PHYTOCHEMICAL AND BIOLOGICAL STUDIES OF THE COMPOUNDS OF AERIAL PARTS OF *SENECIO LYRATUS* (ASTERACEAE)

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AND TECHNOLOGY

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Phytochemical and biological studies of the compounds of aerial parts of *senecio lyratus* (asteraceae)

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A thesis submitted in partial fulfillment for the degree of Master of Science in chemistry in the Jomo Kenyatta University of Agriculture and Technology (JKUAT).

2008

DECLARATION

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

Signature…….. Date……

Philip Ngunyi Ndiritu

This thesis has been submitted for examination with our approval as university supervisors.

1. Signature……. Date……

   Prof. Joseph M. Keriko

   J.K.U.A.T, Kenya

2. Signature……. Date……..

   Prof. Gathu Nyagah

   J.K.U.A.T, Kenya

DEDICATION

To my parents, my wife Nancy, my children, Ndiritu, Mumbi and Wangui, my brothers and sisters.

ACKNOWLEDGEMENT

I wish to sincerely thank my supervisors; Prof. Joseph Keriko and Prof. Gathu Nyagah for their guidance, supervision and continuous support throughout the course of research and writing up of the thesis without which this work could not have been the way it is.

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Above all I am grateful to GOD who gave me life and provided all that was needed in the course of study.

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ABBREVIATIONS

^{13}C NMR	Carbon-13 nuclear magnetic resonance
^{1}HMMR	Proton nuclear magnetic resonance
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
DCM	Dichloromethane
DEE	Diethyl Ether
DMSO	Dimethyl sulphoxide
EtOAc	Ethyl acetate
FT-IR	Fourier Transform Infrared
LD_{50}	Lethal dose at 50%
LD_{50} ip	Lethal dose at 50% Intra Peritoneal
MeOH	Methanol
Mp	Melting point
ppm	Parts per million
PTLC	Preparative Thin Layer Chromatography
TLC	Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
VLC	Vacuum Liquid Chromatography

ABSTRACT

Extracts of the aerial parts of *Senecio lyratus* were phytochemically and biologically studied. The extracts exhibited biological activity against bacterial and fungi. The aerial parts of *S. lyratus* were collected from Kericho District in Kenya. Brine shrimps (*Artemia salina*) lethal toxicity tests were done on *n*- hexane, dichloromethane and methanol extracts. Their LD_{50} were 506.11, 553.21 and 689.44 ppm, respectively. The n-hexane extract was found to be more potent and its column chromatographed fractions were subjected to anti-bacterial and anti-fungal tests *in vitro*. Two fractions RSL9 and RSL5 had equal inhibition diameters of 9.00 mm against *Staphylococcus aureas* and 8.33 mm against *Bacillus subtilis* at 1000 ppm. The isolated pure compounds were also subjected to anti-bacterial and anti-fungal assay test and proved to be relatively active. Compounds Rsle and Rslb had inhibition diameters of 10.00 and 11.67 mm against *Candidas albicans* fungi, respectively. The highest inhibition diameters for Rsle and Rslb against *Pseudomonas aureginosa* bacteria were 8.33 and 7.33 mm, respectively at 1000 ppm. Three compounds were characterized from the *n*-hexane extract by use of physical and spectroscopic data. The isolated compounds were β-amyrin (**24),** β-sitosterol (**25**) and stigmasterol (**26**). A fourth compound is yet to be characterized.

CHAPTER ONE

INTRODUCTION

1.1 Background

Man has, from time immemorial relied on plants as a source of food and shelter for his survival. He has also discovered that plants could be used for treating his various ailments including those of other animals. Moreover, plant extracts could be used to protect other plants from pests. The importance of plant extracts to be used for medicinal, pesticides, fungicides and insecticides cannot be underestimated. For instance, it is estimated that 20,000 plants are used for medicinal purposes (Penos, 1982).

Microbes play an important part in the cyclic changes that the biological elements undergo on earth. In this sense they are of transcendental importance in the terrestrial economy, because without them higher organisms would rapidly cease to exist. Yet they couple these fundamental activities with a number of other functions which may be valuable, trivial or a thorough nuisance to mankind. Microbes, for example, cause most diseases. From a biological point of view, disease is valuable in that it limits excessive animal population, although that can be highly inconveniencing to the civilized World today. Pollution and putrefaction are all very well in their place. Our sewage systems depend on them, but out of control they can be disagreeable and destructive. Microbes ferment foods, yielding delicious delicacies and wines, but tainted food is dangerous.

Microbes aid our digestion and nutrition, but some upset our stomachs. Over geological time microbes formed several of the worlds' most valuable mineral deposits, but when they corrode

steel and concrete we do not welcome their peculiar propensities. Microbes are neither generally good nor generally bad they can be either. The important thing, which is not realised, is that they have an enormous effect on the economy and well being of mankind (Postgate, 1992).

Microorganisms are relentless opportunists. Because of their astronomical numbers, their phenomenal growth rate and their adaptability, they can often exploit new circumstances to grow and cause disease. The bacterium responsible for legionaries' disease, for example, occurs in many streams and lakes, where it has no effect on human health. But when the same microbe is released into the air, as an aerosol by improperly maintained cooling towers, for example, it can prove deadly. Breathed into the lungs, this otherwise innocuous bacterium causes a virulent type of pneumonia, which is often fatal.

Most micro-organisms are free-living and perform useful activities that benefit animal and plant life. Microorganisms that have the ability to cause diseases are called pathogens. They include bacteria, fungi, viruses and protozoa. Infectious diseases account for approximately one-half of all deaths in tropical countries (Iwu *et al.*, 1999). The incidence of epidemics due to drug resistance and emergence of hitherto unknown disease-causing microbes pose public heath concern. Intractable infectious diseases such as amoebic, sexually transmitted diseases, meningitis, respiratory tract infections and opportunistic AIDS infections has renewed interest in infectious diseases in medical and public health communities and renewed strategies on treatment and prevention.

Plants have provided a source of inspiration for novel drugs and pesticidal compounds, as plants derived medicines and pesticides have made large contributions to human health and well being. These compounds may become the base for the development of medicine, a natural blueprint for the development of new drugs or a phytomedicine to be used for the treatment of man (Iwu *et al*., 1999). The toxicological and environmental properties of the compounds must be considered. Not all-natural product compounds are safe and introduction of toxic compounds into the environment would cause adverse effects.

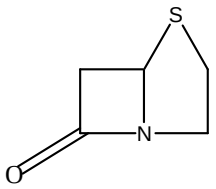
1.2 Anti-bacterial Agents

The aim of anti-microbial therapy is to selectively kill invading bacteria cells. Anti-bacterial agents consist of anti-biotics and non-antibiotic synthetic compounds such as sulfonamides and quinolones. Anti-biotics are microbial metabolites or their synthetic analogs that inhibit growth and survival of other micro-organisms in small doses. They may broadly be divided into β-lactam anti-biotics and non β-lactam anti-biotics. The β-lactam anti-biotics contain a β-lactam ring and share a common mechanism of action. They include the penicillins, cephalosporins and cefamycins, thienamycins, monobactams, nocardicins and beta-lactamase inhibitors. Non-β-lactam antibiotics include macrolides, aminoglycosides, tetracyclines, peptides, lincosamides, chloramphenicol and miscellaneous agents.

1.2.1 Penicillins

Alexander Fleming discovered penicillins through serendipity in 1929 (Nathan *et al., 1968)*. They were initially isolated from the mold *Penicillium chrysogenum (notatum)*. Structurally they consist of a β-lactam ring fused to a thiazolidine ring, together known as a penam (**1**)

structure. The β-lactam ring has a powerful acylating action on bacterial transpeptidase enzymes (transpeptidases) they are involved in cell-wall synthesis. Thus in their mode of action on proteins cause antigenicity.



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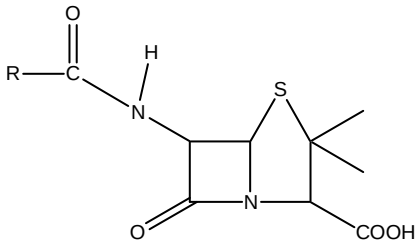

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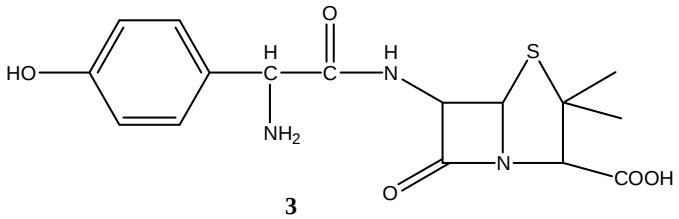
Penicillins are generally well tolerated but about 6% of the population are allergic to them. The penicillin (**2**) structure is the basic structural requirement for biological activity. The side chain R determines anti-bacterial and pharmacological characteristic of particular penicillin. Molecular modification of the penicillin structure and variation in formulation gives a range of penicillins that have different pharmacokinetics and pharmacodynamic properties such as slowed excretion, better tissue diffusion, oral activity, broad spectrum activity and resistance to β-lactamase (Mwagiru, 2005). Amoxillin (**3**) is an example of a useful penicillin.



