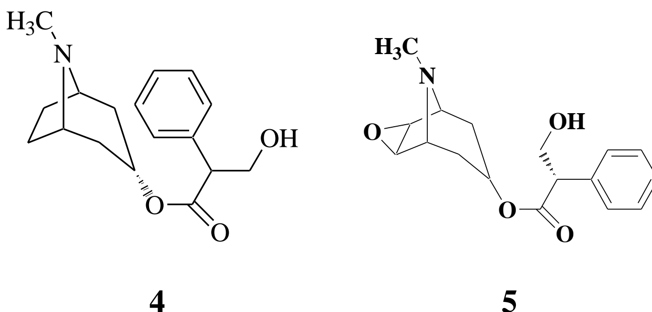
Aframomum melegueta Lemmikkipum (Zingiberaceae) is a spicy edible fruit that is cultivated and occurs throughout the tropics. The medicinal uses include, aphrodisiac, measles, leprosy, for excessive lactation, post partum hemorrhage, purgative, galactogogue,

anthelmintic and haemostatic. It contains essential oils such as gingerol, shagaol and paradol (Iwu, 1993).

Nauclea Latifolia Smith (Rubiaceae) is a shrub or small spreading tree that is a widely distributed savanna plant. It is found in the forest and fringe forest. Medicinal uses are as a tonic and fever medicine, toothaches, dental caries, septic mouth and malaria, diarrhea and dysentery. Key constitutes are indole-quinolizidine alkaloids and glycoalkaoids and saponins. There are studies showing the root has antibacterial activity against gram positive and antifungal activity (Lamidi *et al*., 1995).

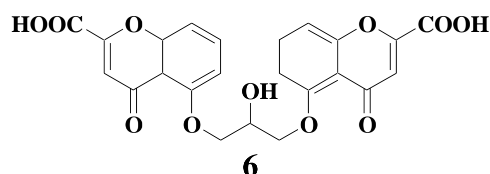
Araliopsis tabouensis Aubrev. (Rutaceae) is a large evergreen tree found throughout west tropical Africa. Its medicinal use is for the treatment of sexually transmitted disease. The back infusion is drunk for gonorrhea in the Ivory Coast (Irvine, 1961). Its major constituents are alkaloids. Seven alkaloids have been isolated from the root and stem bark (Fish *et al*, 1976).

Some of the drug products in use which are based on ethnomedical practices are atropine **(4)** and scopolamine **(5)** isolated from Solanaceae family and has a long history of ethno medical use in the Middle East and Europe to relieve colic, dilate the pupils and as poisons.

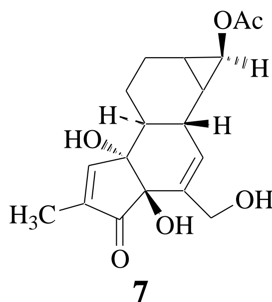


Pharmaceutical drugs are seen increasingly as over prescribed, expensive, and even toxic. Herbal remedies are seen as less expensive and less toxic. Historically, plants derived medicines have made large contribution to human health and well-being. Their role is two fold; first as phytomedicine to be used for the treatment of diseases and secondly, they may become a template for the development of other medicine, a natural blue print for the development of new drugs (Nelson, 1982). It is estimated that plant materials have provided the model for 50% western drugs. Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices or other purposes that suggested potentially usefully biological activity (Robbers *et al*., 1996).

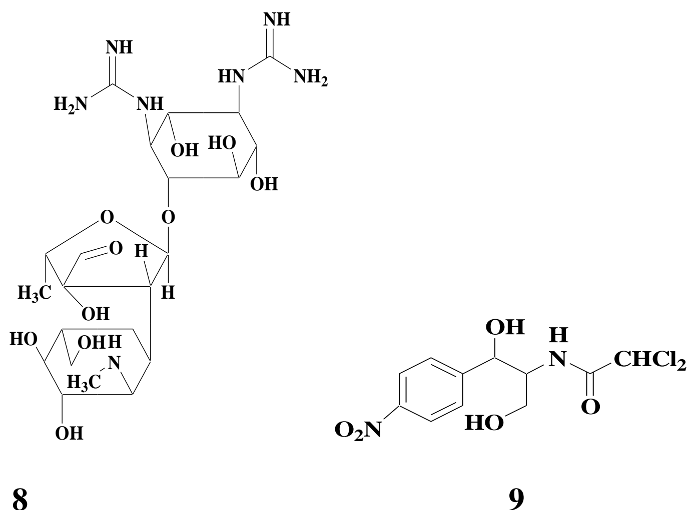
Cromolyn **(6)** from *Ammi visnaga* Lam. was introduced in the 1970s primarily as a preventive for bronchial asthma and other allergies. The plant has been used in traditional medicine in the Eastern Mediterranean for treating bronchial congestion (Pandey, 1998).



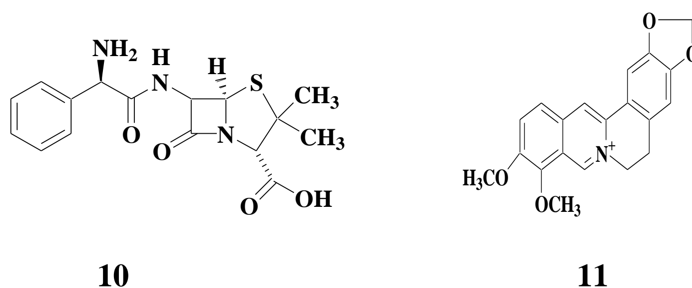
Herbal medicine research has a promising future, screening for HIV inhibitors from plant has been rewarding for example extracts from samples of *Homalanthus* (Euphorbiaceae) were found to contain prostratin **(7)**, an anti AIDS virus HIV-1 (Guastafson *et al.*, 1992).



Medicinal plants contain compounds that are active against microbes (Grein and Brantner, 1994). These bioactive compounds have a broad range of activities and are potentially antibacterial, antifungal, anti-tumor agents, pesticides, herbicides and plant growth stimulants (Suffness and Dodros, 1979). It was the discovery of penicillin that led to later discovery of antibiotics such as streptomycin **(8)**, chloromycetin (**9)** and ampicillin **(10)** (Trease and Evans, 1972).

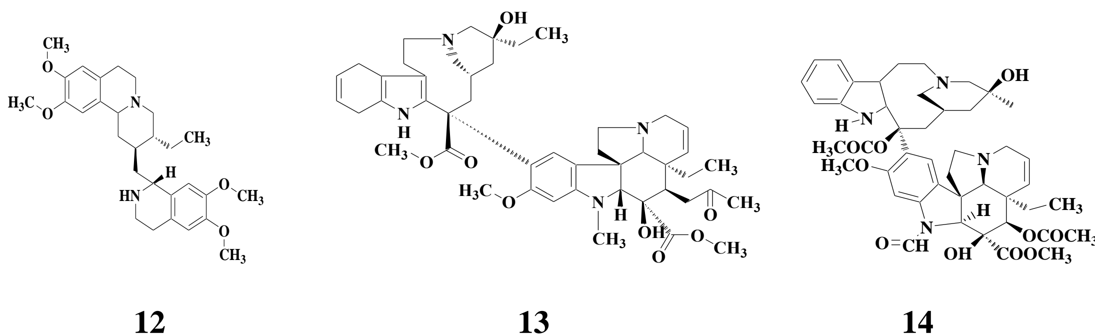


Though most of the clinically used antibiotics are produced by soil micro–organisms or fungi, higher plants have also been a source of antibiotics (Trease, 1972). Examples of these are the bacteriostatis and antifungicidal properties of lichens, the antibiotic action of allinine in *Allium sativum* L. (garlic), or the antimicrobial action of berberine **(11)** in goldenseal (*Hydrastis Canadensis* L.) (Trease, 1972).

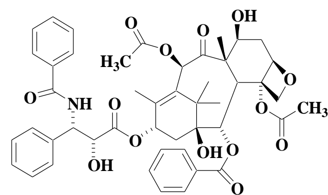


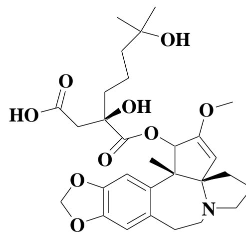
Phytomedicines are effective in treatment of infections diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials. Their actions often act beyond the symptomatic treatment of diseases. For example, *Hydrastis canadensis* not only has antimicrobial activity but also increases blood supply to the spleen promoting optimal activity of the spleen to release mediating compounds (Murrry, 1995). The isoquinoline alkaloid emetine **(12)** obtained from the roots of *Cephalous ipecacuanha* Legros and related species, has been used for many years as amoebicidal drugs as well as for the treatment of abscesses spread by *Escherichia histolytic* infections (Iwu *et al*., 1999).

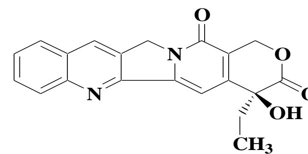
Similarly, higher plants have made important contributions in areas beyond anti-infectives, such as cancer therapies. Examples include the antileukaemic alkaloids; vinblastine **(13)** and vincristine **(14)**, which was both obtained from the Madagascan periwinkle (*Catharanthus roseus* syn. *Vinca roseus* L.) (Nelson, 1982).



Other cancer therapeutic agents include taxol **(15)**, homoharrigtonine **(16)** and several deriratives of camptothecin **(17)** (Nelson, 1982).

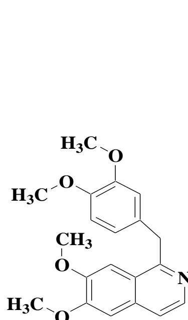


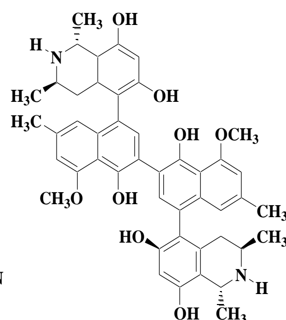


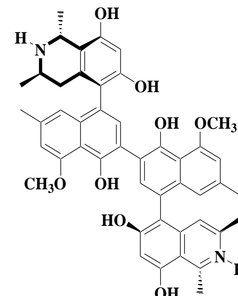


15 **16** **17**

Higher plant's benzylisoquinoline alkaloid; papaverine **(18)** has been shown to have a potent inhibitory effect on the replication of several virus including crytomegalovirus, meascles and HIV. Most recently, three new atropisomeric naphthylisoquinoline alkaloid dimers michellamines A **(19)** and B **(20)** were isolated from a newly described species tropical liana *Ancistrocladus korupensis* Thomas from the rain forest of Cameroon (Turano *et al*., 1989).

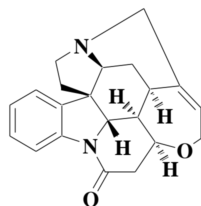


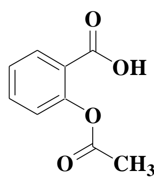


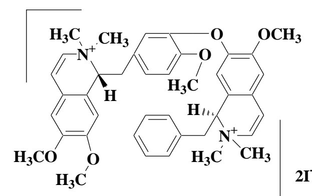


18 **19** **20**

These compounds showed potential anti-HIV with michellamine B being the most potent and abundant member of the series. These compounds were capable of complete inhibition of the cytopathic effect of HIV-1 and HIV-2 on human lymphoblastoid target cell *in vitro* (Boyd *et al*., 1994). Many other drugs including strychnine **(21),** aspirin (**22)** and the active constituent of the drug curare; d-tubocurarin **(23)** is of herbal origin (Farnsworth, 1996).

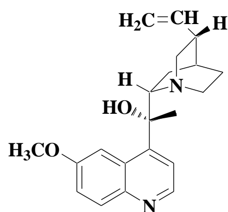


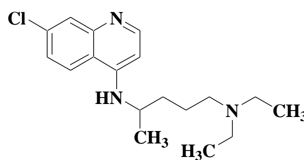


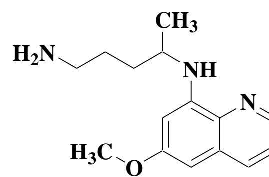


21 **22** **23**

Another important drug of plant origin with a long history of use is quinine **(24)**. This alkaloid occurs naturally in the bark of *Cinchona succiruba* Pavon tree. Apart from its continued usefulness in the treatment of malaria, it can be used to relieve nocturnal leg cramps (Nelson, 1982). The previously, prescribed drugs were analogs of quinine such as chloroquine (**25**) and primaquine (**26**). But their use has been discontinued due to resistance.

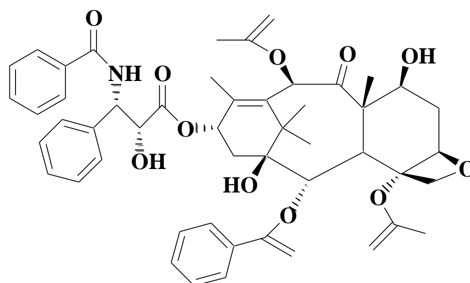






24 **25** **26**

The compound paclitaxel **(27)** isolated from *Taxus brevifolia* Nutt. bark has been showed to have anti cancer activity and is the source of the main constituent of anticancer drug taxol (Pandey, 1998).



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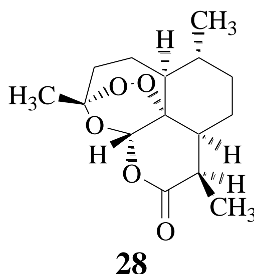
The recent clinical success of artemisinin **(28)** from *Artemisia annua* L. in the treatment of malaria has rekindled interest in medicinal plants as potential sources of novel drug (Di Flumeri *et al*., 2000).



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Some strains of malaria parasites have become resistant to the quinine; therefore antimalarial drugs with novel mode of action are required. Newer anti-microbial agents with activities and structures different from those presently in use could be obtained from medicinal plants hence requiring follow up with attempts to isolate and identify the active compounds (Baker *et al*., 1995).

1.2 Economics of herbal medicine trade

The potential for developing antimicrobials into medicines appears rewarding, from both the perspective of drug development and the perspective of phytomedicine. The immediate source of financial benefit from plant based antimicrobials is from herbal products market. This market offers many opportunities for those cultivating new crops, as many of the plant that are wild grafted today must be cultivated to march the demands of this market. Medicinal plants provide a subsidy from nature that can be of great value to rural people and may be of value to global society as a source of new drugs. About 80% of the world's population relies on these plants as a major source of primary health care. More than 50% of all the drugs in clinical use currently have a natural product origin (Amit and Shailendra,

2005). According to International Union of Conservation of Nature (IUCN) and world health organization (WHO) the cultivation of plants as sources of drugs is the best and most promising way to satisfy market's expanding demand for these raw materials. For wild plant species that are endangered through over exploitation, this is certainly the only applicable way in which we can stop their decline and secure their long-term survival. In addition, there will be an accelerated poverty reduction through creation of rural income and enhancing affore station (Mukonyi *et al.,* 2001).

Hydrastis Canadensis L*.,* one of the top selling antimicrobial in the U.S. herbal market, represent an example of an herb that has undergone domestication. Originally this plant, native to eastern North America, was wild grafted and efforts to cultivate this plant were undertaken in order to supply the demands of the herbal products market and to battle its threatened extinction. It is vital to be in a position to capitalize on the phytomedicine market providing environmentally responsible solutions to public health concerns presented by new trends in infectious diseases. In order to be prepared the industry must be able to sustainably harvest and supply the herbal market (Henry, 2000).

A number of significant global disease including cancer, malaria, tuberculosis and certain viral, fungal and bacterial infections are showing patterns of resistance to known therapeutic agents. The plant kingdom represents an extra ordinary reservoir of novel molecules. Of the estimated 250,000- 500,000 plant species around the globe. Only a small percentage has been investigated phytochemically and the fractions subjected to biological or pharmacological screening (Hostettmann *et al.*, 1995). A survey conducted by WHO (2002) showed that 60% - 80% of the world's population depended on herbal medicine for their health needs, the

majority of this population comes from the developing countries and over 75% of the people infected by AIDS live in Africa (UNAIDS and WHO, 1998). The vast variety of medicinal plants systems are in African countries. Ethnomedicinal studies are therefore the way forward for the continent. Plants which are observed to be efficacious and frequently prescribed by traditional healers may contain compounds that are potential drug candidates and could rightly be recommended for further examinations (Igoli *et al.*, 2005).

1.3 Bacteria and fungal infections

The skin, respiratory tract and gastrointestinal tract are inhabited by a variety of bacteria. These normal bacteria are harmless or even helpful, in protecting their hosts from interference by the growth of harmful bacteria. An opportunistic infection occurs when an organism indigenous to one part of the body invades another part where it is pathogenic. To be pathogenic, bacteria must be able to resist the host defenses, which include bacteriocides and intracellular destruction by leukocytes. Host resistance to infection is lowered in weak and debilitated patients and in those with a decreased ability to mount an effective immune response because of disease or the effect of drugs such as corticosteroids, immuno-suppressive agents or cytotoxic agents (Trease and Evans, 1972).

Fungal infection is an inflammatory condition in which fungi multiply and invade the skin, the digestive tract, the genitals, and other body tissue, particularly, the lungs and liver. Microscopic fungi, which are called dermatophytes, often live exclusively on such dead body tissues as hair, the outer layer of the skin, and the nails. The fungus grows best in moist, damp, dark places with poor ventilation and on skin that is irritated, weakened, or continuously moist. Superficial fungal infections include tinea capitis, an infection of the

neck and scalp; tinea barbae, also called barber's itch, along the beard area in adult males; tinea corporis on parts of the body, such as the arms, shoulders, or face tinea cruris, or jock itch, involving the ground; tinea pedis, or athlete's foot; tinea vesicolor, and tinea unguium, or infection of the nails. The term tinea gladiatorum is sometimes used to describe ringworm infections in atheletes. Tinea gladiatiorum is most common in swimmers, wrestlers, and athletes involved in other contact sports. Fungal infections of the skin and nails are very common in children but they can affect all age groups (Rebecca and Patience, 2005).