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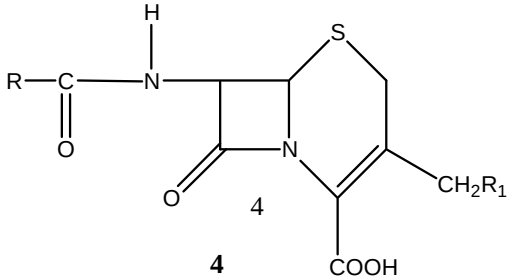
1.2.2 Cephalosporins

Cephalosporins (**4**) are bactericidal broad-spectrum anti-biotics used in a variety of infections including septicemia, meningitis, biliary-tract infections, pneumonia and urinary tract



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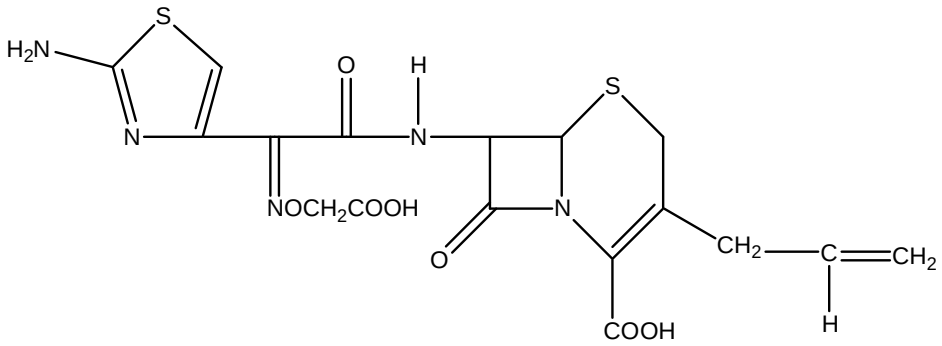
infections. They have greater resistance to β-lactamases than penicillins in addition to more activity against Gram negative organisms (Mwagiru, 2005).

Cefixime (**5**) is an example of a third generation cephalosporin. It is effective against a wider spectrum of Gram negative bacteria's including meningitis (Ashutosh 2000).



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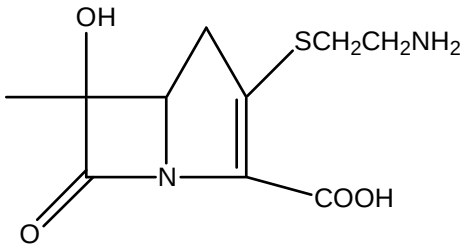
1.2.3 Thienamycin

Thienamycin (**6**) are broad-spectrum anti-biotics having a carbapenem nucleus consisting of a β-lactam ring fused to a pyrroline ring. The presence of a carbon atom at position 4 (instead of sulphur as in penicillins), together with the group at position 3 and the double bond at Carbons 2-3 make thienamycin very reactive and very potent.



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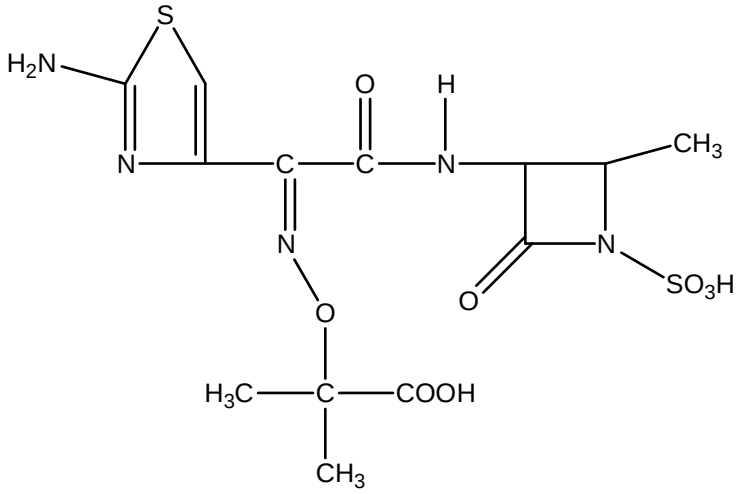
1.2.4 Monobactams

These are monocyclic β-lactam antibiotics produced by various moulds. None of the natural compounds are used clinically but the semi-synthetic agent aztreonam (**7**) is very active against Gram negative bacteria and inactivates β-lactamases. It is mainly used for Pseudomonas infection (Mwagiru, 2005).



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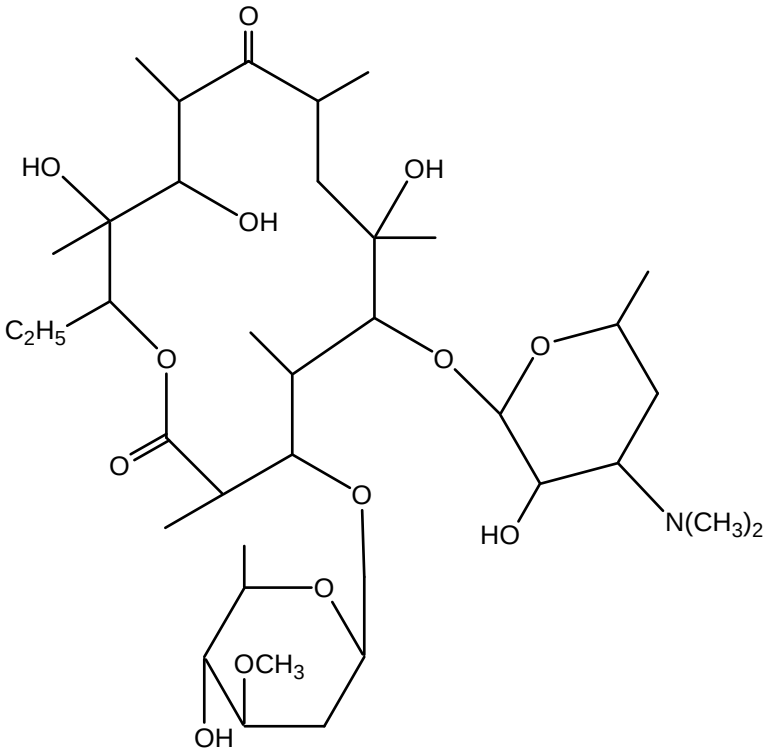
1.2.5 Macrolide Antibiotic

Macrolides are hydroxylated macrocyclic lactones of 12 - 20 carbon atoms. They consist of one or two amino sugars linked glycosidically to a lactone nucleus. They act by inhibiting protein synthesis by binding to bacterial ribosome. They do not bind to mammalian ribosome. Erythromycin (**8**) isolated from *Streptomyces erythreus* is good example (htep://www.britannica.com/eb/article.9032963/erthromycin).



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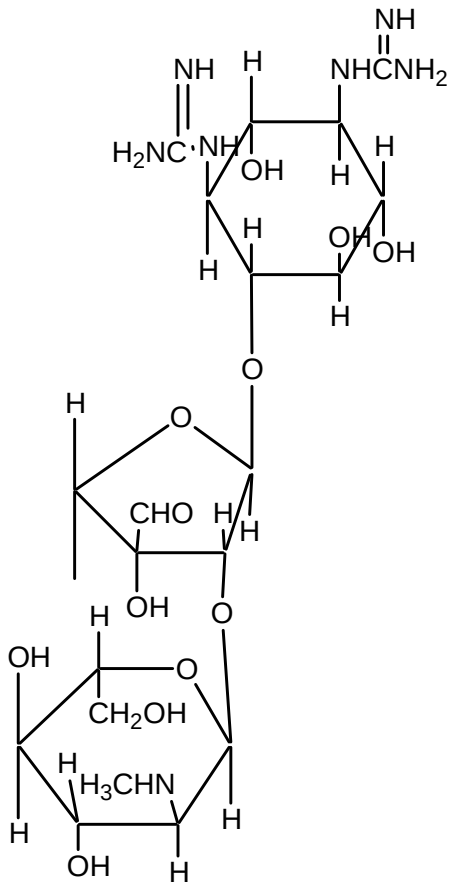
1.2.6 Aminoglycosides

These are anti-biotics originally isolated from Streptomyces an example being streptomycin (**9**). They contain one or more amino sugars linked by glycoside linkages to a basic 6-member cyclical ring. Aminoglycosides inhibit bacterial protein synthesis. They pass into bacterial cells by binding to external lipopolysaccharides, which diffuse into cells. They have a broad spectrum of activity but the risk of toxicity limits their use to serious Gram negative infections such as by *P. aeroginosa*. Streptomycin (**9**) is active against tuberculosis (Ashutosh, 2000).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CHO
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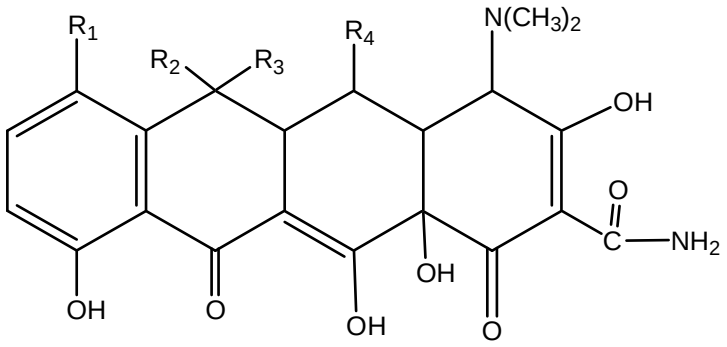
1.2.7 Tetracyclines



	R_1	R_2	R_3	R_4
Tetracycline	H	CH_3	OH	H
Chlortertracycline	Cl	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Minocycline	$-N(CH_3)_2$	H	H	H
Doxycycline	H	CH_3	H	OH
Demecycline	Cl	H	OH	H
Methacycline	$=CH_2$	H	OH	H

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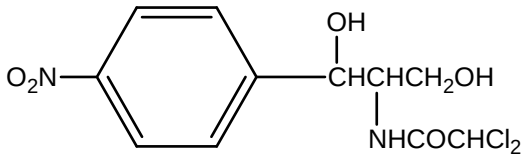
These anti-biotics were initially isolated from Streptomyces. Structurally they may be regarded as derivatives of naphthalene or quinones. Tetracyclines (**10**) are amphoterics.

1.2.8 Chloramphenicol

This is an aromatic anti-biotic originally isolated from *S. venezuelae* (Mwagiru, 2005). Chloramphenicol (**11**) is a broad spectrum antibiotic, however, due to serious side effects including anaplastic anemia, its use has declined.



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It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs, and that higher plants are known to be the main source of drug therapy in traditional medicine. Since about 80% of the world's population reside in developing countries, about 64% of the total population of the world utilize plants as drugs (Farnsworth and Soejarto, 1985).

Many of the plant-derived drugs currently in use are prototypes. Any textbook of pharmacology has morphine as the major topic in any chapter on analgesics or atropine in a chapter on anti-cholinergic agents just to name a few.

These drugs derived from plants mainly alleviate human suffering. It should also be pointed out that many biologically active plant derived substances that have not been useful as drugs *per se* have been models for synthesis template or structural modification to produce useful drugs.

Therefore, there is need for conservation of forests and medicinal plants. Each year large areas of rainforest are destroyed through conversion to agricultural land or for other purposes. These habitats may contain plants that are nowhere else on earth. Therefore, reaforestation may enhance the potential source of new products such as medicines that could be introduced into commercial use. One effect of reaforestation is to increase dramatically the supply of medicinal plants.

CHAPTER TWO

LITERATURE REVIEW

2.1 Family Asteraceae

The family Asteraceae consists of herbs with alternate or opposite, entire or divided leaves, without stipules, flowers (florets) bisexual, monoecious or dioecious, stalkless in heads on an enlarged termination of the stem (receptacle) surrounded by a series of bracts (phyllaries) collectively forming the involucre, and sometimes each with a bract or scale at its base. The sepals are variously reduced frequently to scales or bristles (pappus); commonly the heads are themselves inflorescence, and when held at roughly the same levels this is called a corymb. The florets are of 3 types (1) bilaterally symmetrical with a spreading 3-toothed unilateral limb (ray), with or without 2 upright teeth at apex of petal tube, (2) bilaterally symmetrical with a spreading 5-toothed ray and (3) regular with 4-5 lobes at apex of tube; stamens 5, fused in a ring around style, filaments free and joined to petal tube; anthers often tailed; ovary inferior with 1 erect ovule and an erect style and mostly bifid stigma; fruit a dry indehiscent achene, often winged or dispersed by wind carrying the outspread pappus, rarely fleshy (Agnew and Shirley, 1994).

2.2 Genus Senecio

The leaves are alternate; heads in corymbs or solitary; phyllaries quite coherent, (sub) equal, occasionally with some smaller ones outside. Florets tabular and often with ray florets as well.

In Kenya the Senecio species are normally found in high altitude grassland. Some Senecio species mentioned are below.

2.2.1 *Senecio jacksonii* is a plant with thick creeping stolons and tufts of oblinear, more or less toothed, often hairless leaves; heads solitary or paired, inner phyllaries about 8 mm long.

2.2.2 *Senecio cripatipliosus* is a herb with ovate, heart shaped, finely toothed basal leaves and softly hair stems to 0.5 m high. Heads in a terminal corymb, inner phyllaries 20 - 25 mm, about 8 mm long; rays 12 - 16, about 10 mm long; achenes, pale. Found around the Chania falls in the Bamboo forest at the Aberdares, 2050 – 3000 m.

2.2.3 *Senecio sotikensis* has glandular-hairy, woody herb or soft shrub bearing stalkless, oblong, pinnately lobbed leaves; heads in showy terminal corymbs, large, yellow rayed; phyllaries 16 - 21, 8 - 10 mm long. It is found in the Elgon heathland 3000 – 4500 m.

2.2.4 *Senecio marangunesis* has an erect, hairy, branched shrub or scrambler with stalked, oblong-ovate toothed leaves which are often lobed at base; heads bell shaped, in dense terminal corymbs, yellow rayed; phyllaries about 4 mm long. Rarely found in the upper forest edges of the Aberdares, 2720 – 2950 m.

2.2.5 *Senecio spartaceus* is an erect almost hairless perennial from a woody rootstock; leaves linear to oblanceolate, basal ones often absent at flowering; heads yellow, rayless, on hairy stalks, in a loose corymb; phyllaries 10 - 14, 6 - 9 mm long: achene hairless. Common in the wooded grassland in Western Kenya, 1800 - 2200 m.

2.2.6 *Senecio vulgaris*, all parts of the plant are poisonous to many mammals, including humans. The toxin affects the liver and has a cumulative affect. Some mammals, such as rabbits, do not seem to be harmed by the plant, and will often seek it out. Various birds also eat the leaves and seeds. Groundsel has a long history of herbal use and, although not an officinal plant, it is still often used by herbalists. The whole herb is anthelmintic, anti-scorbutic, diaphoretic, diuretic, emmenagogue and purgative. It is often used as a poultice and is said to be useful in treating sickness of the stomach, whilst a weak infusion is used as a simple and easy purgative. The plant can be harvested in May and dried for later use, or the fresh juice can be extracted and used as required. This plant should not be used by pregnant women see also the notes above on toxicity. A homeopathic remedy is made from the plant. It is used in the treatment of menstrual disorders and nosebleeds. (http://jambo.africa.kyoto-u.ac.jp/kiroku/asm_normal/abstracts/pdf/ 23-2/65-89.pdf).

2.3 *Senecio lyratus*

2.3.1 Plant Description

Senecio lyratus (Asteraceae) is a warily trailing climber, with triangular or ovate toothed leaves lobed at the base (Wahiaula, 1988). Phyllaries are about 4 mm long, achenes cylindrical, hairy expect for those of the ray florets, which are narrowly winged and hairless. The plant is commonly distributed in dry upland and woodland areas at 500 - 2760 m above sea level (Agnew and Shirley, 1994).

It is commonly distributed in upland forest and woodland areas around Mt. Kenya, Cherangani highlands, Mau forests, Kericho and Kisii district among others. In Kericho district, the Kipsigis call *Senecio lyratus* "Menerenet".

2.3.2 Traditional Use

The Kipsigis and the Masai communities of Kenya are reported to have used the plant in the treatment of venereal diseases such as syphilis and gonorrhea. The roots of this plant are washed then ground into fine powder, which is applied, on fresh wounds. The leaves alone are used as emetic (Kokwaro, 1976).

2.4 Phytochemical Compounds of Senecio Species

Nearly all Senecio species contain pyrrolizidine alkaloids as the most characteristic secondary metabolites. Pyrrolizidine alkaloids have been found to possess interesting medicinal properties, some are carcinogenic while others are reported to exhibit anti-tumor activities (Mattocks, 1986; Risk, 1991).

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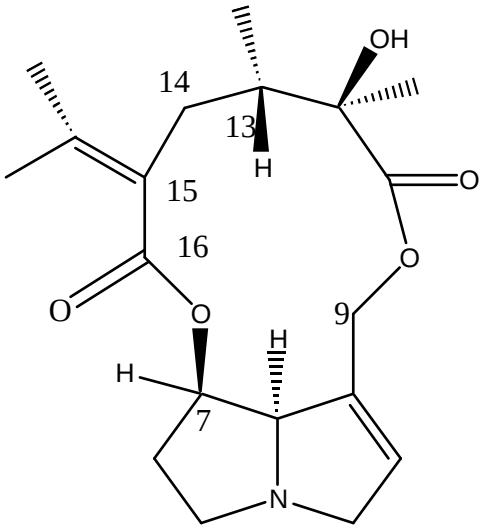
Pyrrolizidine alkaloids are derived from arginine or ornithine and characteristically have two 5-membered rings with nitrogen at one of the common positions. At least 560 alkaloids of this type are known. Pyrrolizidine alkaloids rarely occur in the free form and are found as complex esters and *N*-oxides. Although pyrrolizidine alkaloids occur in all plant parts, the concentration in at least one example, *Senecio vulgaris,* is highest in the inflorescence (Hartmann, 1991).