Systemic fungal infections occur when spores are touched or inhaled or there is an overgrowth of fungi in or on the body. Such infections are most often a serious problem in those with suppressed immune systems. *Candidiasis* is a rather common fungal infection. When it occurs in the mouth, it is called thrush. Less often, it occurs in the mucosa membranes of other parts of the digestive system, or in the vagineam, heart valves, urinary tract, eyes, or blood. Other systemic fungal infections include aspergillosis, which mostly affects the lungs and may also spread to the brain and kidneys; blastomycosis, a lung infection that may spread through the bloodstream; coccidioidomycosis, also known as San Jaquin or valley fever; mucormycosis, which can develop into a very serious, life-threatening infection; and histoplasmosis (Rebecca and Patience 2005).

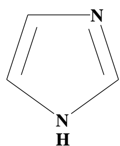
1.4 Antibiotics and antifungals

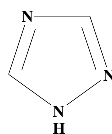
With the discovery of microorganisms as the causative agents of infectious diseases, many substances including those of plant origin became recognized as "antiseptics". These are substances having the ability to inhibit the growth of microorganisms. Later on, this term was

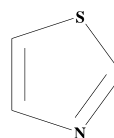
changed to “antibiotics” from the term “antibiosis”, which describes antagonism between different species and even between members of the some species in nature. An antibiotic is a biochemical drug, derived from one or more kinds of microorganisms. It may have the ability to inhibit the growth of (bacteriostatic agent) or to kill (bactericidal agent) a number of other micro-organisms. This makes antibiotics to be of immense value in the treatment of a number of diseases that result from microbial infections (Amit and Shailendra, 2005).

Antibiotics, also known as antimicrobial drugs are therefore drugs that fight infections caused by bacteria, either by inhibiting the growth of or by destroying these microbes. Their therapeutic success relies on their pharmacokinetic behavior and the contribution that the host’s own defenses are able to make towards clearance of the offending microorganism (Amit and Shailendra, 2005).

An antifungal drug is a medication used to treat fungal infections. Such drugs imidazole **(29),** triazole (**30),** and thiazole **(31)** are usually obtained by a doctor’s prescription or purchased over-the-counter. Antifungals work by exploiting differences between mammalian and fungal cells to kill the fungal organism without dangerously affecting on the host cells. Unlike bacteria, both fungi and humans are eukaryotes. Thus fungal and human cells are similar at the molecular level (Lee *et al*., 1999).

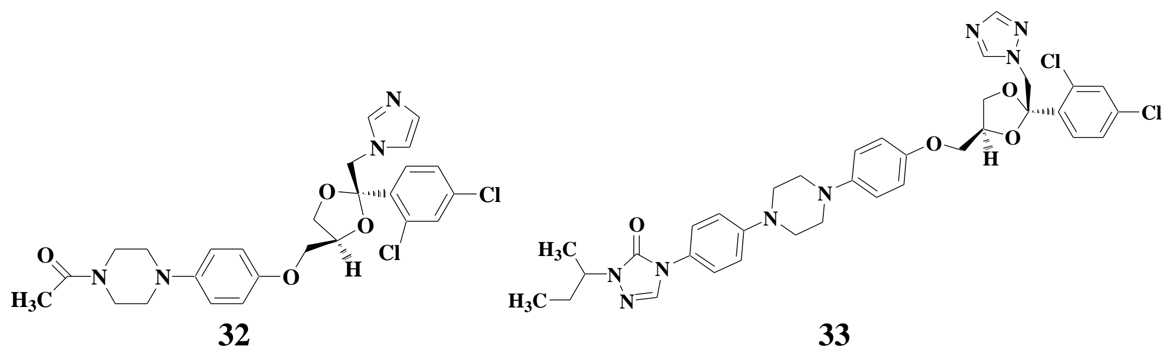






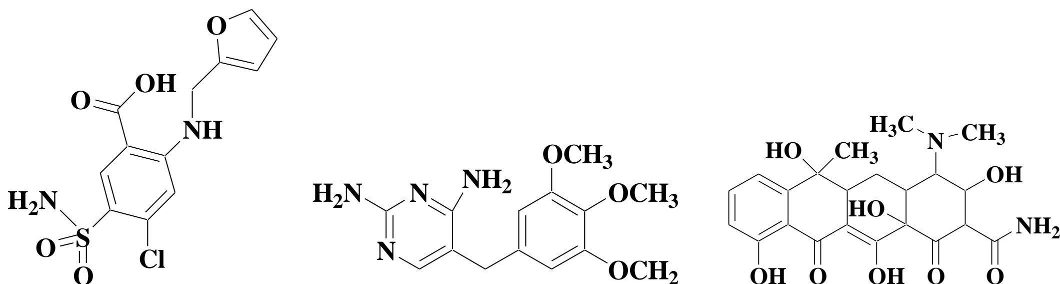
29 **30** **31**

This makes it more difficult to find or design drugs that target fungi without affecting human cells. As a consequence, many antifungal drugs cause side-effects. Some of these side-effects can be life-threatening if the drugs are not used properly. There are also many drug interactions. For example, the azoles antifungals such as ketoconazole **(32)** or itraconazole **(33)** can be both substrates and inhibitors of the p-plycoprotein, which (among other functions) excretes toxins into the intestines (Lee *et al*., 1999).



1.5 Bacteriostatic and fungiostatic agents

Bacteriostatic agents are substances that inhibit the growth of other micro-organisms by interfering with bacterial protein production, bacterial DNA production and bacterial cellular metabolism. Bacteriostatic antibiotics inhibit growth and reproduction of bacteria without killing them; killing is done by bactericidal agents. Examples of bacteriostatic agents include; sulfonamides like furosemide **(34)**, trimethoprim **(35)**, tetracycline **(36)**, chloramphenicol **(37)** and erythromycin **(38)** and their derivatives to mention just but a few (Sritharan and Sritharan, 2004).



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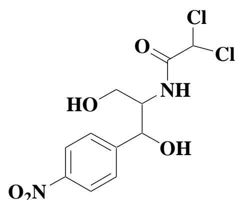
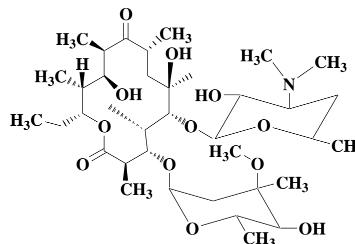
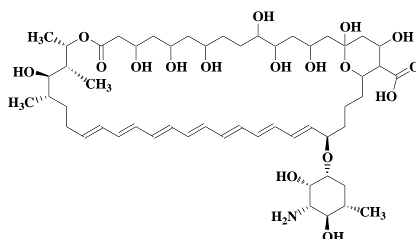




37 **38**

Fungistatic drugs are those that inhibit growth of, whereas fungicidal drugs kill fungal pathogens. The immunocompetent host is usually far better equipped to eliminate fungal pathogens that the immunosupressed host. Therefore, it would be especially desirable to have a truly fungicidal drug, one that absolutely kills all fungi, as a treatment option for the immunosupressed patient. The critical question would be whether a fungicidal drug can be delivered to the target site in a concentration high enough for a sufficient time to reduce the intralesional fungal counts to zero. By this simple definition, there are no fungicidal drugs available today. Fungicial drugs are those that lead to a reduction of 99.9% of the initial inocula. Although this less restrictive *in vitro* standard is more easily met, it has serious limitations. Whether the 99.9% kill should be an acceptable standard remains uncertain. As an alternative, the minimum inhibitory concentration, though indicating static activity, has served well; perhaps it should be the only information reported for fungal susceptibility testing. Amphotericin B **(39)** has been used for more than 30 years, and until very recently, has been the gold standard, Amphotericin B acts within minutes to bind avidly to ergosterol-containing fungal cell membranes and less avidly to mammalian cell membranes (Graybill *et al*., 1997).



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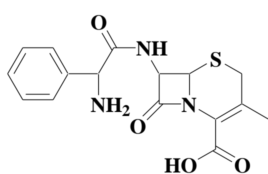
1.6 Bactericidal agents

These are substances that kill micro-organisms and, preferably, nothing else. Bactericides are disinfectants, antiseptics or antibiotics. Members in this class include: penicillin, cephalosporins like cefalexin **(40)** and aminoglycosides like Gentamycin **(41)** (Sritharan and Sritharan, 2004).



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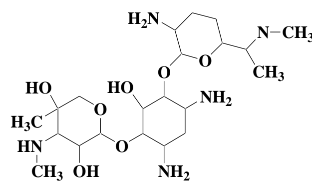
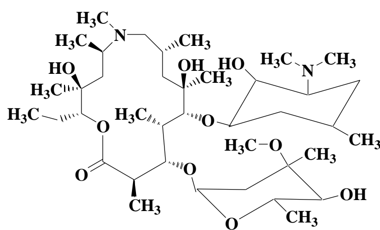


41

Effective chemotherapy in the use of antibiotics depends upon selective toxicity. This may be defined as the ability of the drug to inhibit microorganisms at concentrations tolerated by the host (Amit and Shailendra, 2005). New antibiotics originate from the random or target based screening of microbial products and synthetic compounds, and from rational drug design. Different strategies have been used in the screens to select against known antibiotics and to target specific fungal structures with no mammalian counterparts (Weisbium, 2000).

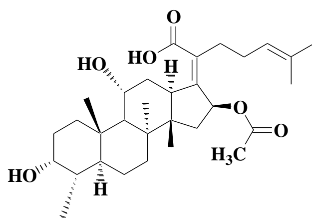
Some of the recent antibiotics that are in use include macrolides. The macrolides are a group of antibiotics that have a large lactone ring structure. These can be 14 or 16 membered rings.

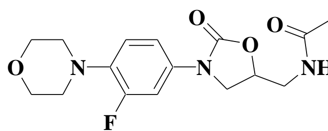
They are relatively non-toxic antibiotics, and most active against Gram- positive bacteria. Members include erythromycin **(38)** and azithromycin **(42)** (Weisblum, 2000).



42

The steroid fusidic acid **(43)** is among the most recent antibiotics in the market. Fusidic acid is also used to treat Gram-positive infections and it acts by preventing translocation of ribonucleic acid (RNA). Fusidic acid is usually administered combination with other antibiotics.

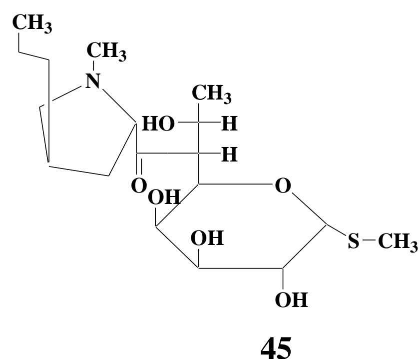


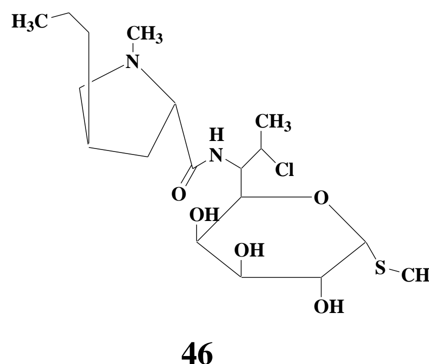


43 **44**

Linezolid **(44)** antibacteria are a type of bacterial protein synthesis inhibitors and it belongs to a group oxazolidinone antibiotics. Its mode of action is to prevent the initiation of protein synthesis. It does this by interfering with the interaction between RNA of the bacteria. It is active against Gram-positive cocci, including meticillin resistant *Staphylococcus aureus* and vancomycin resistant enterococci (Trissel *et al*., 2000).

The lincosamide antibiotics have a similar mode of action like the macrolides. They are relatively non-toxic and are most active against Gram-negative bacteria (Gary *et al*., 1999). Examples include lincomycin **(45)** and its semi-synthetic derivative clindamycin **(46)**





Almost all of these conventional antibacterial agents have exhibited some form of bad side effects that affect the patient. Some of these side effects include among others erosion of cartilage in weight-bearing joints, convulsions, increases intracranial pressure, toxic psychosis, central nervous system stimulation (nervousness, lightheadedness, confusion, hallucinations) hives, difficulty breathing, swelling of the face, lips, tongue, or throat and abdominal pain. Other mild reactions include; burning, stinging, or sensitivity where the medicine is applied and gray or bluish colored skin can also result (Jeljaszewicz *et al*., 2000).

1.7 Antibiotic resistance

A major problem in antimicrobial therapy is the evolution of antibiotic resistant strains of bacteria and fungi, which are an important cause of serious infections. Unnecessary over use of antimicrobial agents speeds up the evolution of resistant strains. The transfer of resistance between different species by plasmids producing multiple drug resistant strains exacerbates the problem (Tait-Kamradt *et al*., 2009). Few laboratories in Kenya test ineffective antibacterial agents before antibiotics are administered to patients. As a result, many antibiotics in Kenya are dispensed indiscriminately. Like the rest of the world therefore antibiotic resistance to several bacteria has become a health problem. Pharmaceutical industries in the developed world have been so versatile with development of new drugs that the society and scientific community have become complacent about the potential of

bacterial resistance. The drugs for which most resistances have developed are β-lactam antibiotics. The continued search for effective antibacterial drugs is therefore still very important (Doern *et al*., 1999).

Antifungal drug resistances have become a major problem in late-stages of AIDS patients. About 5-10% of oral candidiasis is now intractable with the antifungal drug fluconazole and up 33% of oral *Candida* isolates from AIDS patients are resistant to fluconazole (Seattle, 1999). Resistance appears to be correlated with the total cumulative dose, which is a reflection of long-term prophylaxis or therapy. Antifungal drug resistance has recently been identified in systemic candidiasis from bone marrow transplant patient and is starting to appear in strains of *Cryptococcus neoformans* from AIDS patients with a history of cryptococcal infection who are prophylaxed to prevent reactivation. Clearly, antifungal drug resistance is becoming a major problem in the field of pathogenic mycology. In the last few years, progress has been made in identifying the basic mechanisms by which a stratin of *Candida albicans* becomes resistance to antifungal drugs (Seattle, 1999).

Kenya is rich in plant diversity which appears to have potential therapeutic use in modern medicine. Such plants are known to traditional healers and are being used by them in therapy. Identification and isolation of biologically active principles from such herbs may lead to the development of novel drugs for clinical use (Murray, 1995). Among the Kenyan plants that have shown appreciable anti-fungal and anti-microbial activity are members of the families Fabaceae and Menispermaceae, including the plants *Erythrina abyssinica* and *Chasmanthera dependens,* respectively. This research is directed specifically to these plants that are distributed in the Kerio-valley in Keiyo Marakwet County, Kenya.

1.8 Justification

The World Health Organization (WHO) estimates that 4 billion people, 80 % of the world population use herbal medicine for some aspect of their primary health care (Farnsworth *et al*., 1996). Phytochemical investigation of plant species used in folklore medicine to identify the bioactive compounds is important. When these active compounds are isolated, studies can be done so as to synthesize more selective and potent derivatives. This will enhance the medicinal property of these extracts. The sophistication of herbal remedies used around the world varies with the technological advancement of countries that produce and use them. These remedies range from medicinal teas and crude tablets used in traditional medicine to concentrated, standardized extracts produced in modern pharmaceutical facilities and used in modern medicinal system under a physician's supervision. The bioactive compounds can be used as starting point for synthetic pharmacophores and as industrial raw materials.

1.9 Statement of the problem

The antibacterial and antifungal properties of the plants *E. abyssinica* and *C. dependens* have not been exhaustively investigated.

1.10 Hypotheses

(i) Herbs used to treat bacteria and fungal infections can be source of potential natural products with unique chemical structures and can exhibit useful antimicrobial properties.

(ii) The bioactive agents are stable compounds, which can be extracted, isolated and identified.

(iii) These compounds can remain bioactive once isolated from the plant materials.

1.11 Objectives

1.11.1 General objective

The study aims at extraction, isolation, characterization and identification of bioactive compounds with antibacterial and antifungal properties from the plant species *E. abyssinica* and *C. dependens*.

1.11.2 Specific objectives

(i) To investigate antifungal activity against *Candida albicans* and *Trichophyton mentagrophytes* and antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeroginosa* of the crude extracts of *Erythrina abyssinica* and *Chasmanthera dependens*.

(ii) To isolate bioactive compounds from the crude extracts of *Erythrina abyssinica* and *Chasmanthera dependens* using chromatographic techniques; VLC, TLC, CC (Silica gel), Sephadex LH-20 and prep TLC.

(iii) To elucidate the structures of the isolated compounds using spectroscopic techniques; ^{1}H NMR, 13C NMR and 2D NMR.

(iv) To determine antifungal activity against *Candida albicans* and *Trichophyton mentagrophytes* and antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeroginosa* of the isolated pure compounds.

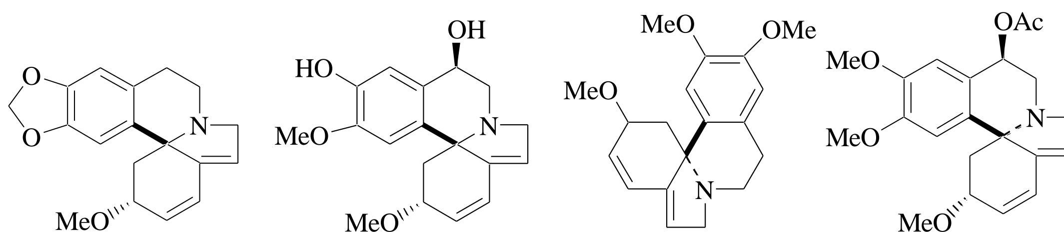
CHAPTER TWO

LITERATURE REVIEW

2.1 Genus *Erythrina*

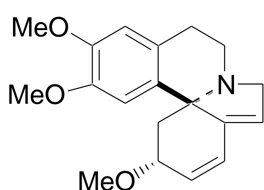
The use of plants by man is an ancient practice, perhaps as man himself. Plants are particularly useful as medicines, flavors, food, insect deterrents, ornamentals, fumigants, spices and cosmetics. The genus *Erythrina*, a member of the family Fabaceae and subfamily Papilionideae comprises of over 110 species of trees, shrubs and herbicaceous plants that are widely distributed throught the tropical warm regions of the world. Seven species of *Erythrina* are found in eastern and southern Africa, that is, *E. caffra* Thunb*, E. decora* Harms*, E. humeana* Sprengel, *E. livingstoniana* Baker*, E. lystemon* Hutch*, E. abyssinica and E. latissima* Meyer (Majinda *et al*., 2001). *Erythrina* species are known to produce flavonoids, isoflavonoids, pterocarpans, terpenoids, saponins and alkaloids. The alkaloids produced are of the erythrina type, some of which has been shown to have curare-like activity on the central nervous system. The bark of *E. lattisima* is burnt and used as dressing for open wounds (Bojase *et al*., 2001).