Cells of *Senecio vulgaris* in tissue culture lack the ability to synthesize pyrrolizidine alkaloids, but retain the ability to take up and accumulate the corresponding *N*-oxides. Cells of non-alkaloid producing plants do not take up pyrrolizidine *N*-oxides (Hartmann, 1991).

Pyrrolizidine alkaloids are biosynthesized in the roots and transported to other parts of the plant. Root cultures of *Senecio vulgaris* synthesize pyrrolizidine alkaloids and accumulate them as *N*-oxides (Herbert, 1985). This synthesis parallels that of intact plant roots. Senecionine (**12**) is biosynthesized and converted to the *N*-oxides and transported throughout the plant. Plants of many species contain only *N*-oxides of pyrrolizidine alkaloids (Hartmann *et al.*, 1989).

A few structural compounds of the pyrrolizidine alkaloids have been illustrated. These alkaloids are found mostly in the Asteraceae family. The genus Senecio is particularly important for containing the macrolide - type pyrrolizidine alkaloids, such as senecionine (**12**).



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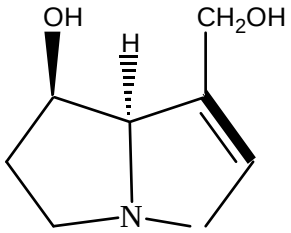
2.5 Pyrrolizidine Alkaloids in Animals

Pyrrolizidine alkaloids are feeding deterrents and insecticide for many species of insects. Some insects such as *Melanchra persicariae* and *Spodoptera littoralls* feed on *Senecio vulgaris,* which contains pyrrolizidine alkaloid, but do not sequester the alkaloids. Certain insects appear to be able to excrete these alkaloids without harm. For example, senecionine (**12**), its *N*-oxide, and hydrolytic products including retronecine (**13**) were found in the honeydew of the aphid *Myzus pericae* when this insect fed on *Senecio vulgaris* (Molyneux *et al.*, 1990).



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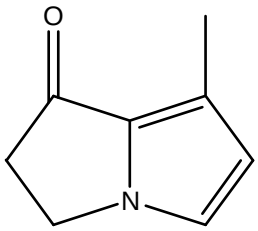
Some moths store Senecio alkaloids without modification. The larvae of the cinnabar moth, *Tyria jacobea,* and related species accumulate such alkaloids in their tissues by feeding on groundsel or ragwort, both rich in pyrrolizidine alkaloids and, thus become distasteful to predators. All stages of these moths are brightly colored and are not eaten by most insectivores (Harborne, 1982).

In addition to use of pyrrolizidine alkaloids as protective chemicals, a number of species of three groups of Lepidoptera (*Danainae, Ithomiinae,* and *Arctiidae*) biosynthesize pheromones from these compounds that they acquire by pharmacophagy or larvae feeding (Schnelder, 1987). Male butterflies of several other species of the genus *Danaus ingest* pyrrolizidine alkaloids and convert them into pheromones such as danaidone (**14**), danaidal (**15**), and

hydroxydanaidal (**16**) that are released from the abdominal hairpencil organs during courtship behavior (Boppre, 1978; Schneider, 1987).



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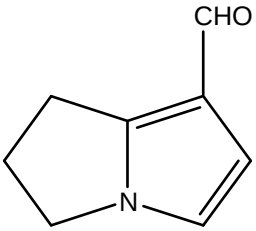

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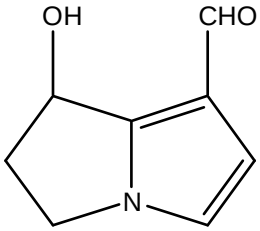

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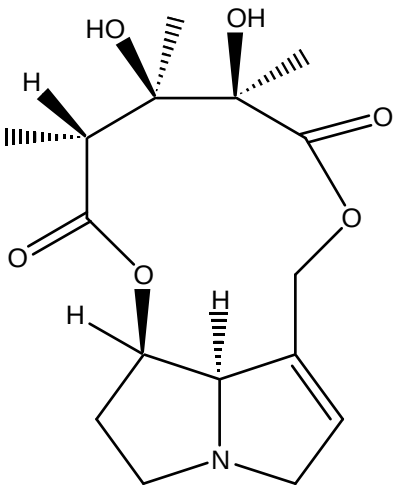
2.6 The Role of Pyrrolizidine Alkaloids in Livestock Poisoning

Many pyrrolizidine alkaloids-containing plants are poisonous to livestock (Hartmann, 1991). Over 300 plants from at least 30 genera and 6 families that contain this group of alkaloid are responsible for livestock poisoning. The most important of these plants are of the genera Senecio, Crotalaria, Heliotropium, and Symphytum (Suffness and Cordell, 1985). Pyrrolizidine alkaloids produce hepatotoxic effects: the action seems to be cumulative. As little as 1 - 5% of the animals weight in plant material is enough to be fatal. The LD_{50} of a variety of pyrrolizidine alkaloids range from about 50 to 1100 mg/kg. Monocrotaline (**17**) has LD_{50} ip in rat of 175 mg/kg; retronecine (**13**) has LD_{50} ip of 634 mg/kg in mouse; senecionine (**12**) has an LD_{50} ip of 50 mg/kg in mouse (Wink, 1993b). The toxicity of the *N*-oxides of these alkaloids may be about the same or less as the alkaloids themselves (Suffness and Cordell, 1985). However, *N*-oxides do not have the bitter taste associated with alkaloids (Wrobel, 1985).



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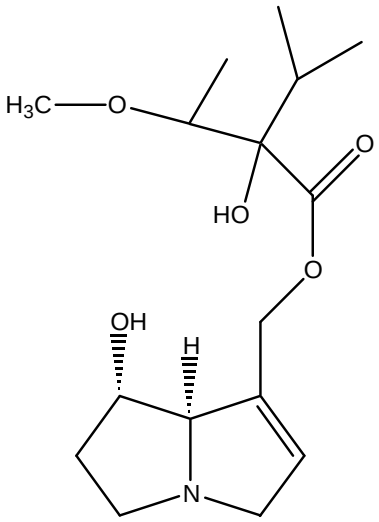
The toxicity may be due to the formation of pyrrole esters that have potent alkylating properties. Hydroxydanaidal (**16**) has been suggested as a major hepatotoxic compound (Harborne, 1986). These esters are produced by metabolism of pyrrolizidine alkaloids in the mammalian liver and are able to bind the certain sites in the liver as well as with macromolecules such as DNA. Some studies suggest that the toxicity of pyrrolizidine alkaloids lie not in their own structures but in the one major metabolite to which they are converted. The dehydrogenation product produced is much more toxic. Hydrolysis of the complex ester groups of these compounds yields retronecine (**13**), which is then dehydrogenated. More recently, however, a breakdown product, E-4-hydroxyhex-2-enal, has been suggested to be the actual reactive metabolite that binds the DNA in the liver (Harborne, 1986).

2.7 Human Poisoning by Pyrrolizidine Alkaloids

Human health problems have been attributed to the accidental consumption of plant materials that contain pyrrolizidine alkaloids (Beir and Nigg, 1992). The consumption of wheat

containing seeds of *Heliotropium popovii* has caused a large outbreak of veno-occlusive disease in Afghanistan. The main alkaloid present was heliotrine (**18**) (Mattocks, 1986).



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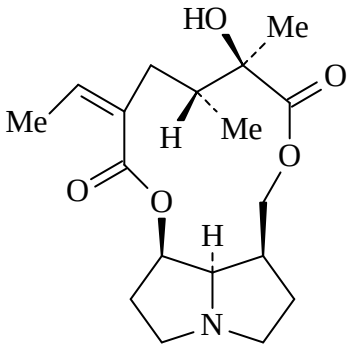
Pyrrolizidine alkaloids also occur in honey made from *Senecio jacobaea* (tansy ragwort) and in milk of cattle that feed on this plant. Herbal teas are responsible for poisoning problems as well (Bier and Nigg, 1992). In Arizona, a number of cases of veno-occlusive disease have been attributed to the consumption of tea from *Senecio longilobus*, whereas a similar poisoning in West Germany is thought to have arisen from senecionine (**12**) in a *Petasites* species (Robins, 1993). Tea from comfrey leaves (*Symphytum* ssp., Boraginaceae) has long been used (Schneider, 1987), however, comfrey leaves and roots cause tumors when fed in the diet of rats. Pyrrolizidine alkaloids have been identified as one of the causes of primary liver cancer in countries of central Africa, where the incidence of this cancer is the highest in the world. Many pyrrolizidine alkaloids are carcinogenic and have been demonstrated to induce tumors in a number of different animal systems.

2.8 Economic Value of Pyrrolizidine Alkaloids

Serious economic losses are sustained annually from deaths of livestock, which have grazed on land containing pyrrolizidine alkaloids (PA). Despite the display of hepatotoxic activity by many pyrrolizidine alkaloids, some have shown activity of therapeutic values. These useful alkaloids usually contain a saturated pyrrolizidine nucleus or are quaternary amines or *N*-oxides.

The macrocyclic diester, platyphylline (**19**), obtained from Senecio species has found use in the past for the treatment of hypertension and internal ulcers (Petrovsky, 1956; Anichkov and Belenkev, 1955). Platyphylline (**19**) is not hepatotoxic because it cannot be easily oxidised to a reactive pyrrole derivative. An ophthalmic drug is reported to contain 1% platyphylline tartrate (Rachev et al., 1982).

Senaetnine (**20**) is a pyrollizidine alkaloid that did not appear to be hepatotoxic to rats, but did cause pulmonary damage when given intravenously to rats (Mattocks and Driver, 1987). It is found in South Africa Senecio species.



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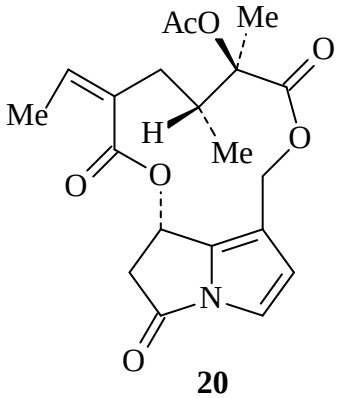
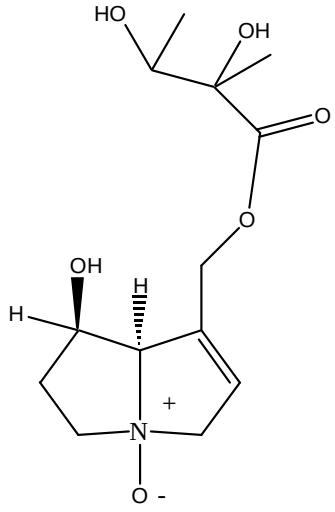


20

2.9 Medicinal Uses of Pyrrolizidine Alkaloids

The medicinal uses of pyrrolizidine alkaloid containing plants have been doubted, because of the cumulative toxic effects of these compounds. Although the toxicity of specific compounds varies, the use of many herbal medicines involving plants of Asteraceae and Boraginaceae that may contain pyrrolizidine alkaloids should be avoided. Some varieties of pyrrolizidine alkaloids and other *N*-oxides have anti-tumor activity (Wink, 1993b). Herbal materials of *Senecio vulgaris* have been used for hundreds of years as a treatment for cancer. The active compounds have been shown to be senecionine (**12**) and senecionine *N*-oxide. Indicine *N*-oxide (**21**) from *Heliotropium indicum* (Boraginaceae) has pronounced anti-tumor activity. Monocrotaline, from several Crotalaria species (Fabaceae), has similar activity (Blasko and Cordell, 1988; Suffness and Cordell, 1985). Alkylation seems to be involved in the ant-tumor activity. As the *N*-oxides cannot serve as the enamines and hence cannot be directly involved in the alkylation, these compounds should only be active to the extent they are converted to the free bases. At least in the case of indicine *N*-oxide, this does not appear to be the case (Suffness and Cordell, 1985). Senecionine and senecionine *N*-oxide have been identified as the antifertility agents from *Senecio vulgaris* (Tu *et al.*, 1988).



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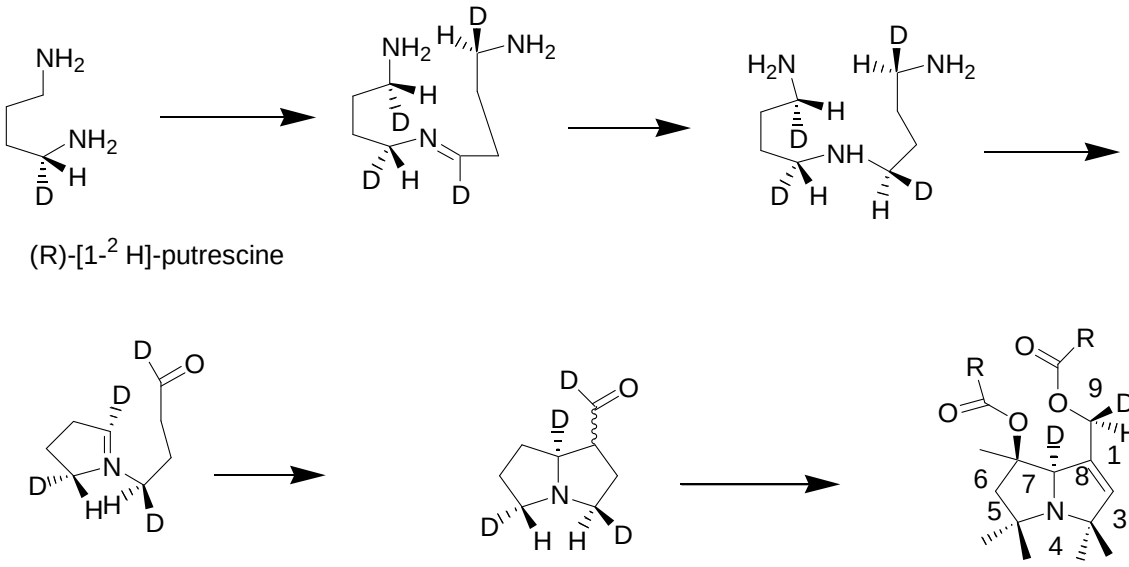
2.10 Biosynthesis of Pyrrolizidine Alkaloids

Different alkaloids may have different biosynthetic pathway. Putrescine is the precursor for biogenesis of retrosine (**22**).



22
Retrorsine

Fig 2.1 Biogenesis of retrorsine

Although most pyrrolizidine alkaloids have been regarded as coming from ornithine by the intermediacy of putrescine, arginine probably is the actual precursor in most instances (Hartmann, 1991). For instance the biosynthetic pathway of retronecine (**23**) uses arginine as the precursor while retrorsine (**22**) uses putrescine.

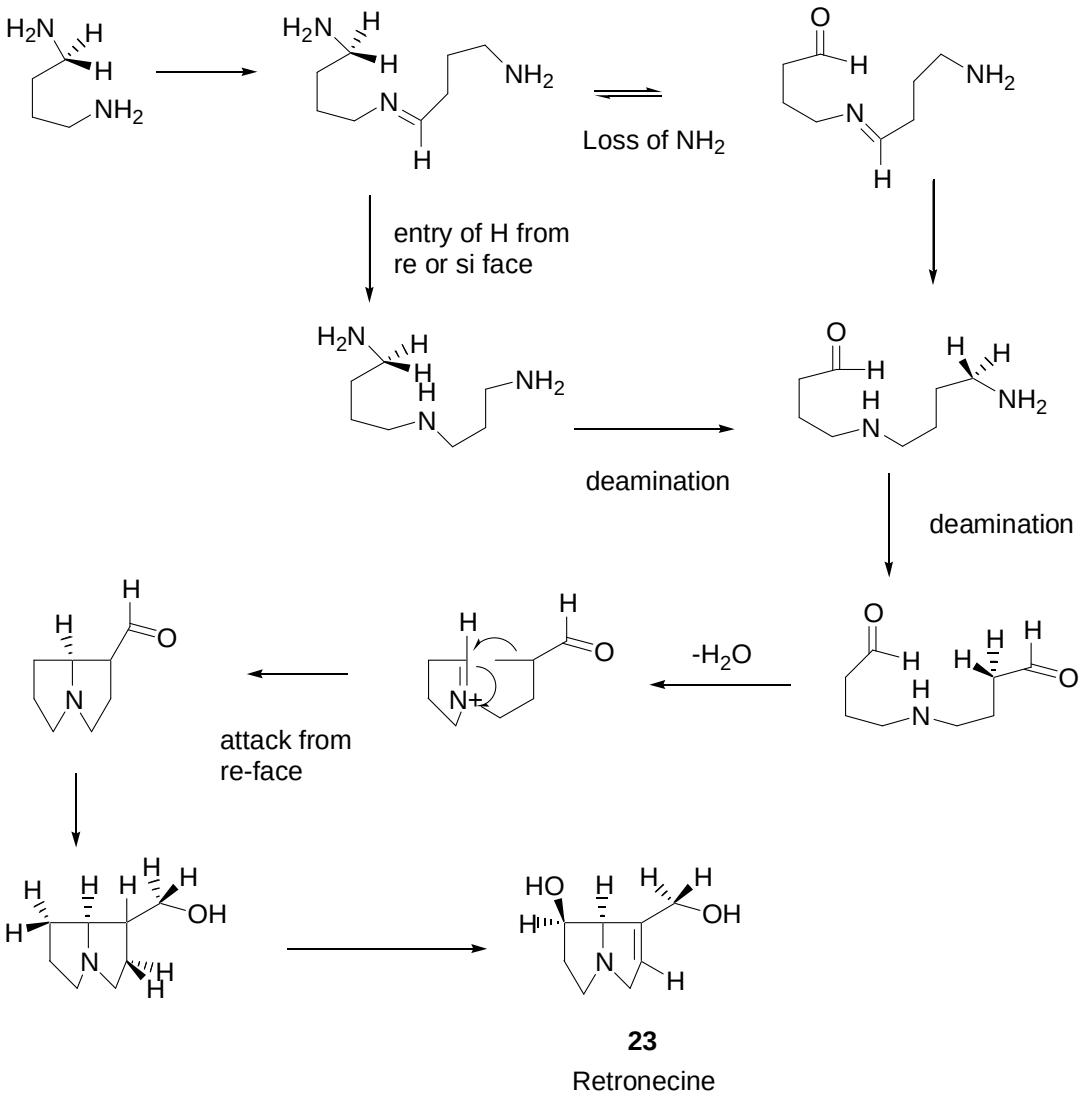


Fig 2.2 Biosynthesis of retronecine

2.11 Terpenoids

Triterpenes constitute a significant portion of the lipid substances of all plants. More than 4,000 triterpenoids have been isolated. These compounds are precursors to steroids in both plants and animals. Steroids are components of membranes in plants. Both Triterpenes and steroids occur free, as glycosides (Boar and Allen, 1973), or in other combined forms.