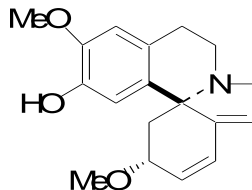
Work on *E. latissima* concentrated mainly on the seeds and a number of erythrina-type alkaloids were isolated. These were erythraline **(47)**, 11-hydroxysodine **(48)**, erysopine **(49)** and erythracine **(50)** (Bojase *et al*., 2001).

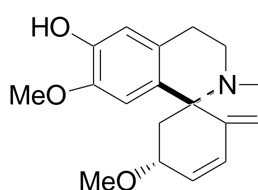


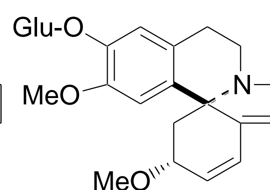
47 **48** **49** **50**

In detailed phytochemical investigation of the seeds erythraline **(47)** was reisolated along with other known alkaloids, erysotrine **(51)**, erysovine **(52)**, erysodine **(53)**, D-glucoerysodine **(54)**, β-oxoerythraline **(55)** and erysotramidine **(56)** (Bojase *et al*., 2001).









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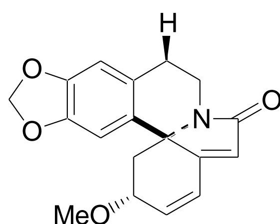
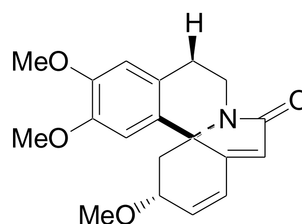


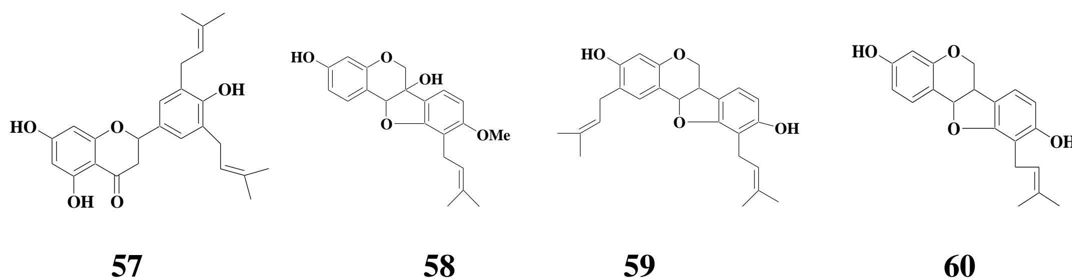


55 **56**

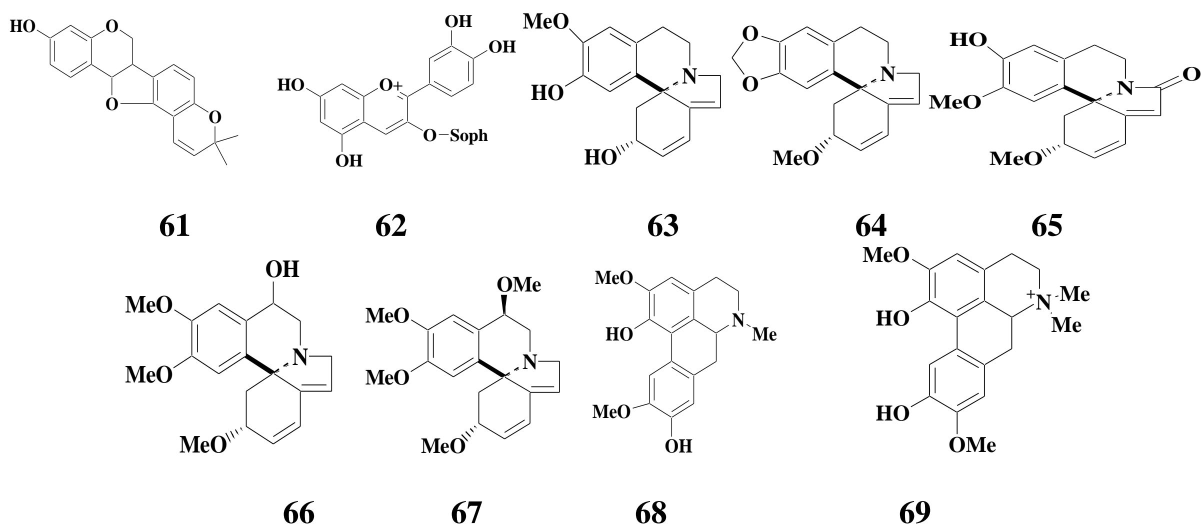
Pounded roots of *E. abyssinica* are used in Kenya to treat diseases such as anthrax, and the bark is boiled with goat meat for treating gonorrhoea. The bark of the green stem may also be pounded and then tied into a fine piece of cloth and the liquid from it squeezed into the eyes to cure inflammation of the lids. The bark may be roasted until black, powdered, and applied to burns and general body swellings. A decoction is taken orally as an anthelmintic and to relieve abdominal pains. The roots are used to treat syphilis and the leaves to cure skin diseases in cattle (ICRAF, 1992).

Plate 1: Aerial parts of *Erythrina abyssinica*

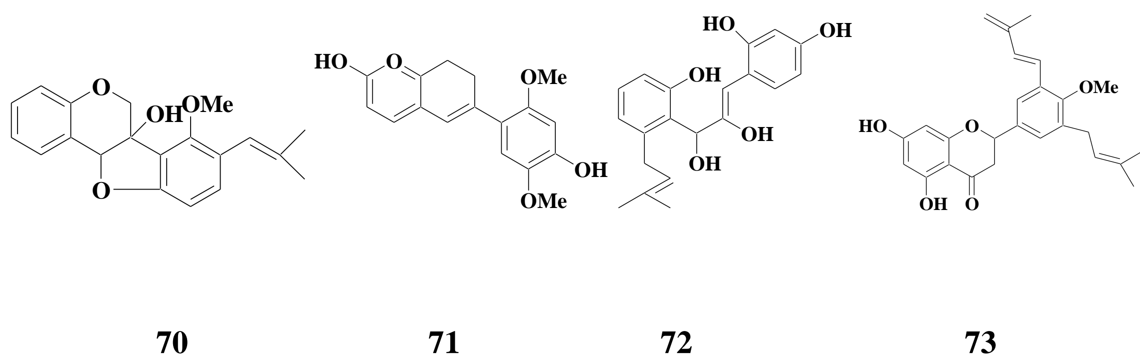
The following flavonoids have been isolated from the roots of *E. abyssinica*; abyssinone **(57)**, cristacarpin **(58)**, erythrabyssin II **(59)**, phaseollidin **(60)** and phaseollin **(61)** (Bisby *et al*., 1994).



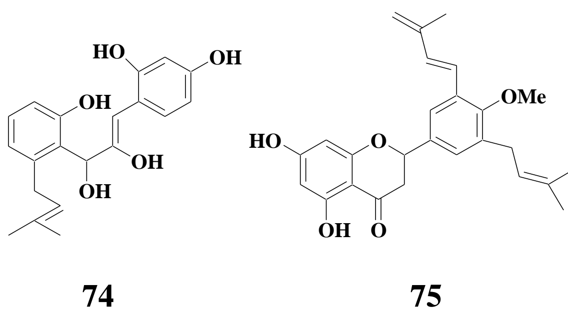
From flowers callistephin and cyanidin-3-sophoroside **(62)** were isolated. From seeds the following erythrina-type alkaloids have been identified, erythravine **(63), Glycoerysodine,** 11-methoxyerysovine **(64)** and 11-oxoerysodine (**65**). From the leaves, erythratine **(66)**, erythristemine **(67)**, isoboldine **(68)**, and orietaline **(69)** have been reported (Bisby *et al*., 1994).



The acetone extract of the roots of *E. abyssinica* showed potent anti-plasmodial activity against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *Plasmodium falciparum.* Chromatography of this extract yielded a new pterocarpene [3-hydroxy-9-methoxy-10-(3,3-dimethyallyl)pterocarpene **(70)** and a new isoflav-3-ene [7,4′-dihydroxy-2′,5′-dimethoxyisoflav-3-ene] **(71)** along other known compounds. While the ethyl acetate extract of the stem bark of this plant showed anti-plasmodial activity against chloroquine sensitive (D6) and chloroquine resistant (W2) strains of *Plasmodium falciparum* with 1C_{50} values of 7.9 ± 1.1 and 5.3 ±0.7 g/ml, respectively. From this extract, a new chalcone, 2′,3,4,4′-tetrahydroxy-5-prenylchalcone (trivial name 5-prenylbutein) **(72)** and a new flavanone, 4′,7-dihydroxy-3′-methoxy-5-prenylflavanone (trivial name, 5-deoxyabyssinin II **(73)** have been isolated (Derese *et al*., 2003).



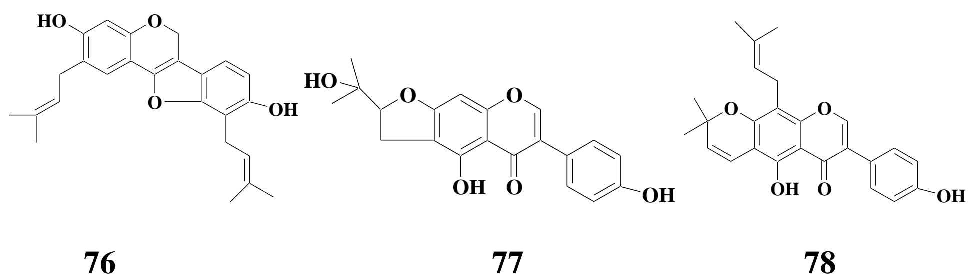
From the stem bark of *E. burttii* Baker a new isoflavone, 5,2′,4′- trihydroxy-7-methoxy-6-(3-methlbut-2-enyl) isoflavone **(74)** and a new flavanone, 5,7-dihydroxy-4′-methoxy-3′-(3-methylbutadienyl)-5′-(3-methylbut-2-enyl)flavanone (trivial name, burttinonedehydrate) **(75)** were isolated (Derese *et al*., 2003).



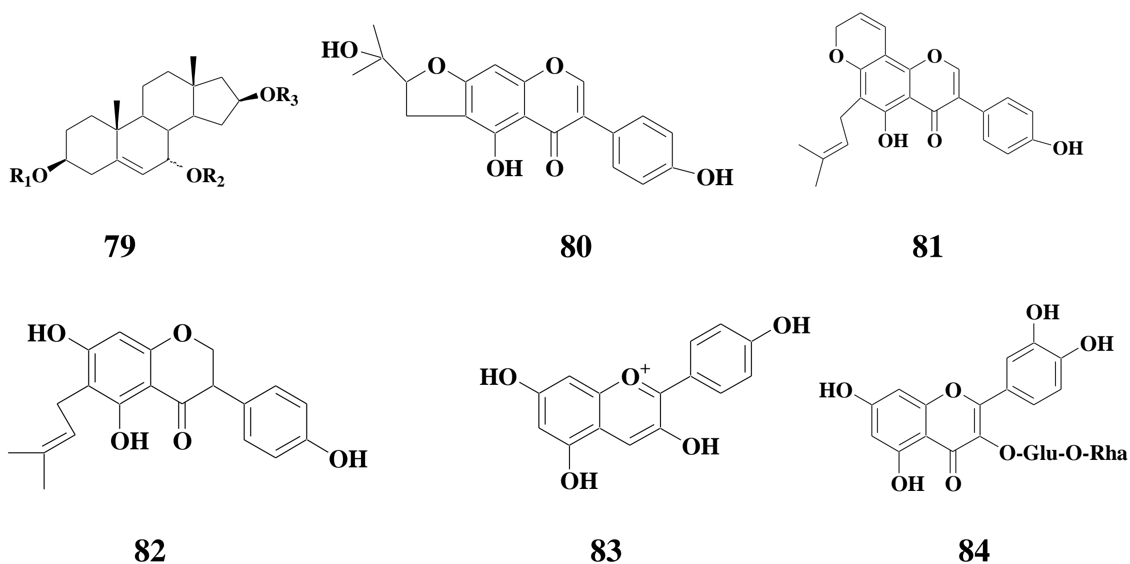
74 **75**

Erythrina variegata is native to the coast of India and Malaysia. It has a reputation for medicinal properties in India, China and South East Asia. The bark and leaves used in many traditional medicines including paribhadra, an Indian preparation said to destroy pathogenic parasites and relieve joint pain. Juice from the leaves is mixed with honey and ingested to kill tapeworms, roundworms and threadworm women take this juice to stimulate lactation and menstruation. It is also commonly mixed with castor oil to cure dysentery. A warm poultice of the leaves is applied externally to relieve rheumatic joints. The bark is used as a laxative, diuretic and expectorant (Hedge, 1993).

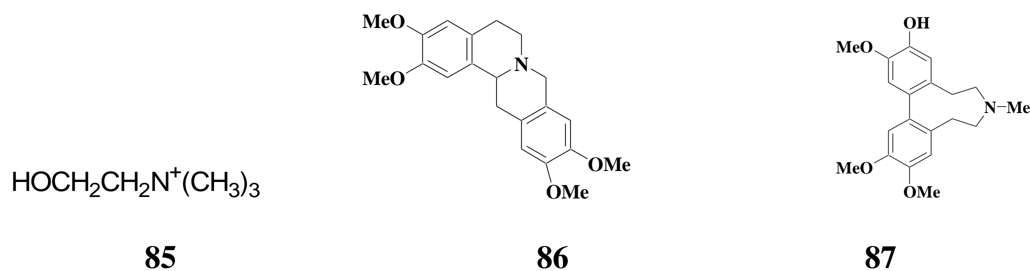
Flavonoids isolated from *E. variegate* Linn. are; from the roots erycristagallin **(76)**, erythrabyssin II, eryvariestyrene, isobavachin **(77)**, phaseollidin, phaseollin and scandenone **(78)**, from the bark alphinumisoflavone **(79)**, erythrinin A, erythrinin C **(80)**, osajin **(81)** and wighteone **(82)** (Chawla *et al*., 1988).

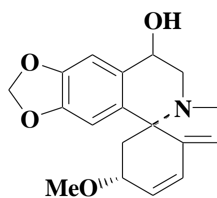


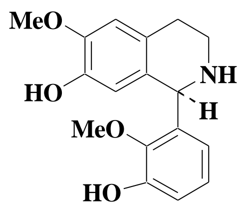
From flowers pelargonidin **(83)** and quercetin-rutinoside **(84)** were identified (Chawla *et al*., 1988).

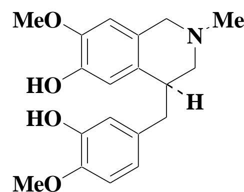


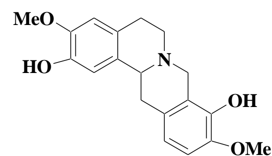
The alkaloids found in leaves were choline **(85)**, coreximine **(86)**, erybidine **(87)**, erysopine, erythrinine **(88)**, nororientaline **(89)**, retickululine **(90)** and scoulerine **(91)**. From the seed and pod, erysodienone **(92)**, and erythratidine **(93)** were reported (Chawla *et al*., 1988).



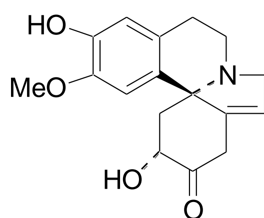


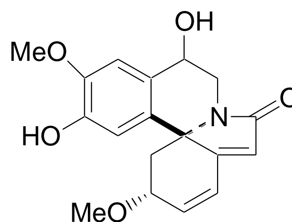






88 **89** **90** **91**





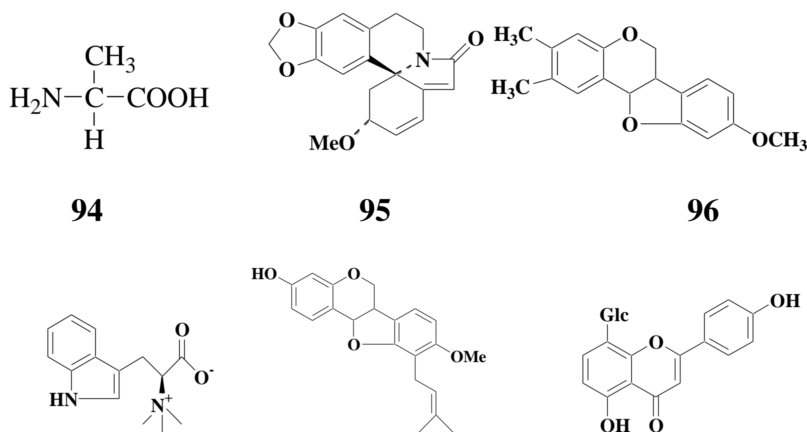
92 **93**

E. mulungu Vell. has long been used in Brazil by indigenous people as a natural sedative; it has been used to calm an over excited nervous system and promote a restful sleep. In both North and South America herbal medicine systems *E. mulungu* is considered to be an excellent sedative to calm agitation and to treat nervous problems including insomnia and anxiety. It is also widely used for asthma, bronchitis, gingivitis, and hepatitis, inflammation of the liver and spleen, intermittent fevers and to clear obstructions in the liver. In both Peru and Brazil *E. mulungu* is used for epilepsy (Vasconcelos, 2006). Herbalist and practitioners in the United State use the *E. mulungu* to kill hysteria from trauma or shock, as a mild hypnotic sedative to calm the nervous system, to treat insomnia and promote healthy sleeping patterns, to regulate heart palpitations, and to treat hepatitis and liver disorders. Chiropractors recommend *E. mulungu* for hernias, stomachaches, and epilepsy (Vasconcelos, 2006).