Triterpenes arise via dimerization of two farnesyl pyrophosphate units to produce an intermediate compound squalene. Squalene may then be cyclised by several mechanisms from different conformations on an enzyme surface to produce the parent skeletal types of many different kinds of triterpenes that undergo subsequent modification (Banthorpe and Charwood, 1980). The structures of many triterpenes and steroids may be explained by the "biogenetic isoprene rule". Squalene is derived from two farnesyl pyrophosphate units joined in tail–to–tail (1-1) condensation.

A number of plant sterols have been reported to possess growth – regulating activity and developmental modification properties in plants (Heftman, 1975a; 1975b; Mandava, 1979) for example, β-sitosterol initiate's flower buds in *Chrysanthemum* species.

Sesquiterpenes isolated from the family Asteraceae are an important source antifeedant against Colorado Potato Bettle (CPB) (Hough-Goldstein, 1990). Gonzalez-Coloma *et al.* (1995) discovered that an ethanolic extract of *Senecio palmensis* had strong anti-feedant activity against CPB larvae. Two compounds have been isolated from *Senecio palmensis*, one from the chemical class of bisabolenes and the other a siliphinene sesquiterpene (Gonzalez-Coloma *et al.*, 1995). Both of these chemicals may alter the host selection process through adult behavioral avoidance because adults are highly mobile and are the primary finders of host plants (Hough-Goldstein, 1990). Bisabolenes serve as effective anti-feedants by causing feeding inhibition. The modes of action for siliphinene sesquiterpenes are both anti-feedant and toxic effects. These modes of action result in detrimental effects such as a decreased growth rate in CPB larvae (Gonzalez-Coloma *et al*., 1995).

2.12 Biological Activity of Triterpenes

Although triterpenes have been considered to be relatively innocuous plant constituents, several have been established to pronounce physiological activity. Many triterpenes have antiherbivore activity. Although phytosterols are required for insect and fungal growth, triterpenoid compounds may interfere with these process and exhibit anti-herbivore or anti-microbial effects (Croteau and Johnson, 1985). In general, those that are highly oxygenated seem to be more active in this regard (Seigler, 1983).

2.13 Triterpenes Derived via a Chair-Chair-Chair-Boat Transition State

Cyclization of squalene 2,3-epoxide via a chair-chair-chair-boat transition state gives rise to many of the most common plant triterpenes. Pentacyclic triterpenoid compounds such as α- and β-amyrin, oleanolic acid, taraxerol, taraxasterol, lupeol, betulin, euphol, friedelin, and ursolic acid may constitute several percent of the dry weight of many plants. Biogenetic schemes for the formation of most of these groups of triterpenes have been proposed (Coates, 1976). Most of these involve shifts on methyl and hydride groups along with skeletal rearrangements to achieve the final structures. The cyclization of 2,3-epoxysqualene to β-amyrin (**24**) by microsomal fractions from pea cotyledons, which involves the enzyme 2,3-oxidosqualene: β-amyrin (**24**) cyclase has been demonstrated (Abe *et al*., 1989a; 1989b; Croteau and Johnson, 1985).

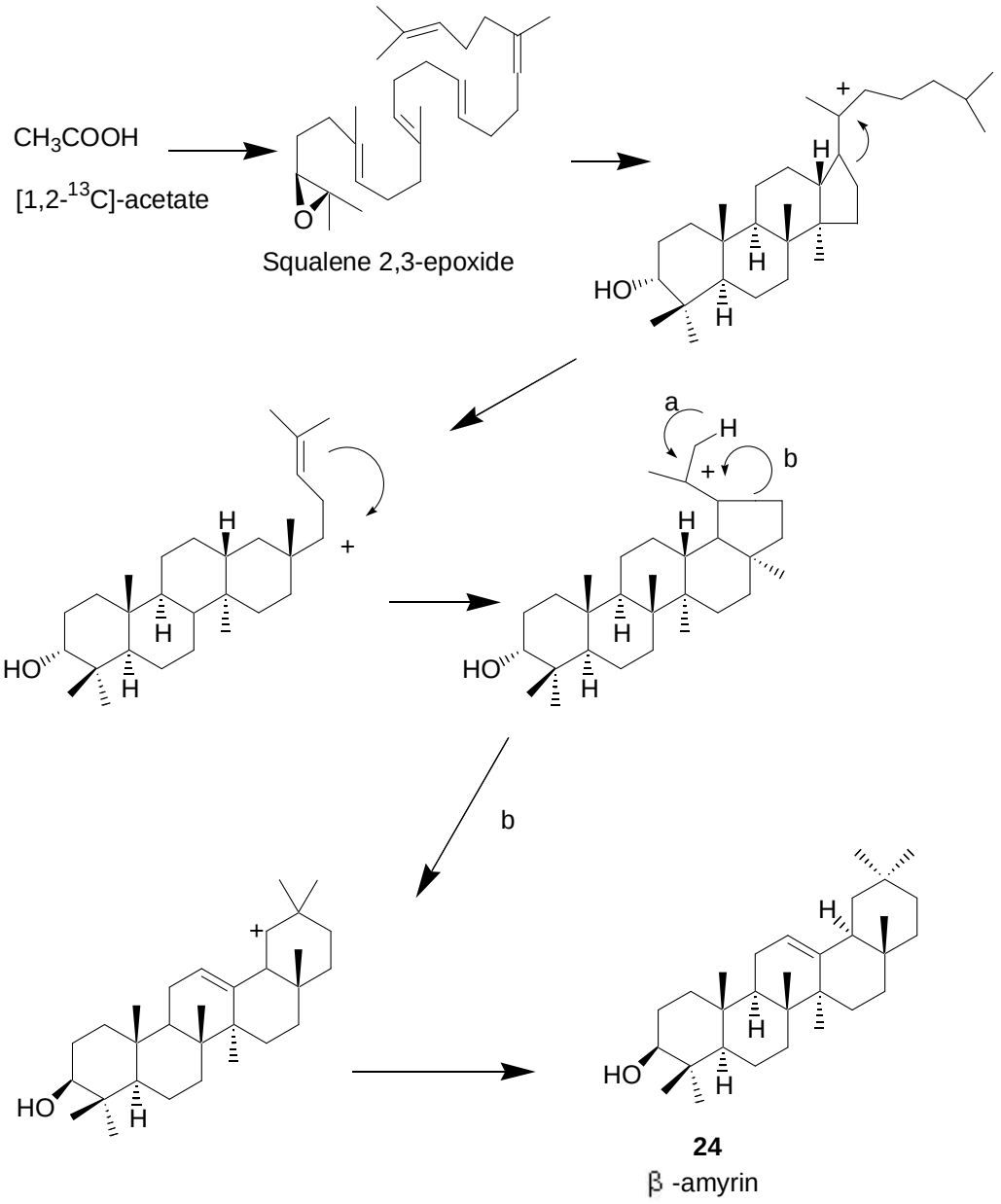
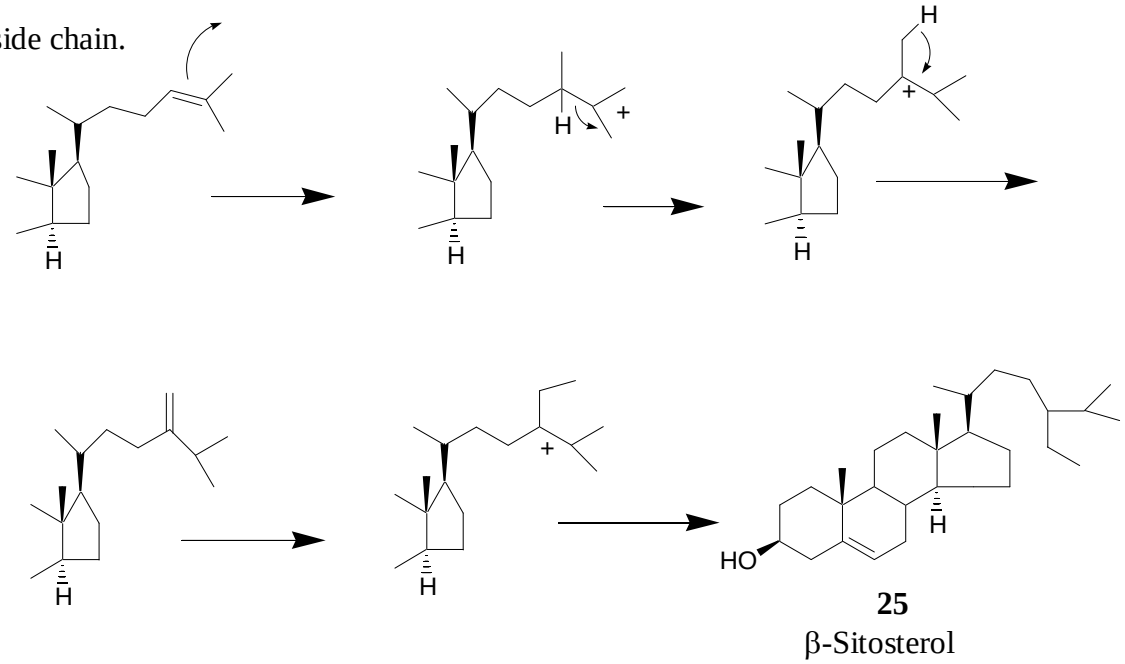


Fig 2.3 Biogenesis of pentacyclic triterpene, β-amyrin, from cyclization of 2,3-epoxysqualene

2.14 Formation of Plant Sterols

In most plants, β-sitosterol, campesterol, and stigmasterol are the most abundant phytosterols. These compounds arise from cycloartenol by a series of reactions including modification of the side chain.



25
β-Sitosterol

Fig 2.4 Proposed biogenesis of β-sitosterol

2.15 Biological Activity of Plant Sterols

A number of plant sterols have been reported to posses growth-regulating activity and development modification in plants (Heftman, 1975a; 1975b; Mandava, 1979). For example, β-sitosterol initiates flower buds in *Chrysanthemum* species; exogenously applied lanosterol (not a plant product) also stimulates flowering in these plants. Estrone (oestrone) and related compounds reported to increase the number of flowers in *Ecballium elaterium* and to influence sex expression. None of this processes are well understood (Mandava, 1979). Despite the reports of *in vitro* growth regulating activity of progestagens in plants, there is no evidence for *in vivo* activity.

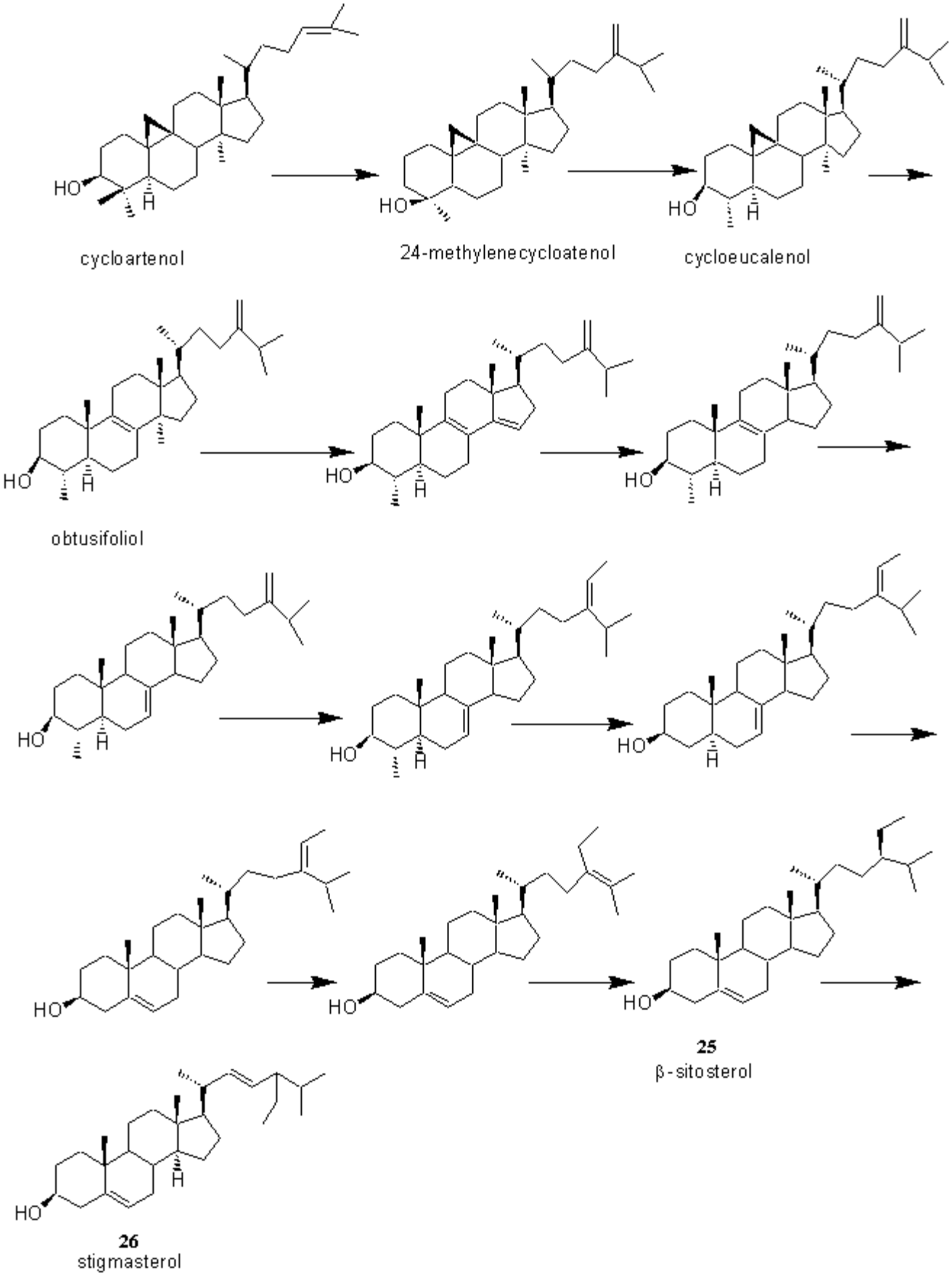


Fig 2.5 Biosynthesis of stigmasterol

Many animals convert steroidal precursors to vitamin D and related compounds (DeLuca and Schnoes, 1983). Vitamin D is involved in the assimilation of calcium ions from the diet, stimulates transport of calcium ions *in vivo*, and in conjunction with parathyroid hormones, mobilizes calcium ions from bones (Williams *et al*., 1989).

2.16 Micro-organisms

There are enormous species of bacteria and fungi. Some bacteria used in this research include *Staphylococcus aureus, Pseudomonas aeruginosa* and *Escherichia coli*. One fungus has been used, that is *Candida albicans*.

2.16.1 *Staphylococcus aureus*

Staphylococcus aureus causes boils, styes, pustules, impetigo infections of wounds (cross-infection), ulcers and burns, osteomyelitis, martitis, septicaemia, meningitis, pneumonia and pleural empyema, toxic food poisoning, toxic shock syndrome and toxic skin exfoliation. Staphylococcus species are gram positive. Antibiotics with activity against *S. aureus* include penicillins, macrolides, fusidic acid, vancomycin and cephalosporins. However, *S. aureus* has strains that are resistant to penicillins and others are multiple drug resistant.

2.16.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa can be found in the intestinal tract, water, soil and sewage and is frequently found in moist environments in hospitals (sinks, cleaning buckets, drains, humidifiers).

Infection caused by *P. aeruginosa* include skin infections, especially burn sites, wounds, pressure sores and ulcers, urinary infections, respiratory infections and external ear and eye infections. *P. aeruginosa* is resistant to most of the commonly used anti-biotics. Anti-microbials that usually show activity against Pseudomonas include aminoglycosides, polymyxin, some penicillins and cephalosporins (Monica, 2000).

2.16.3 *Escherichia coli*

Escherichia coli belong to the gram-negative rods referred to as enterobacteria. They are naturally found in the intestinal tract, in the soil and water. *E. coli* causes watery diarrhoea in infants and adults. It also causes vomiting, dysentery, fever and colitis, with blood, mucous and pas cells in faecal specimens. Anti-microbial agents that are used to treat *E. coli* are sulphomamides, trimethoprime, cotrimoxazole nalidixic acid, nitrofuraritoin, tetracycline, ampicillin, amoxycillin, cephalosporins and aminoglycosides (Monica, 2000).

2.16.4 *Candida albicans*

Candida albicans, a fungus, is the commonest cause of candidiasis. The yeast is a common commesal of gastrointestinal tract. Most C*andida* infections are opportunistic, occurring in delibitated persons (Monica, 2000).

2.17 Research Rationale and Justification

The cost of imported fungicides, pesticides and medicinal drugs is quite high and consumes a large proportion of the foreign exchange allocation. If such fungicides, pesticides and

medicines were produced in our developing country like Kenya, then there would be a lot of savings on foreign exchange and cost of production from the plants would be relatively low. Moreover, most synthetic drugs and pesticides are non-biodegradable and have adverse effects to the environment. Products of plants are biodegradable and therefore would be environmentally friendly.

Furthermore, the continued use of synthetic drugs and chemicals to target organisms develop resistance. Hence there is need to search for alternative compounds from plants particularly novel compounds that have not been tested against harmful organisms. Therefore, there is need to search for cheap, more potent anti-bacterial and anti-fungal that are environmentally friendly.