The chemicals in *E. mulungu* have been studied extensively; they have been found to comprise large amounts of novel flavonoids, triterpenes, and alkaloids. Studies have been done on erythrina alkaloids in the last decade, as they represent a group of very active

chemicals with various properties and are almost always present in *Erythrina* species. Thus far, alkaloids have been found in 78 of 107 species in the genus *Erythrina; E. mulungu* is documented to contain 20 isoquinoline alkaloids. Many of these alkaloids have demonstrated anti-inflammatory, cardio active, narcotic, and sedative activities. One novel alkaloid discovered in *E. mulungu* is called cristamidine **(95)**. Its positive effect on the liver was demonstrated in a 1995 clinical study with rats. The hypertensive and heart-regulatory activity were studied and attributed to its alkaloids. Another alkaloid in *E. mulungu* (and other *Erythrina* plants), erysodine **(53)**, has been documented with neuromuscular effect characteristics of curare arrow poisons two studies also indicate that it might be useful as an anti-nicotine drug, as it demonstrated action as a competitive antagonist and to block nicotine receptors. Interestingly, both of these studies were published by major (and competing) pharmaceutical companies (Vasconcelos, 2006).

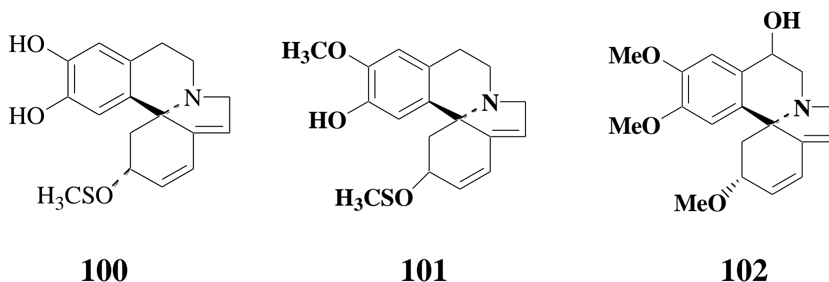
The main plant metabolites in *E. mulungu* include: alanine **(94)**, cristamidine **(95)**, dimethylmedicarpin **(96)**, erysodine **(53)**, erysopine **(49)**, erysotrine **(51)**, erysovine **(52)**, erythratine **(66)**, hypaphorine **(97),** phaseollidin **(60)**, sandwincensis **(98)**, and vitexin **(99)** (Marchioro, 2005).



97 **98** **99**

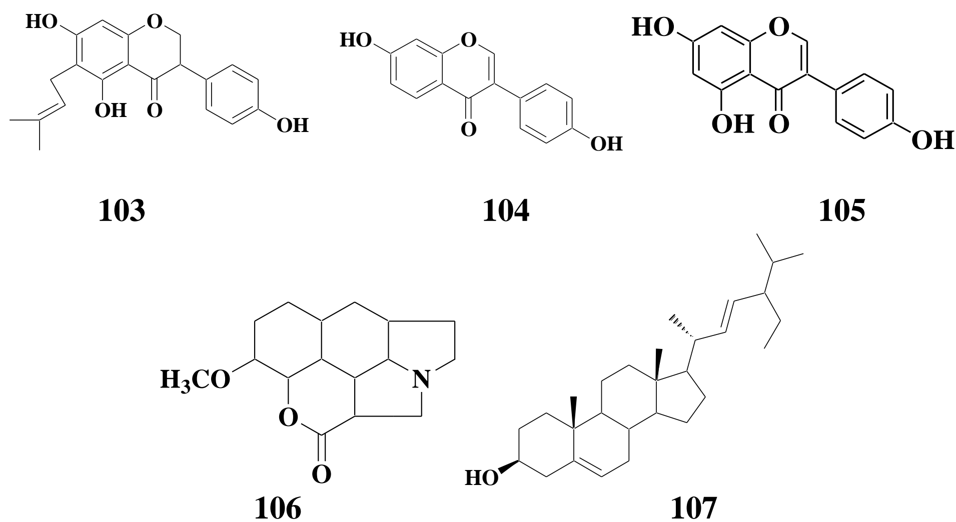
Erythrina berteroana Urban is reported be narcotic, piscicidal, poisonous, and soporific, coralbean is a folk remedy for dysmenorrheal (Duke and Wain, 1981). According to Morton (1981), the sedative flower decoction is used for dysentery hemorrhages and nervousness. Guatemalans believe that tucking in ones pillow will make one sleep well (Allen and Allen, 1981). Seeds contain erysodine **(53)**, erysovine **(52)**, and hypophorine **(97)**. Hypophorine is the betaine of tryptophane and is a curare-like convulsive poison (Chawla *et al*., 1988).

According to Hartwell (1971), *Erythrina fusca* Lour. seed are used in folk remedies for cancer in Annam and Bengal. *E. Fusca* is reported to have the same medicinal attributes as *E. indica* whose bark is used for fever, hepatosis, malaria, rheumatism, toothache, also for boils and tractures. Perry (1980) cites many more uses for E*rythrina indica* L.. The bark is used for poulticing fresh would in Malaysia, boiled roots are taken internally or externally for beri-beri and grafted woods for hematuria (Perry, 1980). The root is used for rheumatism, bark and leaves as a vermifuge. The seeds of *E. fusca* contain the alkaloids erythraline **(47)**, erysopine **(49)**, erysothiopine **(100)**, eryso-thiovine **(101)**, erythratine **(102)** and hypaphorine **(97)**. The similarity in alkaloid and amino acid patterns in *E. fusea* and *E. glauca* Willd were considered in rendering these species synonymous (List and Horhammer, 1979).



100 **101** **102**

From the Studies of chemical constituents of *Erythrina arborescens* Roxb., six compounds were isolated from the plants and identified as alpinumisoflavone, weighteone **(103)**, daidzein **(104)**, genistein **(105)**, vittadinoside **(106)** and stigmasterol **(107)** (Yu *et al.*, 2000).



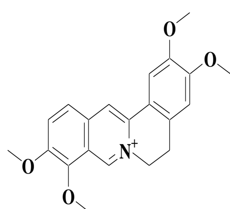
2.2 Genus *Chasmanthera*

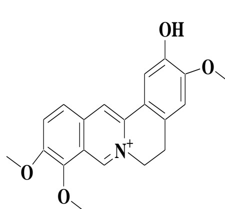
Lianas from the family Menispermaceae, which are spread in the tropics and subtropics all over the world, are very important for the traditional medicine of the indigenous people. The role of these drugs can be explained at least partially by their ingredients. Till now the most investigated ingredients are alkaloids which are derived from the benzylisoquinoline group. The typical bitter tasting isoprenoids are hardly investigated especially if they are glycosides. Folk medicinal indications of Menispermaceae drug all over the world nearly the same, for example: fever, cough, jaundice, cholera, gastrointestinal diseases, rheumatism, veneral diseases and snake bite. *C. dependens* is a woody climber that grows wild in forest margins and savanna. The plant is used medically for venereal disease, topically on sprained joints

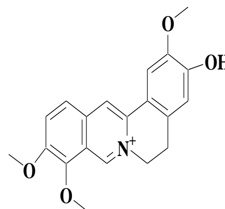
and bruises and as a general tonic for physical and nervous debilities (Barbosa-Filho *et al*., 2000).

Plate 2: ***Chasmanthera dependens***

The constituents include berberine type alkaloids, palmitine **(108)**, columbamine **(109)**, and jatrorhizine **(110**) (Barbosa-Filho *et al*., 2000).



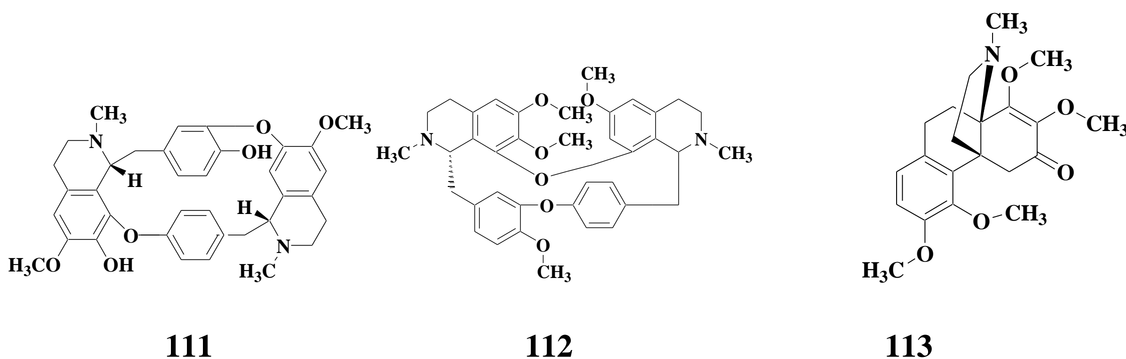




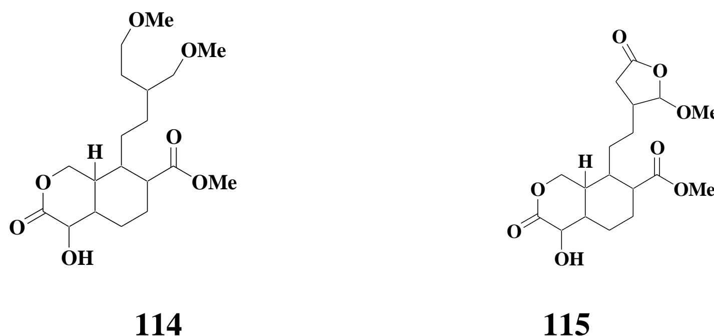
108 **109** **110**

Different genera of Menispermaceae are being used by herbal manufacturers in the formulation of the drug patha. The important source of patha is roots of *Cissampelos pariera* Var.*, Cyclea peltata* Lam *and Stephania japonica* Thunb.. These genera are being used to treat ailment associated with parexia in Ayurvedic system of medicine. The genera are known as patha in Ayurveda and have been used for the treatment of fever, urinary problems and skin infection. *Cissampelos pariera* is found commonly in semi dry forests of tropics,

Cyclea peltata in Western Ghats and Decca region whereas *S. Japonica* occurs in wet deciduous to semi evergreen forests of tropical temperate Asia. Various alkaloids and different pharmacological activities of the plants have been reported. Bisbenzylisoquinoline alkaloids, cissamperine **(111)** with tumor inhibitor activity, tetrandrine **(112).** Various alkaloids were isolated from roots of *S. japonia* such as tertiary phenolic biscoclaurine type alkaloid hasubanonine **(113).** (Yoganarasimhan , 2002).



A phytochemical study of the roots of *Tinospora capillipes* Gagnep. (Menispermeceae) resulted in the characterization of two new diterpenoids; tinocapilactones A **(114)** and B **(115)** (Yoganarasimhan *et al.,* 2002).



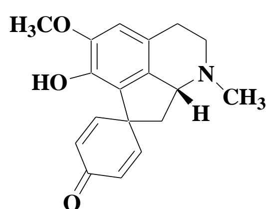
Tinospora cordifolia Lour. (Menispermaceae) is a deciduous climbing shrub distributed throughout tropical India and China. It is widely used in veterinary fork medicine and in ayurvedic system of medicine for its general tonic, anti-periodic, anti-inflammatory, anti-arthritic, anti-allergic and anti-diabetic properties. The roots of this plant are known for its

anti-stress, anti-leprotic and anti-malarial. The chemical constituents reported from this shrub belong to different classes such as alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoids, phenolics, aliphatic compounds and polysaccarides (Kirtikar and Basu, 1976).

The family Menispermaceae is highly specialized in its extraordinarily rich diversification of benzyltetrahydroisoquinoline and aporphine derivatives, coupled with the accumulation of these alkaloids in dimeric form, in numbers unparalleled by any other angiosperm family. Up to the end of 1996, 1858 alkaloids have been described from 244 species of the family (Barbosa-Filho *et al.,* 2000).

These alkaloids include many important discoveries in the field of medicine and pharmaceutically active compounds (Dewick, 2002) such as the bisbenzylisoquinoline alkaloid known as tubocurarine. This is the principle active component in the arrow poison curare, isolated from *Chondrodendron tomentosum* Ruiz, which acts as a muscle relaxant. Because of its richness in alkaloids this family is used worldwide in traditional medicine to treat a variety of ailments. The ethnobotanical survey on the Menispermaceae family confirmed its importance for medicinal uses. World wide species from 41 genera were recorded to be used for various ailments. In Southern Africa and Africa, *Cissampelos* species were by far the most frequently used followed *Stephania, Tinospora and Triclisia*. The genus *Cissampelos* is used in South Africa, Africa and the rest of the world for stomach problems, menstrual problems, pregnancy related problems, as a diuretic, for wounds, ulcers and as a tonic (Van Wyk and Gericke, 2000).

The family Menispermaceae contains 22 different alkaloids types of which the bisbenzylisoquinoline, aporphines and protoberberines are considered good chemical makers for this family. The major alkaloids in seven species of the genera *Albertisia, Antizoma* and *Cissampelos* were isolated and identified by means of NMR and HPLC, seventeen alkaloids were isolated: glaziovine **(116)**, crotsparine **(117),** bulbocapnine **(118)**, decentrine **(119)**, lauroscholtzine **(120)**, reticuline, methylcurine **(121)**, cocsoline **(122)** and insularine **(123).**



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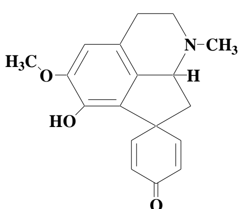

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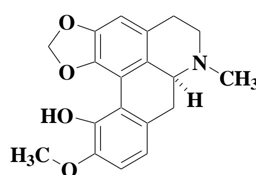

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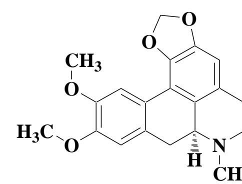

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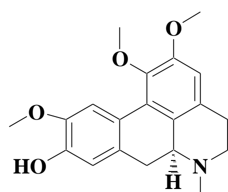

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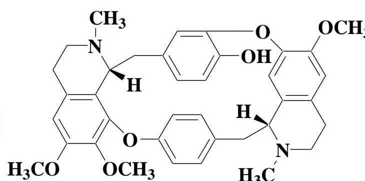

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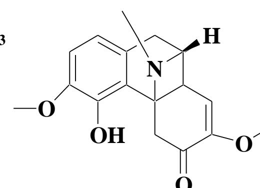

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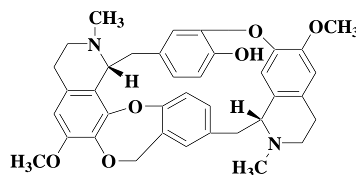
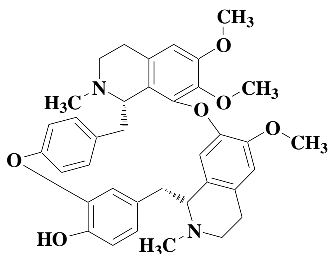
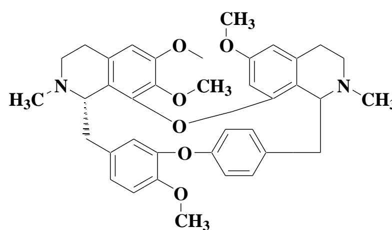


123

The genus *Cocculus* is distributed throughout the tropical and the subtropic regions. About 35 species have been found throughout the world. Two species of this genus are found in Pakistan, *Cocculus hirsutus* Diels and *Cocculus pendulus* Forst. This genus is a rich source of different types of isoquinoline and bisbenzylisoquinoline alkaloids. Some of them possess biological properties, for example oxycanthine **(124)** are highly effective against human

tuberculosis and leprosy, while tetrandrine **(125)** has shown tumor inhibiting properties. The juice of leaves, mixed with water, coagulates into a green jelly like substance, which is taken with sugar as a tonic and is useful against gonorrhea (Bhakuni and Joshi, 1974).



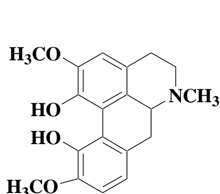


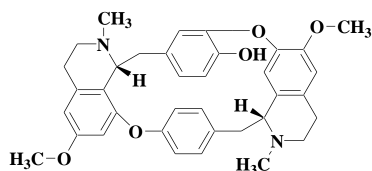
124 **125**

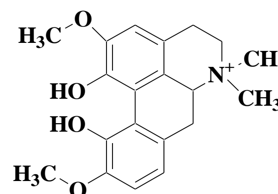
Powder of the leaves, mixed with water, is applied to eyes, for its cooling effect. The root is bitter alterative, laxative, refrigerant, sudorific, demulcent and is used with other ingredients in bilious, dyspepsia and stomach ache in children. The roots of *Cocculus pendulus* are used as a febrifuge and a tonic prepared from them is also substituted for the fruits of *Anamiia cocculus*. Juice of the plant was used by the Arabs in the preparation of fermented beverage (Bhakuni *et al.*, 1974).

Cissampelos pareira is found throughout the Amazon in Peru, Brazil, Equador and Colombia and it is cultivated by many to beautify their gardens. In Brazil, this plant is well known as abutua and in Peru as “abuta”. *Cissampelos pariera* is commonly referred to as the midwives herb throughout South America because of its long history of use. All types of abuta are used in tropical countries to prevent a threatened miscarriage and to stop uterine hemorrhages after childbirth. Abuta is also believed to aid poor digestion, drowsiness after meals and constipation (Caceres, 1987).

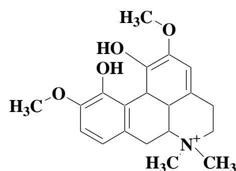
Cissampelos plants including *Cissampelos pariera* contain isoquinoline alkaloids. Out of 38 alkaloids that have been isolated from "Abuta" tetrandrine **(126)** is the best documented. Clinical research over the years has found tetrandrine **(126)** to have pain relieving, anti inflammatory and fever reducing properties. The main chemicals in Cissampelos pariera are alkaloids, corytuberine **(127),** curine **(128),** magnoflorine **(129),** menismine **(130)** and quercitol **(140)** (Caceres, 1987).

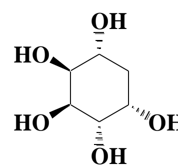






126 **127** **128**





129 **130**

Chasmanthera dependens is widely distributed from Sierra Leone to Eritrea and Somali and from the Eastern DRC Congo and Tanzania to Angola, Zambia and Zimbabwe. It is commonly planted in home gardens especially in Ghana. In West Africa leaf and stem sap are locally applied to cure sprains and bruises, as a dressing for fractures or mixed with shea butter as an embrocation to treat pain and stiffness. The bark is chewed as a remedy for venereal discharges or as a general tonic for physical or nervous weakness in inflammatory and exhausting diseases. In Nigeria stem maceration together with stems and roots of several other plants is drunk against convulsions. In Kenya the stem is roasted and eaten to treat

convulsions in infants. In Uganda the plant is used against dementia, snakebites and epilepsy. A decoction of freshly pounded roots mixed with roots of *Vernonia sp*. is drunk to cure malaria. A decoction of pounded roots mixed with leaves of *Tagetes sp*. is drunk by children to treat cough. In DR Congo the leaf sap is applied as first aid to stop bleeding of wounds. In Ethiopia, Borana pastoralists eat the roots and leaves (Adekunle and Okole, 2002).

Ethanol extracts and crude water extracts of the roots showed significant antifungal activity against *Aspergillus flavus*, *Aspergillus fumigatus*, *Aspergillus niger*, *Candida albicans*, *Microsporum audonii*, *Trichoderma viride* and *Trichophyton mentagrophytes*. The ethanol extracts of the plants were more active than the water extracts. *Chasmanthera* is closely related to *Tinospora* and *Jateorhiza*; these genera have been combined in the past. *Chasmanthera* is an African genus which comprises two species: *Chasmanthera dependens* and *Chasmanthera welwitschii* Troupin. The species appear to be ecologically different, although their areas of distribution overlap in the Central Africa Republic and intermediates have been found there. Further research is needed to decide if the two species should be combined into a single species (Almeida *et al*., 2001).

C. dependens is widely used in traditional medicine, but very few pharmacological tests have been done. Pharmacological tests on the alkaloids present in *C. dependens*, but obtained from other species, indicate that additional research is warranted. Kenyan species *C. dependens* has not been systematically studied phytochemicaly, though it is widely used in ethnomedicine. This study was done to investigate and establish the structure of its metabolites and their antimicrobial bioactivity.

CHAPTER THREE

METHODOLOGY

3.1 Glass ware

All the glassware used in this study were thoroughly washed with water and detergent. They were rinsed with water followed by acetone and finally dried at 110˚C in an electric oven for one hour. The stained glasswares were soaked in chromic acid overnight before being washed with detergent and water.

3.2 Laboratory equipments and instruments

The plant materials were ground using a motor grinding Laboratory mill (Christy and Norris Ltd., Chelmsford, England) and weighed using a top-loading analytical balance (Denver instrument Colorado, U.S.A). The weight of the isolated compounds was taken on an ADA 180/C machine (Adam Equipment Co. Ltd. New Milford, U.S.A). The melting points of the pure compounds were determined on a Gallen kamp melting point apparatus (Sanyo, West Sussex, UK) and were uncorrected. Ultraviolet lamp, ENF-240 C/F (Supertonics Corporation

Westbury, UK) of long and short wave length (365 nm and 254 nm, respectively) was used for visualizing the spots on a developed thin layer chromatography (TLC) plate.

3.3 Nuclear magnetic resonance (NMR)

The NMR spectra were obtained from Varian Gemini 300 and 400 MHz machines ^{1}H NMR spectra were run in $CDC1_3$ (deuterated chloroform) or CD_3OD (deuterated methanol) depending on solubility of the isolated compounds. Chemical shifts (δ) were recorded in part per million (ppm) relative to tetramethyl silane (TMS). The peak multiplicity; *s*-singlet, *d*-doublet, *q*-quartet and *m*- multiplet, were also recorded. Coupling constants were recorded in hertz (Hz). The ^{13}C NMR spectra were run on the same machines at 75 and 100 MHz and the multiplicity determined by DEPT experiments.

3.4 Chromatographic materials

Pre-coated plastic sheets (polygram Sil G/UV $_{254}$) and aluminum sheet (Alugram Sil G/UV $_{254}$) of 20 by 20 cm (Matcher-Nagel Gmbh and Co. Frankfurt, Germany) were used for TLC. Vacuum liquid chromatography (VLC) was carried out using slurry packing with Kiesegel silica gel 60 G (Merck, Germany). The normal column chromatography was packed with Kiesegel silica gel 240 G or Sephadex LH 20 (in case of sieve material).

3.5 Chemicals

The solvents, *n*-hexane, dichloromethane (DCM), chloroform ($CHCI_3$), ethyl acetate (EtOAc), acetone (MeCOMe) and methanol (MeOH) were of the laboratory grade (Kobian Kenya Ltd, Nairobi) and hence they were freshly distilled before use. Sulfuric acid, basic

bismuth nitrate, potassium iodide, glacial acetic acid, *p*-anisaldehyde and dimethylsulfoxide (DMSO) used were of analar grade (Kobian Kenya Ltd, Nairobi).

3.6 Spray reagents

The spray reagents used in this research in detecting and visualizing the separated out compounds on the TLCs include:

i. Anisaldehyde – sulfuric acid prepared by mixing 0.5 ml of *p*-anisaldehyde, 10ml of acetic acid, 85 ml of MeOH and 5 ml of concentrated sulfuric acid (Krishnaswamy, 2003).

ii. Sulfuric acid / MeOH mixture was prepared by mixing 5 ml of concentrated sulfuric acid and 95 ml of distilled MeOH (Krishnasswamy, 2003).

iii. Dragendorff reagent prepared by mixing 0.85 g of basic bismuth nitrate in 40 ml of water, 10 ml acetic acid and 8 g potassium iodide to form solution A and 20 ml acetic acid and 100 ml water formed solution B. The spraying reagent was made immediately before spraying by taking 1 ml of A and mixing it with 10 ml of solution B. Larger quantities were made in the same ratios (Krishnaswamy, 2003).

3.7 Collection and preparation of plant materials

The roots, stem bark and leaves of *E. abyssinica* DC and *C. dependens* Hoschst were obtained from Kerio valley in Elgeyo Marakwet County, 60 km East of Eldoret town Rift valley province where the two plants are well distributed. The plants materials were dried under shade for 14 days except the root tubers of *C. dependens* which was dried for 28 days. They were ground into fine powder in preparation for extraction. The finely powdered materials were weighed separately.

3.8 General procedure of extraction

The finely ground material of *E. abyssinica* roots 1534.4 g was soaked in *n*-hexane a least polar solvent for 48 hours with occasional swirling to ensure thorough extraction. The soaked materials were filtered and the crude extract collected in a clean container. The crude extract was then concentrated and solvent recovered by distillation using rotary evaporator, under reduced pressure and low temperature of about 45°C to give the crude extracts. The *n*-hexane extract after concentration was dried in a fume chamber and weighed.

The residue after extraction with *n*–hexane was soaked in DCM solvent for 48 hours with occasional swirling to ensure thorough extraction. The extract was filtered. The filtrate was concentrated and solvent recovered by distillation using rotatory evaporator. The concentrated extract was dried and weighed. The residue after extraction with DCM was soaked in EtOAc solvent for 48 hours with occasional swirling. This was followed by filtering, concentration, drying and weighing. The residue after extraction with EtOAc was soaked in MeOH for 48 hours with occasioned swirling. The same procedure was followed and the mass obtained. The above procedure was repeated with the 1600.3 g of *E. abyssinica* stem.

The same procedure of extraction was followed with *C. dependens* root tuber (1681.0 g), stem (1417.9 g) and leaves (1114.1 g).

3.9 Fractionation of the extracts

The VLC column was dry packed with silica gel (Kiesegel 60 G, Merck, Germany) and consolidated by applying *n*-hexane to the packing and then sucking by use of a vacuum to

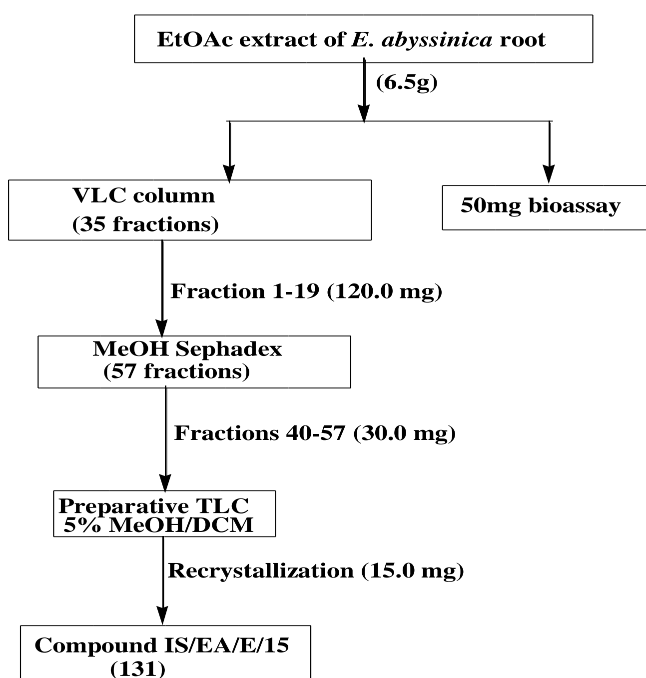
dry and to effect good packing. Care was taken so that the packing was kept uniform and making an evenly flat surface. The crude extracts to be worked on were first mixed with some silica gel and applied as dry powder onto the top of the well packed VLC. Elution was done using various proportions of a solvent gradient of increasing polarity, starting with 100 % *n*-hexane, then DCM, EtOAc and finally 100 % MeOH. 50 ml fractions were collected for each solvent system.

The fractions were further subjected to normal column chromatography and other chromatographic techniques. The purity was continuously monitored by use of TLCs at each stage. The pure compounds that were analyzed were finally obtained either through further purification by use of a normal column packed with Sephadex LH 2O (a sieve gel method) or by use of preparative TLC.

Analytical pre-coated TLC plates were used throughout the purification process for the establishment of optimum solvent systems for separations, complexity of the extracts and purity of the isolated compound. Spots on the chromatograms were detected under UV light at 254 nm and 366 nm for UV active compounds. Visualization was also done upon development by separately spraying the TLC plates with *p*-anisaldehyde and heating for 10 minutes at 110°C in an oven or Dragendorffs reagent for alkaloids. Fractions that showed homogeneity were combined and concentrated together to give pure compounds or partially pure compounds for further purification. The purification of the four compounds generally followed the procedures and schemes specified below.

3.10 Compound IS/EA/E/15 (131)

The EtOAc extract of *E. abyssinica* root barks 10.0 g was loaded in a VLC column and eluted with 200 ml of 100 % *n*-hexane, then DCM, EtOAc and finally 100 % MeOH. About 50 mls fractions were collected. A total of 35 fractions were obtained. The fractions were spotted on a TLC plate to establish optimum solvent system for separations in which 5 % MeOH/DCM solvent system was identified. Fractions 1-19 (120 mg) were loaded in Sephadex LH 2O column and eluted with MeOH to give 57 fractions. Fractions 40-57 (30 mg) were pooled and loaded in a preparative TLC to give the pure compound IS/EA/E/15 (131). Crystallization in methanol yielded 15.0 mg as presented in the scheme 1.

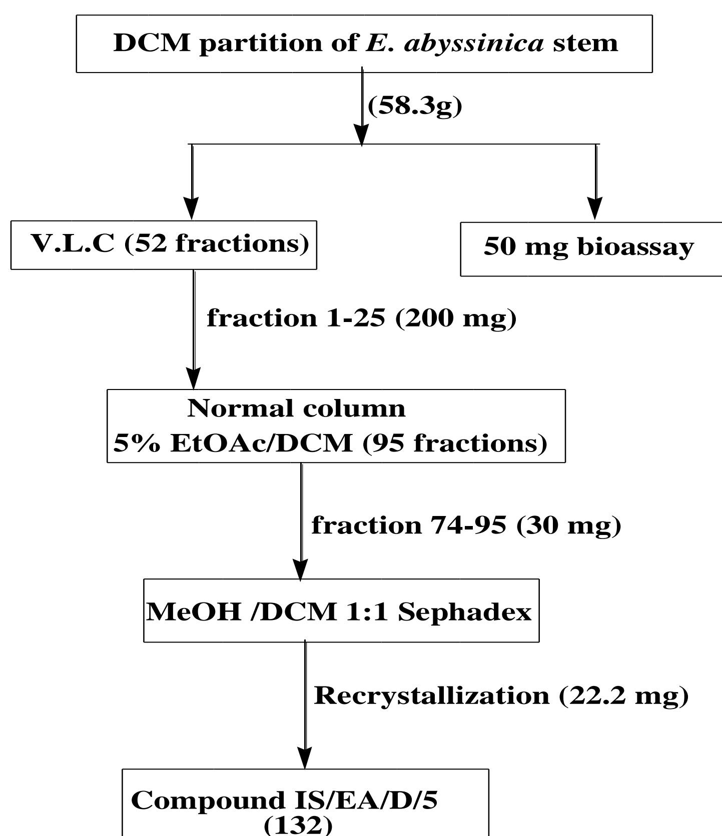


Scheme 1: Isolation of compound IS/EA/E/15 (131) from EtOAc roots barks extract of *E. abyssinica*

3.10.1 Compound IS/EA/D/5 (132)

The DCM extract 58.3 g of *E. abyssinica* stem barks from the VLC gave 52 fractions. The fractions 1-25 were positive to *p*-anisaldehyde and were combined to give 200 g. This was

loaded in a normal column and eluted with solvent system of increasing polarity, starting with 100 % *n*-hexane, then DCM, EtOAc and finally 100 % MeOH. The procedure gave 95 fractions. The fractions were spotted on a TLC plate to establish optimum solvent system for separations in which 5% EtOAc/DCM solvent system was identified. Fractions 74-95 had same R_f were recombined to give 30 mg. This was packed in 1:1 methanol /DCM Sephadex (LH 20) to give a pure compound IS/EA/D/5 (132). It was recrystallized in methanol yielding 22.2 mg as presented in the scheme 2.

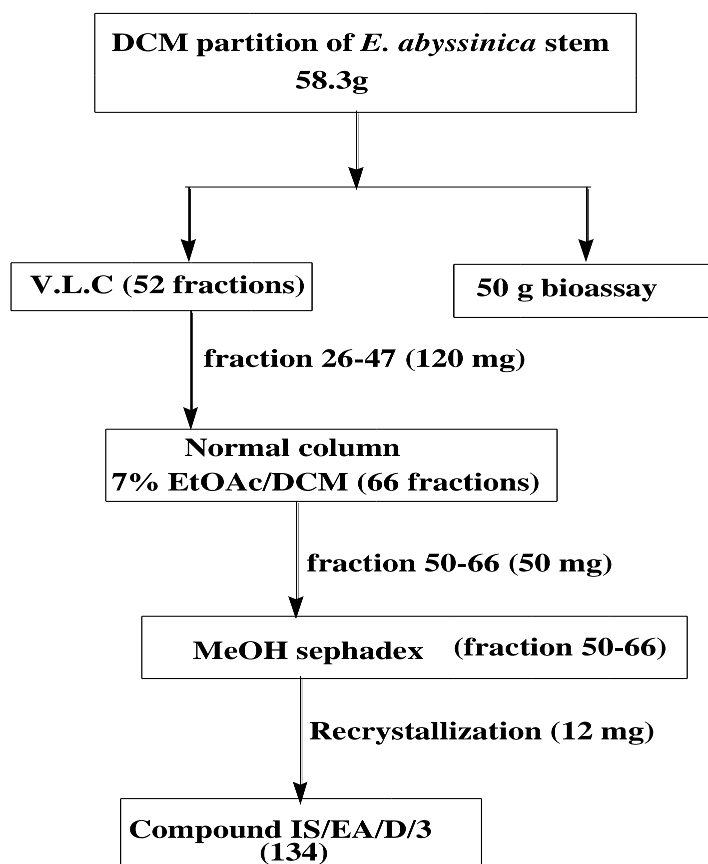


Scheme 2: Isolation of compound IS/EA/D/5 (132) from DCM stems barks extract of *E. abyssinica*

3.10.2 Compound IS/EA/D/3 (134)

The DCM partition of *E. abyssinica* stem barks from the VLC gave 52 fractions. The fractions 26-47 (120 mg) were positive to spray reagent *p*-anisaldehyde and were combined. This was loaded into a normal column and eluted with solvent system of increasing polarity, starting with 100 % *n*-hexane, then DCM, EtOAc and finally 100 % MeOH giving 66 fractions. The fractions were spotted on a TLC plate to establish optimum solvent system for separations in which 7 % EtOAc/DCM solvent system was identified. Fractions 50-56 had same R_f and were recombined to give 50 mg. This was purified in a MeOH Sephadex (LH 20) column to give compound IS/EA/D/3 (134).

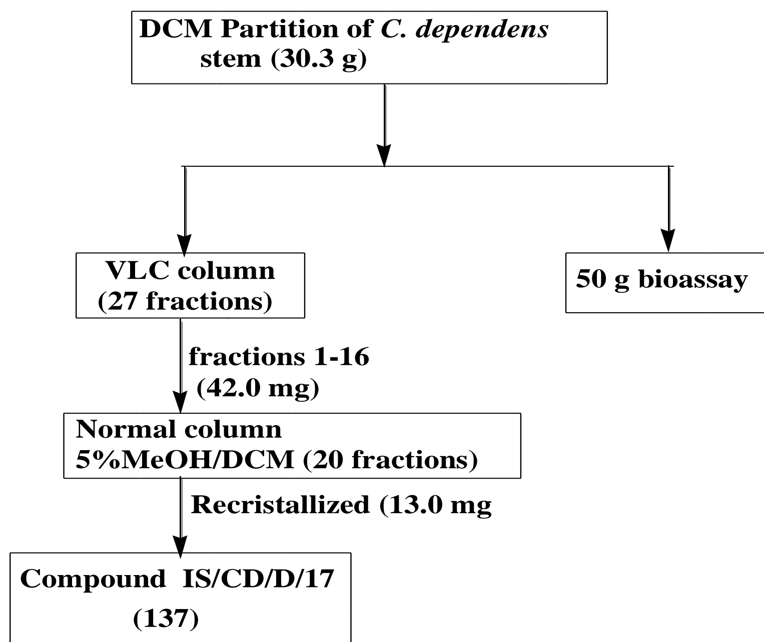
The pure compound was recrystallized with methanol to give 12 mg as presented in the scheme 3.



Scheme 3: Isolation of compound IS/EA/D/3 (134) from DCM stem barks extract of *E. abyssinica*

3.10.3 Compound IS/CD/D/17 (137)

The DCM extract of *C. dependens* stem (30.3 g) was loaded in a VLC column and eluted with 200 ml of 100 % *n*-hexane, then DCM, EtOAc and finally 100 % MeOH. A total of 27 fractions were obtained. The fractions were spotted on a TLC plate to establish optimum solvent system for separations in which 5% EtOAc /DCM solvent system was identified. Fractions 1-16 (42.0 mg) had same R_f and were pooled together. This was again loaded in a smaller normal column chromatography packed with Kiesegel silica gel 240 G and eluted with 5 % EtOAc /DCM solvent system. Fractions with same R_f were pooled together, recrystallized in $CHCl_3$ to give 13.0 mg of compound IS/CD/D/17 (137) as presented in the scheme 4.



Scheme 4: Isolation of compound IS/CD/D/17 (137) from DCM extract of the stem of *C. dependens*

3.11 Microbial test cultures

Microorganisms used were obtained from Kenya Medical Research Institute (KEMRI). These included standard and local clinical isolates from patients. Microorganisms that were used included: A Gram-positive bacteria *Staphylococcus aureus* (ATCC 25922), a Gram-negative bacteria *Pseudomonas aeroginosa* (ATCC 25923) and two fungi, *Candida albicans* (ATCC 90028) and *Trichophyton mentagrophytes* (clinical isolate) were used as fungal test microorganisms. Bacterial strains were maintained on nutrient agar (NA) petri dishes at 4^oC while fungi were maintained on sabouraud dextrose agar (SDA) petri dishes. Antifungal and antibacterial *in-vitro* assays were done using disc diffusion method. All the procedures were done according to clinical laboratory standard procedures and quality control (Chhabra and Uiso, 1992; McChesney *et al*., 1991). Briefly, fresh cultures were obtained by growing the test strains overnight at 37^oC for bacteria while fungi were grown at 28^oC for 48 hours.

3.12 Anti-bacterial screening tests

The plate diffusion method was used to test the extracts and isolated compounds against the bacteria. Antibacterial sensitivity and resistance were confirmed by use of standard discs containing tetracycline. Nutrient agar 14 g was dissolved in 0.5 litre of distilled water. This was sterilized by autoclaving at 121°C and 15 psi pressure for 15 min. A Filter paper (Rund MN 615) of 9 cm diameter was punched and the paper pieces sterilized. Nutrient agar (15 ml) was poured into petri dishes in a lamina flow apparatus under sterile conditions. Then 0.1 ml of bacterial suspension was added to it. Filter paper pieces containing 2000 µg of the test extract were put on petri dish and then finally incubated at 37ºC for bacteria. The results were

observed by measuring the diameter of the zone of inhibition from original 6 mm (Chhabra and Uiso, 1992; McChesney *et al*., 1991).

3.13 Anti-fungal screening tests

Trichophyton mentagrophytes fungi and a yeast *Candida albicans* were used for the antifungal screening. Standard antifungal agent fluconazole was used for positive control (Chhabra and Uiso, 1992; McChesney *et al*., 1991). The Agar diffusion method was used to test the plants extracts against fungi and yeast, where 2000 µg/ml of the extracts were dissolved in 1 ml DMSO and distilled water. Potatoe dextrose agar (PDA) autoclaved for 15 min at 121ºC temperature and 15 psi pressure poured into the petri dishes. The fungal solutions were poured into the holes of diameter 6 mm containing the PDA. 0.1 ml of the plant extracts solution, 100 ml of dimethyl sulphoxide (DMSO) and dilute water was added into the holes. DMSO solvent was used as a control for each petri dish. The petri dishes were covered and incubated at 27 ºC for 72 hrs. The results were taken on the third and forth day by measuring the diameter of zone of inhibition from original diameter of 6 mm (McChesney *et al*., 1991; Chhabra and Uiso, 1992).

3.14 Minimum inhibitory concentration (MIC) and minimum bactericidal/fungal Concentrations (MBC/MFC)

Minimum Inhibitory Concentration (MIC) is the lowest concentration of antimicrobial agents found to inhibit growth of a particular test organism (Aulton, 1990). The active extracts from the antimicrobial screening were tested for MIC and MBC/MFC. The MIC was determined using two-fold serial dilution method in a peptone water solution for bacterial and PDA broth for yeast and fungal of the active extracts. Each tube was then inoculated with 0.1 ml of

standardized bacterial suspension ($1x10^8$CFU/ml) and fungal suspension (1×10^8 spores/ml). The cultures were then incubated at 37°C for 24 hours for bacteria, 48 hours for yeast and at 30°C for 72 hrs for fungi. MBC and MFC were determined by sub-culturing 0.1 ml of all the tubes showing no growth on nutrient agar (NA) for bacteria and PDA plates for yeast and fungi. After 24 hours incubation at 37°C, the first plate showing no growth was the MBC, while after 48 hours at 37°C and 5 days for yeast and fungi, respectively. The first plate showing no growth was the MFC (Michael *et al*., 2003).

3.15 Disc Diffusion and MIC rating of the extracts

The negative controls of the disc diffusion testing was done by use of methanol that showed no inhibition, while positive control was done by use of standard antibiotics discs (Oxoid). The average zone of inhibition was calculated for the 3 replicates. A clearing zone of 9 mm for gram-positive and gram-negative bacteria and 10 mm for fungi or greater was used as the criterion for designating significant antibacterial and antifungal activity (Faizi *et al*., 2003). The extracts that displayed MIC lower than 100 µg/ml, the antimicrobial activity was considered very high; from 100-500 µg/ml, high; 500-1000 µg/ml, moderate; 1000-4000 µg/ml, low and anything above this, the extracts were considered inactive for both bacteria and fungi.

3.16 Structure elucidation

Structural elucidation was done on the basis of physical data such as melting point, and the spectroscopic techniques: ^{1}H NMR and ^{13}C NMR, 2D NMR techniques were used for complete elucidation of the compound.

^1H NMR spectra were on measured Varian Gemini 400 MHz in $CDCl_3$ or CD_3OD. The multiplicity of the peaks were denoted as follows; *s* = singlet, *d* = doublet, *t* = triplet, *q* = quartet and *m* = multiplet. The chemical shift values (δ) were recorded in parts per million (ppm) and residual solvent peaks used as reference. Proton noise decoupled (pnd), ^{13}C NMR, DEPT, COSY, and HSQC spectra were determined on Varian Gemini 100 MHz in CD_3OD. HMBC and NOESY were run on Varian 300 and 600 MHz machines. The multiplicities of ^{13}C signals were determined from DEPT analysis.

3.19 Physical and spectroscopic data of the isolated compounds

Compound 131

Yellow amorphous solid, mp 120-122 °C, ^1H NMR (CD_3OD, 400 MHz), δ 6.25 (1H, *d*, J = 2.3 Hz, H-3), δ 6.39 (1H, *dd*, J = 2.3 Hz, 8.9 Hz, H-4), δ 7.91 (1H, *d*, J = 15.3 Hz, H-6), δ 7.72 (1H, *d*, J=15.3 Hz, H-α), δ 7.48 (1H, *d*, J=15.3 Hz, H-β), δ 7.44 (1H, *dd*, J = 8.4 Hz, 2.0 Hz, H-2′), δ 6.78 (1H, *d*, J = 8.4 Hz, H-3′), δ 7.40 (1H, *d*, J = 2.0 Hz, H-6′), δ 3.28 (2H, *d*, J = 8.9 Hz, H-1″) δ 5.31 (1H, *t*, J = 7.3 Hz, H-2″), δ 1.72 (3H, *s*,H-4″), δ1.72, (3H, *s*, H-5″). ^{13}C NMR (CD_3OD, 100 MHZ), δ 114.7 (C1), δ 167.5 (C2), δ 103.0 (C3), δ 166.7 (C4), δ 109.0 (C5), δ 133.3 (C6), δ 193.5 (C=O), δ 146.1 (Cα), δ 129.1 (C β), δ 130.2 (C1'), δ 131 (C2'), δ 116 (C3'), δ 159.5 (C4'), δ 127.7 (C5'), δ 131.9 (C6'), δ29.3 (C1''), δ 123.6(C2'').

Compound 132

Yellow amorphous solid, mp 77-80 °C, ^1H NMR (CD_3OD, 400 MHz), δ 5.31(1H, *t*, J = 3.2 Hz, H-2), δ 2.96 (1H, *t*, J = 4.5 Hz, H-3), δ 2.63 (1H, *d*, J = 3.2 Hz, H-3), δ 7.68 (1H, *d*, J = 5.9 Hz, H-5), δ 6.46 (1H, *d*, J = 5.9 Hz, 2.6 Hz, H-6), δ 6.30 (1H, *d*, J = 2.6 Hz, H-8), δ 6.98 (1H, *s*, H-6′), δ 3.28, (2H, *d*, H-1″), δ 5.31 , (1H, *t*, H-2″), δ 1.67(3H, *s*, H-4″), δ 1.67, (3H, *s*,

H-5″). ^{13}C NMR (CD_3OD, 100 MHz), δ 81.2 (C2), δ 49.7 (C3), δ 193.6 (C4), δ 115 (C4a), δ 130.1 (C5), δ 111.7 (C6), δ 167.0 (C7), δ 103.9 (C8), δ165.5 (C8a), δ 130.1 (C1'), δ 131.7 (C2'), δ 133.7 (C3'), δ 153.7 (C4'), δ 131.7 (C5'), δ 126.4 (C6'), δ 29.7 (C1''), δ 123.6 (C2''), δ 129.8 (C3''), δ 17.9 (C4''), δ 26.0 (C5'').

Compound 134

Deep yellow amorphous powder, mp 80-83 °C, ^{1}H NMR (CD_3OD, 400 MHz), δ 4.12 (1H, *t*, J = 11.0 Hz, H-2α), δ 3.97 (1H, *dd,* J = 11.0 Hz, 5.5 Hz, H-2β), δ 3.72 (1H, *dd*, J = 11.0 Hz, 5.5 Hz, H-3), δ 7.32 (1H, *d*, J = 8.8 Hz, H-5), δ 6.07 (1H, *dd*, J = 8.8 Hz, 2.3 Hz, H-6), δ 5.89 (1H, *d*, J = 2.3 Hz, H-8), δ 6.05 (1H, *s,* H-3′), δ 6.31(1H, *s,* H-6′), δ 5.81 (1H, *d*, J = 9.8 Hz, H-1″), δ 5.08 (1H, *d*, 9.8 Hz, H-2″), δ 0.89 (3H, *s,* H-4″), δ 3.30 (3H, *s,* H-5″), δ 3.30 (3H, *s*, 2′-OCH_3). ^{13}C NMR (CD_3OD, 100 MHz), δ 72.0 (C2), δ 49.7 (C3), δ 194.3 (C4), δ 115.7 (C4a), δ 130.2 (C5), δ 112.2 (C6), δ 165.2 (C7), δ 103.7 (C8), δ 167.1 (C8a), δ 117.2 (C1'), δ 159.7 (C2'), δ 101.2 (C3'), δ 155.3 (C4'), δ 115.7 (C5'), δ 129.2 (C6'), δ 122.7 (C1'').

Compound 137

Clear needle-like crystals, mp 191-193 °C, ^{1}H NMR ($CDCL_3$, 400 MHz), δ 3.62 (1H, *d*, J = 2.0 Hz, H-2), δ 3.69 (1H, *dd*, J = 2.0 Hz, 21.6 Hz, H-3), δ 4.83 (1H, *d*, J = 21. 6Hz, H-4), δ 1.40 (1H, *m,* H-6), δ 1.66 (1H, *m,* H-6), δ 2.00 (1H, *m,* H-7), δ 2.64 (1H, *m,* H-7), δ 1.90 (1H, *t,* H-8), δ 2.39 (1H, *s,* H-10), δ 2.36 (1H, *m,* H-11), δ 1.95 (1H, *m,* H-11), δ 5.45 (1H, *t,* H-12), δ 6.42 (1H, *d*, J = 15.8 Hz, H-14), δ 7.45 (1H, *d*, J = 15.8 Hz, H-15), δ 7.42 (1H, *s,* H-16), δ 1.33 (3H, *s,* H-18), δ 1.33 (3H, *s,* H-19). ^{13}C NMR ($CDCl_3$, 100 MHz), δ 80.5 (C1), δ 50.6 (C2), δ 49.5 (C3), δ 70.6 (C4), δ 34.9 (C5), δ 25.5 (C6), δ 16.6 (C7), δ 47.5 (C8), δ 41.5

(C9), δ 44.0 (C10), δ 41.2 (C11), δ 71.0 (C12), δ 124.6 (C13), δ 108.3 (C14), δ 144.0 (C15), δ 139.0 (C16), δ 173.0 (C17), δ 23.4 (C18), δ 28.5 (C19), δ 172.2 (C20).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Crude extract yields

Dry powdered roots and stem barks of *E. abyssinica* weighed 1534.5 and 1600.3 g, respectively while root tubers, stem and leaves of *C. dependens* weighed 1681.0, 1417.9 and 1145.1 g, respectively as shown in table 1.

Table 1: Masses of plant material obtained

Plant material	Plant parts	Mass in grammes
E. abyssinica	Root barks	1534.4
	Stem barks	1600.3
C. dependens	Root tubers	1681.0
	Stem	1417.9
	Leaves	1114.1

They were sequentially soaked in the four solvents in order of increasing polarity starting from hexane, dichloromethane, ethyl acetate and finally methanol. The amount of crude extracts and percentage yields obtained was recorded and tabulated in table 2 and 3.

Table 2: Masses of sequential extraction of *E. abyssinica* and percentage yields

Root bark	**Extraction solvent**	**Mass (g)**	**% Yield**
	Hexane	10.0	0.7
	DCM	28.0	1.8
	Ethyl acetate	6.5	0.4
	Methanol	29.5	1.9
Stem bark	**Extraction solvent**	**Mass (g)**	**% Yield**
	Hexane	2.8	0.2
	DCM	58.3	3.6
	Ethyl acetate	17.8	1.1
	Methanol	58.3	3.6

Table 3: Masses of sequential extraction of *C. dependens* and percentage yields

Stem	**Extraction solvent**	**Mass (g)**	**% Yield**
	Hexane	15.8	0.7
	DCM	30.3	1.7
	Ethyl acetate	12.9	1.1
	Methanol	9.6	0.7
Root tubers	**Extraction solvent**	**Mass (g)**	**% Yield**
	Hexane	12.0	0.7
	DCM	30.0	1.7
	Ethyl acetate	19.0	1.1

	Methanol	20.8	1.2
Leaves	**Extraction solvent**	**Mass (g)**	**% Yield**
	Hexane	3.0	0.2
	DCM	5.0	0.4
	Ethyl acetate	8.3	0.2

Extraction of *C. dependens* leaves with methanol was not done. The methanol extracts of both plant parts of *E. abyssinica* while the DCM extracts of stem and root tubers of *C. dependens* had the highest percentage yields.

4.2 Antibacterial and antifungal assay for the extract

All the crude extracts were tested against two bacteria strains and two fungi to determine their activity. The pathogens used were *Staphylococcus aureus* (ATCC 259222) (gram-positive bacteria), *Pseudomonas aeroginosa* (ATCC 25923) (gram-negative bacteria), *Candida albicans* (ATCC 90028) and *Trichophyton mentagrophytes*. The zones of inhibition of bacterial and fungal growth were measured after 48 hours and the measurements were done (in mm) from the end of the growth of one side of the disc to the beginning of growth of the other side including the diameter of the disc. Results were recorded and tabulated as shown in table 4.

4.2.1 Antibacterial activity of *E. abyssinica*

The DCM stem extract of *E. abyssinica* showed activity against gram-positive bacteria *Staphylococcus aureus* with zone of inhibition of 10 mm. The methanol stem extract of *E.abyssinica* also showed activity against gram-positive bacteria *Staphylococcus aureus* with

zone of inhibition of 11 mm. There was no activity against the gram-negative bacteria

***E. abyssinica* extract**	***S. aureus***	***P. aeroginosa***	***C. albicans***	***T. mentagrophytes***

Pseudomonas aeroginosa in all the *E. abyssinica* extracts as shown in table 4.

4.2.2 Antifungal activity of *E. abyssinica*

The DCM stem barks extract of *E. abyssinica* had activity against the fungi *Tricophyton mentagrophytes* with zone of inhibition of 16 mm. The hexane stem extract had activity on the same fungi with zone of inhibition of 10 mm. The DCM root extract gave activity on same fungi with zone of inhibition of 12 mm as shown in table 4.

4.2.3 Antibacterial activity of *C. dependens*

The hexane root of *C. dependens* showed activity on *Staphylococcus aureus* with zone of inhibition of 16 mm. Other crude extracts under test had no activity on the test bacteria. There was no activity against gram-negative bacteria tested as shown in table 4.

4.2.4 Antifungal activity of *C. dependens*

The hexane root extract of *C. dependens* showed activity on *T. mentagrophytes* with zone of inhibition of 23 mm. Other crude extracts under test had no activity against the tested fungi as shown in table 4.

Table 4: The inhibition zones (in mm) of the crude extracts

Hexane root	6	6	6	6
Ethyl acetate root	6	6	6	6
Methanol root	6	6	6	6
DCM stem	**10**	6	6	**16**
Hexane stem	6	6	6	**10**
Methanol stem	**11**	6	6	6
DCM root	6	6	6	**12**
Ethyl acetate stem	6	6	6	6
***C. dependens* extract**				
hexane stem	6	6	6	6
Hexane leaves	6	6	6	6
DCM stem	6	6	6	6
DCM root	6	6	6	6
DCM root	6	6	6	6
DCM leaves	6	6	6	6
Hexane root	**16**	6	6	**23**
Ethyl acetate leaves	6	6	6	6
Stem ethyl acetate	6	6	6	6
Control experiment (+ve)	20 (Tet)	18 (Tet)	19 (Flu)	15 (Flu)

Key: *Staphylococcus aureus* ATCC 25922, *Pseudomonas aeroginosa* ATCC 25923, *Candida albicans* ATCC 90028, *Trichophyton mentagrophytes,* Tet = tetracycline, Flu = fluconazole.

The fact that there was no activity toward the gram-negative bacteria indicates their difficulty to be inhibited by many antibiotics (Tait-Kamradt *et al*., 2009). The results from the two tests (antibacterial and antifungal) indicate the bioactivity of the extracts from *E. abyssinica* and *C. dependents* hence the need for a systematic phytochemical analysis of the plants.

4.3 Antibacterial and antifungal activity of the isolated compounds

The antibacterial and antifungal assay for the pure compounds was carried out using the same procedure as discussed for the crude extracts, following the method used by Chhabra and Usio (1991). The inhibition zone was measured as in the crude extracts using the same

pathogens. The control experiments were done using standard antibiotics tetracycline and fluconazole. Results were recorded and tabulated as shown in table 5.

Table 5: The inhibition zones (in mm) of the isolated compounds

***E. abyssinica* extract**	***S. aureus***	***P. aeroginosa***	***C. albicans***	***T. mentagrophytes***
IS/EA/E/15 (131)	6	6	6	6
IS/EA/D/5 (132)	6	6	6	6
IS/EA/D/3 (134)	**15**	6	6	6
IS/CD/D/17 (137)	6	6	6	6
Control experiment (+ve)	20 (Tet)	18 (Tet)	19 (Flu)	15 (Flu)

The pure compounds from *Erythrina abyssinica* DCM extract of the stem bark coded IS/EA/D/3 was found active against *Staphylococcus aureus* ATCC 25922 with zone of inhibition of 15 mm at a concentration of 100 µg/ml. There was activity exhibited earlier towards fungi *T. mentagrophytes* by the crude extracts which is not evident with any of the compounds isolated. This can be attributed to among other factors, the synergic effects which would have boosted the activity of the compounds in the crude state, which is not present after isolation. Separation of the extracts by a combination of the chromatography techniques followed by crystallization yielded the four compounds. The other extracts could not be worked on because of limitations of especially time and other resources.

4.6 Minimum inhibitory concentration (MIC) of active crude extracts and the pure compound

The active DCM extract of *E. abyssinica* against *Staphylococcus aureus* ATCC 25922 had an MIC of 5000 µg/ml and against fungi *Trichophyton mentagrophytes* with an MIC of 3330 µg/ml. *E. abyssinica* methanol extract from the stem bark showed an MIC of 5000 µg/ml

against *Staphylococcus aureus*. *C. dependens* hexane extract of roots tubers was active against bacteria *Staphylococcus aureus* with an MIC of 3330 µg/ml and against fungi *Trichophyton mentagrophytes* with an MIC of 2000 µg/ml. The active pure compound isolated from the stem bark of *E. abyssinica* showed an MIC of 50 µg/ml. These results were as shown in table 6.

Table 6: The MIC of active extracts

Plant Extract	**Bacteria**		**Fungi**	
	S. aureus	***P. aeroginosa***	***C. albicans***	***T. mentagrophytes***
E. abyssinica DCM stem	5000	nd	nd	3330
E. abyssinica Hexane stem	nd	nd	nd	5000
E. abyssinica Methanol stem	5000	nd	nd	nd
E. abyssinica DCM root	nd	nd	nd	5000
C. dependens Hexane root	3330	nd	nd	2000
IS/EA/D/3 (134)	50	nd	nd	nd

4.7 Disc diffusion and MIC rating of the extracts

All the active crude extracts showed antibacterial and antifungal activity with a clearing zone above 9 mm for Gram-positive bacteria and above 10 mm for fungi showing a moderate activity. The pure compound code IS/EA/D/3 isolated from the stem bark of *E. abyssinica* had a very high MIC (50 µg/ml) indicating a relatively high activity. The crude extracts from both plants had a low MIC rating (1000-4000 µg/ml).

4.8 Structure elucidations

4.8.1 Compound 131

The compound was isolated as a yellow amorphous solid from a fraction of EtOAc partition of *E. abyssinica* root barks extract in 5% MeOH / DCM. It was active under UV–light (254 nm) and turned yellow on spraying with *p*-anisaldehyde.

Analysis of ^{1}H NMR (appendix 1a and 1b) spectrum gave 12 signals. The two doublet peaks at δ 7.72 and 7.48 were assigned to position α and β of a chalcone structure. They had similar splitting constants of 15.3 Hz indicating that they were adjacent to each other. The signals appearing at δ 6.25 (*d*), 6.39 (*dd*) and 7.91 (*d*) correlated in their splitting constants (2.3 Hz) (2.3 Hz, 8.9 Hz) and (8.9 Hz) and were assigned to position 6, 4 and 3, respectively. The 2.3 Hz splitting is associated with *meta* coupling while 8.9 Hz is for the *ortho* coupling. The peaks at δ 7.44 (*dd*, J = 8.4 Hz, 2.0 Hz) and δ 6.78 (*d*, J = 8.4 Hz) were adjacent to each other at positions 2′ and 3′, respectively. The singlet signal at δ 1.72 was of methyl hydrogen atoms at positions 4″ and 5″. Table 7 shows the chemical shifts, multiplicity and coupling constants of compound 131.

Table 7: ^{1}H NMR data (400 MHz, CD_3OD) for compound 131

Position	δ H (ppm)	Multiplicity	J (Hz)	Integral
3	7.91	*d*	2.3	1H
4	6.39	*dd*	2.3, 8.9	1H
6	6.25	*d*	8.9	1H
α	7.72	*d*	15.3	1H
β	7.48	*d*	15.3	1H
2'	7.44	*dd*	8.4, 2.0	1H
3'	6.78	*d*	8.4	1H
6'	7.40	*d*	2.0	1H
1''	3.28	*d*	8.9	2H

2"	5.31	*t*	7.3	1H
4"	1.72	*s*	-	3H
5"	1.72	*s*	-	3H

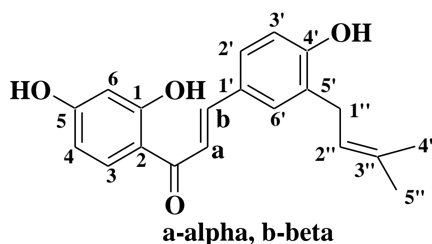
The ^{13}C NMR spectrum (appendix 1c) showed 20 signals. The DEPT experiment (appendix 1d) revealed that the compound has two methyl carbons at δ 26.0 and 17.9. They were assigned to positions 4″ and 5″. The signal at δ 193.5 indicated the presence of a carbonyl group at in the compound. This was given position 2a. There are nine methines at δ 103.0 (C3), δ 109.0 (C5), δ 133.3 (C6), δ146.1 (Cα), δ 129.1 (Cβ), δ 131.9 (C2′), δ 116.3 (C3′), δ 131.9 (C6′) and δ 123.6 (C2″). The DEPT experiment also showed one methylene signal at δ 29.3 and was assigned to positions 1″. There were three hydroxyl groups appearing at δ 166.7, δ 167.5 and δ 159.5 and were allocated positions 4, 2 and 4′, respectively. HSQC spectra (appendix 1e,f and i) was used to assign the protonated carbon atoms to the respective protons. These indicated the following correlation; (δ_C 103.0, δ_H 6.25), (δ_C 109.0, δ_H 6.3), (δ_C 133.3, δ_H 7.91), (δ_C 146.1, δ_H 7.72), (δ_C 131.9, δ_H 7.48), (δ_C 131.9, δ_H 7.44), (δ_C 116.3, δ_H 6.78), (δ_C 131.9, δ_H 7.40), (δ_C 29.3, δ_H 3.28), (δ_C 123.6, δ_H 5.31), (δ_C 26.0, δ_H 1.72) and (δ_C 17.9, δ_H 1.72) (Table 8).

HMBC spectra (Appendices 1j and 1k) were used to assign C→H three bond correlations. The following carbon atoms to proton correlations were observed. C1 (H-3, H-6), C2 (H-6), C4 (H-6), C5 (H-3), C=0 (H-6, H-β, H-α), C-β (H–2′,H-6′), C1′ (H-α, H-3′), C2′ (H-β), C4′ (H-2′, H-6′), C6′ (H-2′, H-β), C1″ (H-6′), C2″ (H-4″,H–5″), C4″ (H-2″), C5″ (H-2″). The COSY spectra (Appendices 1g,h,l and m) was used to identify neighboring protons in the structure in which the following protons were observed as neighboring each other; (H-1″ and H-2″), (H-α and H-β), (H-5 and H-6) and (H-2′ and H-3′). Appendices 1n and 1o gives the spectra of the NOESY experiment. These observations are summarized in table 8.

Table 8: ^{13}C NMR data (100 MHz, CD_3OD) of compound 131

Position	δ C (ppm)	HSQC	HMBC
1	114.7	-	H-3, H-6
2	167.5	-	H-6
3	103.0	6.25	H-4
4	109.0	6.39	-
5	166.7	-	H-3
6	133.3	7.91	-
C=O	193.5	-	H-6, H-β, H-α
α	146.1	7.72	-
β	129.1	7.48	H-2',H-6′
1'	130.2	-	H-α, H-3'
2'	131.9	7.44	H-β
3'	116.3	6.78	-
4'	159.5	-	H-2', H-6'
5'	127.7	-	H-β, H-1"
6'	131.9	7.40	H-2', H-β
1"	29.3	3.28	H-6'
2"	123.6	5.31	H-4", H-5"
3"	118.0	-	H-4", H-5"
4"	26.0	1.72	H-2"
5"	17.9	1.72	H-2"

The structure of this compound was proposed to be 1,5,4′-trihydroxy-5′-prenylchalcone and is isolated from *E. abyssinica* root barks for the first time.



131

4.8.2 Compound 132

This compound was isolated as a yellow amorphous solid from a fraction of DCM partition of *E. abyssinica.* It was fluorescing under UV-Light (254 nm) and turned yellow on spraying with *p*-anisaldehyde. Analysis of the ^{1}H NMR spectrum (appendix 2a and 2b) suggested a prenylated flavanone. The spectrum revealed 11 chemical shifts. The methyl hydrogens appearing at δ 1.67 as singlet were assigned to positions 4'' and 5''. Two aromatic hydrogens appearing at δ 7.68 and 6.46 with a coupling constant of 5.9 Hz indicated that they are adjacent to each other. They were assigned to position 5 and 6 respectively. The signal at δ 6.30 had a splitting correlation with signal at δ 6.46 (position 6) with a splitting constant of 2.6 Hz and was assigned position 8. The singlet at δ 6.98 was assigned position 6'. The assignment of ^{1}H NMR signal to this compound is summarized in table 9.

Table 9: ^{1}H NMR data (400MHz, CD_3OD) for compound 132

Position	δH (ppm)	Multiplicity	J (Hz)	Integral	δH of compound 133
2	5.31	*d*	3.2	1H	5.43, 1H, *dd* (12.7, 3.0)
3a	2.96	*dd*	4.5,3.2	1H	3.00, 1H, *dd* (16.7, 12.7)
3b	2.63	*dd*	4.5, 3.2	1H	2.71, 1H, *dd* (16.7, 3.0)
5	7.68	*d*	5.9	1H	7.73, 1H, *d* (8.6)
6	6.46	*dd*	5.9, 2.6	1H	6.59, 1H, *dd* (8.6, 2.2)
8	6.30	*d*	2.6	1H	6.45, 1H, *d* (2.2)
2′					6.97, 1H, *d* (2.0)
6'	6.98	*s*	-	1H	6.87, 1H, *d* (2.0)
1''	3.28	*d*	-	2H	3.38, 2H, *d* (7.2)
2''	5.31	*t*	-	1H	5.31, 1H, *t* (7.2)
4''	1.67	*s*	-	3H	1.75, 3H, *s*
5''	1.67	*s*	-	3H	1.73, 3H, *s*
$-OCH_3$					3.82, 3H, *s*

The ^{13}C NMR spectrum presented in appendix 2c gave 20 signals for this compound (Table 10). The signals at δ 193.6 indicated the existence of a carbonyl group in the structure at position 4. The DEPT experiment (appendix 2 d) suggested two methyl groups having

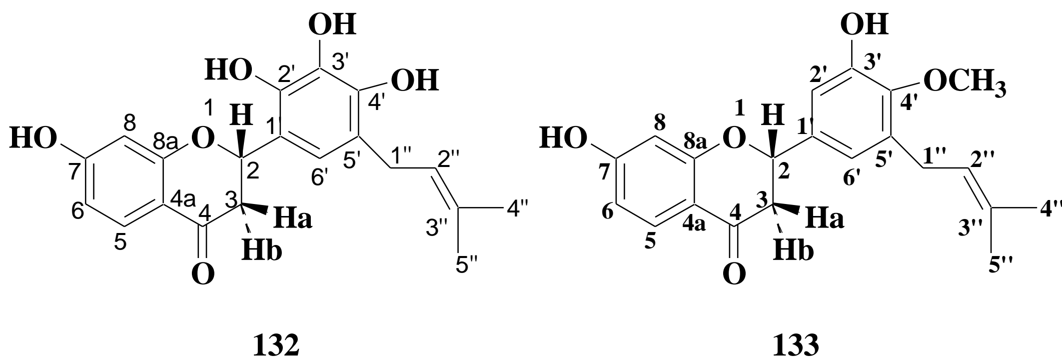
chemical shifts of δ 17.9 and 26.0 were assigned to position 4'' and 5″, respectively. The DEPT experiment also indicated two methylene carbons at δ 49.7 and δ 29.7. They were allocated positions 3 and 1'', respectively. There were six methine carbon atoms at δ 130.1 (C5), δ 111.7 (C6), δ 103.9 (C8), δ 81.2 (C2), δ 126.4 (C6') and δ 123.6 (2''). Quaternary carbon atoms appeared at δ 115.0 (C4a), δ 165.5 (C8a), δ 130.1 (C1'), δ 131.7 (C5') and δ 129.8 (C3''). There were four hydroxylated carbons at δ 167.0 (C7), δ 131.7 (C2'), δ 133.7 (C3') and δ 153.7 (C4'). HSQC spectra were used to assign the protonated carbon atoms to the respective protons. Appendix 2e indicated the following correlation; (δ_C 81.2, δ_H 5.31), (δ_C 49.7, δ_H 2.96, δ_H 2.63), (δ_C 130.1, δ_H 7.68), (δ_C 111.7, δ_H 6.46), (δ_C 103.9, δ_H 6.30), (δ_C 126.4, δ_H 6.98), (δ_C 29.7, δ_H 3.28), (δ_C 123.6, δ_H 5.31), (δ_C 17.9, δ_H 1.67), and (δ_C 26.0, δ_H 1.67).

HMBC spectra (Appendix 2f, 2g, 2h and 2i) were used to assign C→H long - range correlations. The following carbon atoms to proton correlations were observed. C2 (H-6, H-a), C4 (H-2, H-5, H-b), C4a (H-8, H-6), C6 (H-8), C7 (H-5), C8 (H-6), C8a (H-5), C1′ (H-3), C2′ (H-2), C4′ (H-6′, H-1″), C5′ (H-2″), C6′ (H-2, H-1″), C1″ (H-6′), C2″ (H-4″, H-5″), C3″ (H-1″), C4″ (H-2″) and C5″ (H-2″). The COSY spectra (appendix 2j and 2k) was used to identify neighboring protons in the structure in which the following protons were observed as neighboring each other; (δ_H 7.68 and δ_H 6.46), (δ_H 2.96 and δ_H 5.31), (δ_H 2.63 and δ_H 5.31), (δ_H 2.96 and δ_H 2.63). Appendix 2l gives the spectrum of the NOESY experiment. These observations are summarized in table 10. The ^{13}C spectral data of this compound was compared with those of 7,3′-dihydroxy-4′-methoxy-5′-prenylflavanone (**133**) carried out at

75 MHz in acetone-d6 as shown in table 10. Compound **(133)** was isolated from *Erythrina latissima* E.Meyer (Dewick, 1994).

Table 10: ^{13}C NMR data (100MHz, CD_3OD) for compound 132

Position	Chemical shift (δ)	HSQC (δ)	HMBC (δ)	δC of compound 133
2	81.2	5.31	H-6; H-a,	79.9
3	49.7	2.96, 2.63	-	44.2
4	193.6	-	H-2, H-5, H-b,	190.0
4a	115.0	-	H-8, H-6	114.3
5	130.1	7.68	-	128.9
6	111.7	6.46	H-8	110.8
7	167.0	-	H-5	165.3
8	103.9	6.30	H-5	103.2
8a	165.5	-	H-5	163.9
1′	130.1	-	H-3	135.6
2′	131.7	-	H-2	112.9
3′	133.7	-	-	150.0
4′	153.7	-	H-6′, H-1″	146.2
5′	131.7	-	H-2″	135.7
6′	126.4	6.98	H-2, H-1″	119.1
1″	29.7	3.28	H-6″	28.7
2″	123.6	5.31	H-4″, H-5″	123.4
3″	129.8	-	H-1″	131.8
4″	17.9	1.67	H-2″	17.4
5″	26.0	1.67	H-2″	25.4
$-OCH_3$				60.2



The structure of compound (**132**) was proposed to be that of 2′,3′,4',7-tetrahydroxy-5'-prenylflavanone and was isolated for the first time from *E. abyssinica*.

4.8.3 Compound 134

This compound was isolated as a deep yellow amorphous powder from a fraction of DCM partition of *E. abyssinica* stem extract in 7% EtOAc/DCM. It was fluorescing under UV-light (254 nm) and turned yellow on spraying with *p*-anisaldehyde. The ^{1}H-NMR data (appendix 3a) revealed 13 chemical shifts (Table 11). The methyl hydrogens appeared at δ 0.89 as singlets were assigned to positions 4″ and 5″. Two aromatic hydrogens centered at δ 7.32 and 6.07 with a coupling constant of 8.8 Hz indicated that they are adjacent to each other. They were assigned to positions 5 and 6, respectively. Signals at δ 5.08 and 5.81 had similar coupling constants of 9.8 Hz and appeared as doublets. They were assigned to position 2″ and 1″. The methoxy hydrogens appearing at δ 3.30 as a singlet were assigned position 2′. The complete assignment of the ^{1}H NMR Signals to this compound is summarized in table 11. The ^{1}H spectral data of this compound was compared with those of semilicoisoflavone or erylatissin B. (**135**) isolated from *Erythrina latissima* E.Meyer and carried out at 300 MHz in acetone-d6 as in Table 11 (Nkengfack *et al*., 1994).

Table 11: ^{1}H NMR data (400 MHz, CD_3OD) for compound 134

Position	δH (ppm)	Multiplicity	J(Hz)	Integral	δH of compound 135
2					8.18, *s*
2a	4.12	*t*	11.0	1H	
2b	3.97	*dd*	11.0,5.5	1H	
3	3.72	*dd*	11.0,5.5	1H	
5	7.32	*d*	8.8	1H	8.08, *d* (8.8)
6	6.07	*dd*	8.8,2.3	1H	7.01, *dd* (8.7, 2.2)
8	5.89	*d*	2.3	1H	6.92, *d* (2.2)
2′					7.06, *d* (2.0)
3′	6.05	*s*	_	1H	
6′	6.31	*s*	_	1H	6.87, *d* (2.0)
1″	5.81	*d*	9.8	1H	6.43, *d* (9.8)
2″	5.08	*d*	9.8	1H	5.77, *d* (9.8)
4″	0.89	*s*	_	3H	1.46, *s*
5″	0.89	*s*	_	3H	1.46, *s*,
2′-OCH_3	3.30	*s*	_	3H	

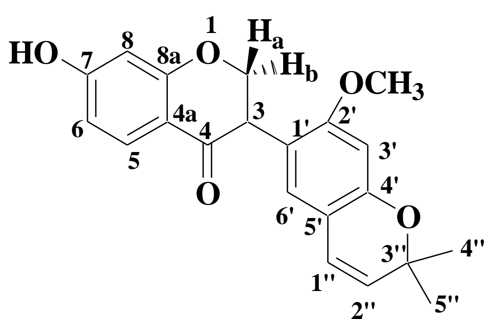
^{13}C-NMR data presented in appendix 3b gave 21 signals for this compound. The signal at δ 194.3 indicated existence of a carbonyl group in the structure at position 4. The DEPT experiment (Appendix 3c) suggested two methyl groups having chemical shifts of δ 28.3 and were assigned to positions 4″ and 5″. The DEPT spectrum also indicated one methylene group at δ 72.0 (position 2), eight methine carbon atoms at δ 130.2 (C-5), δ 112.2 (C-6), δ 103.7 (C-8), δ 49.7 (C-3), δ 101.2 (C-3′), δ 129.2 (C-6′), δ 122.7 (C-1″), and δ 129.2 (C-2″). Quaternary carbon atoms appeared at δ 115.7 (C-4a), δ 117.2 (C-1″), δ 155.3 (C-4′), δ 115.7 (C-5′) and δ 77.6 (C-3″). There was a hydroxylated and a methoxylated carbons appearing at δ 165.2 (C-7) and δ 159.7 (C-2′), respectively. The HSQC experiment (appendix 3d) confirmed the following correlation; C-2 (δ_H 4.12, δ_H 3.97), C-3 (δ_H 3.72), C-5 (δ_H 7.32), C-6 (δ_H 6.07), C-8 (δ_H 5.89), C-3′ (δ_H 6.05), C-6′ (δ_H 6.31), C-1″ (δ_H 5.81), C-2″ (δ_H 5.08), C-4″ (δ_H 0.89), C-5″ (δ_H 0.89), – OCH_3 (δ_H 3.30).

HMBC spectra (Appendix 3e and 3f) were used to assign C→H three bond correlations. The following carbon atoms to proton correlations were observed; H-3 (δ_C 115.7, δ_C 129.2), H-5 (δ_C 194.3, δ_C 165.8, δ_C 167.1), H-6 (δ_C 103.7, δ_C 115.7), H-8 (δ_C 112.1, δ_C 115.7), H-3′ (δ_C 117.2, δ_C 115.7), H-6′ (δ_C 122.7, δ_C 155.3, δ_C 159.7), H-1″ (δ_C 77.6, δ_C 115.7, δ_C 155.3, δ_C 129.2), H-2″ (δ_C 115.7, δ_C 28.3), H-4″ (δ_C 129.2, δ_C 28.3), – OCH_3 (δ_C 159.7). The COSY spectrum (Appendix 3g) was used to identify neighboring protons in the structure in which the following protons were observed as neighboring each other; (δ_H 7.32, δ_H 6.07), (δ_H 3.72, δ_H 4.12, δ_H 3.97), (δ_H 5.08, δ_H 5.81). Appendix 3h gives the spectrum of the NOESY experiment. The complete assignment of the ^{13}C NMR Signals to this compound is summarized in table 12. The ^{13}C spectral data of this compound was compared with those of semilicoisoflavone or erylatissin B (**135**) carried out at 75.4 MHz in acetone-d6 as shown in table 12. Compound **(135)** was isolated from *Erythrina latissima* E.Meyer (Nkengfack *et al*., 1994).

Table 12: ^{13}C NMR data (100MHz, CD_3OD) of compound 134

Position	δ C (ppm)	HSQC (δ)	HMBC (δ)	δC of compound 135
2a	72.0	4.12,	C117.2, C167.1, C194.3	152.9
2b	72.0	3.97	C117.2, C167.1, C194.3	
3	49.7	3.72	C115.7, C159.7, C129.2	125.3
4	194.3			175.1
4a	115.7			118.1
5	130.2	7.32	C194.3, C165.8, C167.1	128.0
6	112.2	6.07	C103.7, C115.7	115.2
7	165.2			162.7
8	103.7	5.89	C112.1, C115.7	102.7
8a	167.1			158.3
1′	117.2			121.8
2′	159.7			116.9
3′	101.2	6.05	C117.2, C115.7	145.5

4′	155.3			140.0
5′	115.7			124.6
6′	129.2	6.31	C122.7, C155.3, C159.7	118.3
1″	122.7	5.81	C77.6, C115.7, C155.3, C129.2,	122.6
2″	129.2	5.08	C115.7, C28.3	131.4
3″	77.6			76.9
4″	28.3	0.89	C129.2, C28.3, C129.2, C28.3	27.5
5″	28.3	0.89	C129.2, C28.3	27.5
2′-OCH_3	56.1	3.30	C159.7	



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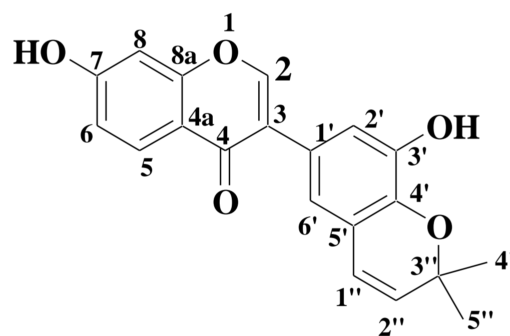
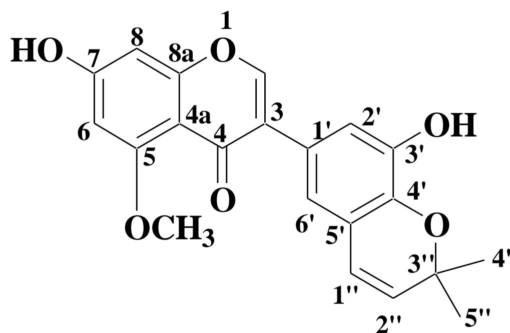


135

The structure of compound (**134**) was assigned the structure of 2'-methoxy-nor-glycyrrisoflavanone, based on glycyrrisoflavanone **(136)** isolated from Glycyrrhiza species (Hatano *et al*., 1988).



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4.8.4 Compound 137

This compound was isolated as a clear needle like crystal from a fraction of hexane extract of *C. dependens.* It was fluorescing under UV- light and showed pink to purple colour on spraying with *p*-anisaldehyde, suggesting a terpenoid structure. Analysis of the ^{1}H NMR (appendix 4a and 4b) gave 17 proton signals (Table 13). The double peak at δ 3.62 was adjacent to the peak at δ 3.69 both having a splitting constant of 2.0 Hz. They were assigned to position 2 and 3, respectively. The doublet peak at δ 4.83 was also adjustment to the peak at δ 3.69 having splitting constant of 21.6 Hz. It was assigned position 4. The singlet peak at δ 7.42 was assigned position 16. The doublet peaks at δ 6.42 and δ 7.45 had a similar coupling constant of 15.8 Hz. This suggested that they were adjacent to each other and were allocated position 14 and 15, respectively. The singlet peaks at δ 1.33 were associated with hydrogens of the methyl group and were assigned to position 18 and 19. Table 13 gives a complete assignment.

Table 13: ^{1}H NMR data (400MHz, $CDCl_3$) for compound 137

Position	δ H	multiplicity	Hz	Integral
2	3.62	*d*	2.0	1H
3	3.69	*dd*	2.0, 21.6	1H
4	4.83	*d*	21.6	1H
6	H_a = 1.40, H_b = 1.66	*m*	-	2H
7	H_a = 2.00, H_b = 2.64	*m*	-	2H
8	1.90	*t*	-	1H
10	2.39	*s*	-	1H
11	H_a = 2.36, H_b = 1.95	*m*	-	2H
12	5.45	*t*	-	1H
14	6.42	*d*	15.8	1H
15	7.45	*d*	15.8	1H
16	7.42	*s*	-	1H
18	1.33	*s*	-	3H
19	1.33	*s*	-	3H

The [13] C NMR spectra (appendix 4d) indicated 20 peaks (Table 14). The carbon chemical shifts at δ 124.6, δ 108.3, δ 144.0 and δ 139.0 confirmed the structure of a furanoditerpene. The peaks were assigned C13, C14 and I6. The peaks at δ 173.0 and δ 172.2 indicated the presence of ester carbonyl groups assigned position 17 and 20. The DEPT experiment (appendix 4c) indicated two methyl groups appearing at δ 23.4 and δ 28.5. They were assigned position 18 and 19, respectively. The DEPT spectrum also showed three methylene groups appearing at δ 25.5, δ 16.6 and δ 41.2. They were allocated position 6, 7 and 11, respectively.

The DEPT experiment indicated nine methines appearing at δ 50.6 (C2), δ 49.5 (C3), δ 70.6 (C4), δ 47.2 (C2), δ 44.0 (C10), δ 71.0 (C12), δ 108.3 (C14), δ 144.0 (C15), and δ 139.0 (C16). There was only one hydroxylated carbon appearing at δ 80.5 and was assigned position 1. Quaternary carbon atom appearing at δ 34.9, δ 41.5 and δ 124.6 were allocated to positions 5, 9, 13, respectively. The peaks at δ 50.6 (C2) and δ 49.5 (C3) suggested epoxy connectivity. HSQC spectra were used to assign the protonated carbon atoms to the respective protons. Appendices 4e and 4f indicated the following correlation; (δ_C 50.6, δ_H 3.65) ,(δ_C 49.5, δ_H 3.69) (δ_C 80.5, δ_H 4.83), (δ_C 25.5, δ_H 1.40, δ_H 1.66), (δ_C 16.6, δ_H 2.00, δ_H 2.64), (δ_C 47.2, δ_H 1.90), (δ_C 44.0, δ_H 2.39), (δ_C 41.2, δ_H 2.36, δ_H 1.95), (δ_C 71.0, δ_H 5.45), (δ_C 108.3, δ_H 6.42), (δ_C 139.0, δ_H 7.45), (δ_C 144.0, δ_H 7.42) (δ_C 23.4, δ_H 1.33) and (δ_C 28.5, δ_H 1.33).

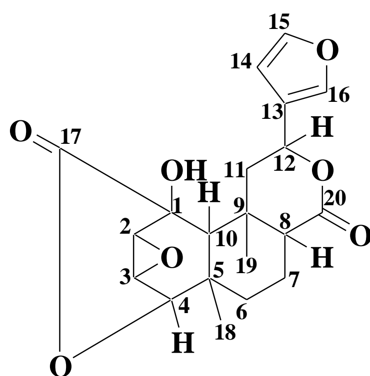
The HMBC spectrum (Appendix 4g) was used to assign C→H three bond correlations. The following carbon atoms to proton correlations were observed; δ_C 70.6 (H-3), δ_C 50.6 (H-4,

H-10), δ_C 80.5 (H-10, H-6), δ_C 25.5 (H-4, H-10, H-8), δ_C 47.2 (H-6, H-10), δ_C 41.5 (H-7, H-12), δ_C 44.0 (H-2, H-11, H-8), δ_C 41.2 (H-10, H-8), δ_C 71.0 (H-14, H-16), δ_C 124.6 (H-15, H-11), δ_C 108.3 (H-12, H-16), δ_C 139.0 (H-16), δ_C 144.0 (H-12, H-14), δ_C 173.0 (H-2, H-4, H-10), δ_C 23.4 (H-4, H-6, H-10), δ_C 28.5 (H-8, H-10, H-11), and δ_C 172.2 (H-7,H-12). The COSY spectrum (Appendices 4h, 4i and 4j) was used to identify neighboring protons in the structure in which the following protons were observed as neighboring each other; (δ_H 3.62 and δ_H 4.83), (δ_H 1.66 and δ_H 2.64), (δ_H 1.40, δ_H 1.66, δ_H 2.00 and δ_H 2.64), (δ_H 1.90 and δ_H 2.64), (δ_H 6.42 and δ_H 7.45), (δ_H 5.45, δ_H 1.95 and δ_H 2.36). Appendices 4k and 4l) gives the spectra of the NOESY experiment. Table 14 gives a complete assignment of this compound. The ^{13}C spectral data of this compound was compared with those of columbin (**138**) isolated from *Tinospora sagittata* var. *yunnanensis* (Castro and Coll, 2008).

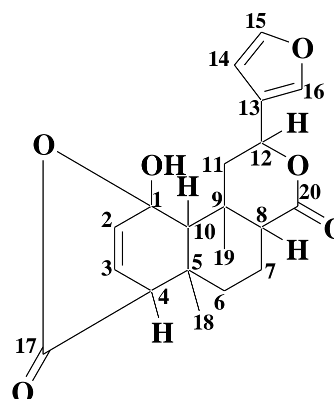
Table 14: ^{13}C NMR data (100MHz, $CDCl_3$) for compound 137

Position	δ C	HSQC	HMBC	δ C literature data of columbin
1	70.6	-	H-3	74.2

2	50.6	3.65	H-4, H-10	128.7
3	49.5	3.69	-	136.8
4	80.5	4.83	H-10, H-6	80.5
5	34.9	-	-	37.2
6	25.5	1.40, 1.66	H-4, H-10, H-8	25.5
7	16.6	2.00, 2.64	-	17.3
8	47.2	1.90	H-6, H-10	47.6
9	41.5	-	H-7, H-12	35.3
10	44.0	2.39	H-2, H-11, H-8	44.5
11	41.2	2.36, 1.95	H-10, H-8	41.9
12	71.0	5.45	H-14, H-16	70.7
13	124.6	-	H-15, H-11	124.8
14	108.3	6.42	H-12, H-16	108.4
15	139.0	7.45	H-16	139.7
16	144.0	7.42	H-12, H-14	144.0
17	173.0	C=0	H-2, H-4, H-10	175.5
18	23.4	1.33	H-4, H-6, H-10	24.3
19	28.5	1.33	H-8, H-10, H-11	27.0
20	172.2	C=0	H-7, H-12	172.4



137



138

Compound (**137**) was assigned the structure 2,3-epoxycolumbin (furano diterpenoid).

CHAPTER FIVE

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

Phytochemicals may be important tools for the effective control of infectious diseases caused by bacteria and fungi. Phytochemicals are currently receiving more attention because they are effective in treatment of infectious diseases while simultaneously mitigating many of the side effects caused by convectional antmicrobials. This study focused on the antimibacterial and antifungal effects of plants *Erythrina abyssinica* and *Chasmanthera dependens*. The following observations were made during the study:

i. *E. abyssinica* DCM crude extract of the stem had a low activity against *S. aureus* and the fungi *T. mentagrophytes* with inhibition zones of 10 and 16 mm, respectively.

ii. The hexane extract of *E. abyssinica* stem had a low activity against the fungi *T. mentagrophytes* with inhibition zone of 10 mm.

iii. The methanol extract of *E. abyssinica* stem had a low activity against *S. aureus* with inhibition zones of 11 mm.

iv. The hexane extract of root tuber of *C. dependens* was highly active against *S. aureus* and *T. mentagrophytes* with inhibition zones of 16 and 23 mm, respectively.

v. Three flavonoids 2',4′,4-trihydroxy-5'-prenylchalcone, 2',3′,4′,7-tetrahydroxy-5'-prenylflavanone and 2'-methoxy-nor-glycyrrisoflavanone were isolated from *Erythrina abyssinica* and 2, 3-epoxycolumbin (furano diterpenoid) from *C. dependens.*

vi. The compound 2'-methoxy-nor-glycyrrisoflavanone isolated from *E. abyssinica* was active against *S. aureus* with inhibition zone of 15 mm.

vii. The standard antibiotics had no activity against *S. aureus* (MDRS) except oxaccilin with zone of inhibition of only 8 mm while from this study *C. dependens* hexane extract from the roots tubers and the isolated compound 2'-methoxy-nor-glycyrrisoflavanone were highly active with zone of inhibition of 16 and 15 mm, respectively.

viii. The standard antibiotic, fluconazole had an activity against *T. mentagrophytes* with 15 mm zone of inhibition while the hexane root extract of *C. dependens* had an activity as high as 23 mm zone of inhibition.

5.2 Recommendations

This study has demonstrated that there is need for further investigation and bioassay guided isolation of pure compounds from *E. abyssinica, C. dependens* and other plants from the same family in addition to the following recommendations;

i. The crude extracts of both plants need to be subjected further to other disease causing microbes (bacteria and fungi).

ii. Further pure compounds need be isolated from both plants and subjected to bio-assay tests.

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