2.18 Statement of Problem

Organisms that causes diseases to man and animals are constantly increasing everyday. Strains of bacteria and viruses keep changing when exposed to chemotherapy compounds. Therefore, search for alternative compounds that are active against these organisms should be enhanced. Plants can provide such needed chemicals for the cure of numerous diseases that affect mankind.

2.19 Hypothesis

The plant *Senecio lyratus* has been used traditionally to treat some sexually transmitted diseases like syphilis and gonorrhea. Therefore, compounds from *S. lyratus* might have potency for treating other related diseases. This research will be geared towards isolating more

compounds that are active against microbial organisms and add value to the current chemotherapeutic compounds. The compounds isolated from *Senecio lyratus* are not toxic and therefore can be used for chemotherapeutic purposes.

2.20 Objectives of Study

2.20.1 General Objective

The main objective of this study was to screen, isolate and characterize bioactive compounds from the aerial part of *Senecio lyratus* (Asteraceae).

2.20.2 Specific Objectives

(i) To obtain crude extracts from aerial parts of *Senecio lyratus,* by sequential solvent extraction.

(ii) To carry out biological activity tests on these crude extracts (toxicity, anti-bacterial and anti-fungal) and on pure compounds.

(iii) To isolate the active compounds of the plant extract using various chromatographic techniques (column chromatography (CC), vacuum liquid chromatography (VLC) and preparative /analytical thin layer chromatography (PTLC/TLC).

(iv) To characterize and elucidate the structure of the isolated compounds using various spectroscopic techniques (IR, UV, MS, and NMR).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Plant Materials

The aerial part of *Senecio lyratus* were collected from Sotik in Kericho district. The authentication of the plant species was carried out by a taxonomist of botany department JKUAT to ensure plants collected were properly identified.

3.2 General Experimental Procedures

All glassware were washed in hot water and soap, rinsed with distilled water and then dried in the oven at 110 ^{0}C. The solvents (dichloromethane, ethyl acetate, methanol and n-hexane) used in the study were laboratory grade obtained from Kobian (K) Ltd and Chemrectic Ltd, Nairobi and were freshly distilled before use.

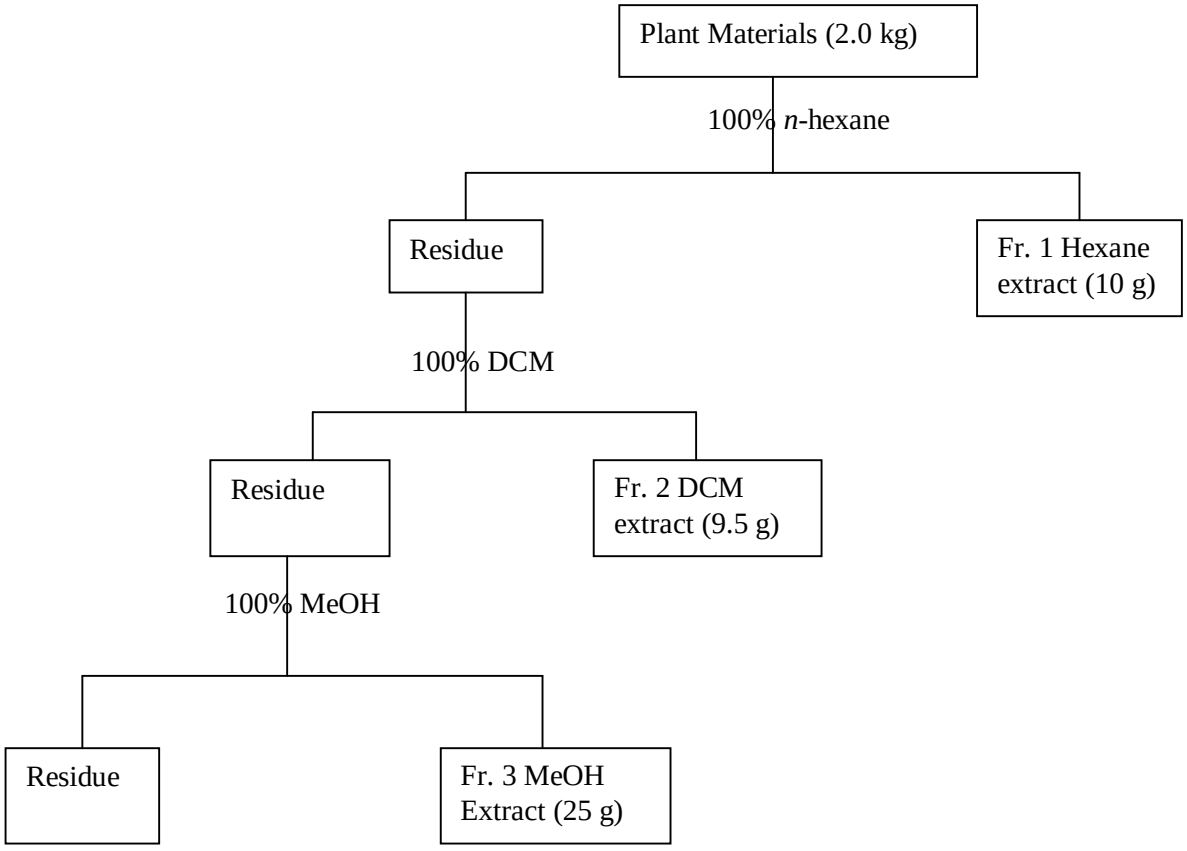
3.3 Extraction

The aerial part of the plant was dried under shade and then ground into fine powder using a grinding machine stationed at Kenyatta University. About 2 kg of the fine powder was soaked in *n*-hexane for three days with occasional shaking, filtered and the filtrate concentrated using a vacuum evaporator (BUCHI Rotavapor R-200). The residue was subjected to the same process using the same *n*-hexane for another two times, making a total of nine days to get the *n*-hexane crude extract. The residue was then extracted with dichloromethane and methanol sequentially and the extracts collected as in the case of *n*-hexane above. The yields for crude extracts were 10 g, 9.5 g and 25 g for *n*-hexane, dichloromethane and methanol, respectively. Extraction process is summarised in Scheme 1.

3.3.1 Extraction of Pyrolizidine Alkaloids

About 10 g of methanol extract were dissolved in 100 ml of 0.25 M sulphuric acid at a pH of 3.0. Chlorophyll and waxes were removed by repeated extraction with petroleum ether and diethyl ether. Zinc dust was added to reduce the *N*-oxides to tertiary alkaloids. The solution was filtered and brought to a PH > 10 by adding ammonia. Washing was done with dichloromethane to extract the pyrolizidine alkaloids.

Scheme 1: General Extraction by Soaking Aerial Parts of *Senecio lyratus*



3.4 Bioassay Techniques

3.4.1 Brine shrimp lethal toxicity tests

Toxicity to brine shrimp (*Artemia salina*) was performed. Artificial sea water was prepared by dissolving 33.0 g of sea salt (Sigma Chemicals Co., UK) in 1.0 L of distilled water. Brine shrimp eggs were added to 500 ml of the artificial sea water in a hatchery where brine shrimp larvae hatched after incubation for 48 hr at 24 ^{o}C (Keriko *et al.*, 1995).

The three crude extracts were tested at various concentrations (100 - 1000 ppm) in calibrated vials containing artificial seawater and 10 brine shrimps. Three replica tests were done for each. A control test with similar specifications without the crude extract was set. The surviving brine shrimps were counted after 24 hr. The LD_{50} was determined by subjecting data to probit analysis, SAS program version 8.2.

3.4.2 Anti-Bacteria Bioassay

To determine the ability of the crude extracts, fractions and pure compounds to inhibit growth of bacteria Gram positive (*S. aureus* and *B. subtilis*) and Gram negative (*E. coli* and *P. aeruginosa*), was carried out in a nutrient agar culture media. The medium was prepared by dissolving 28 g of nutrient agar in 1000 ml of hot distilled water and allowed to cool. The mixture was sterilized by autoclaving at 120 ^{0}C for 15 min. at 15 psi pressure and cooled to 50 ^{0}C.

The medium was dispensed into pre-sterilized Petri dishes to yield a uniform depth of 4 mm. Bacteria culture (100 μl) was introduced in the nutrient agar in the Petri dishes. Filter paper

discs (6 mm diameter) were prepared and sterilized by autoclaving. Crude extracts, fractions and pure compounds (250, 500 and 1000 ppm) were prepared in DCM. Three discs were soaked into each dosage while the fourth one being soaked in DCM as control and allowed to dry before introducing them into the Petri dish. The Petri dishes were inverted and incubated at 37^{o}C for 24 hours. The presence of a clear circular region around the disc loaded with test sample was used as an indicator of inhibition. The inhibition zone was determined by measuring the diameter in millimeters of the circular region around each disc using a ruler (Brooks *et al.*, 1991).

3.4.3 Anti-Fungal Bioassay

Potato dextrose agar media was prepared in a similar way as the nutrient agar media. The fungi (*Candida albicans)* were grown on a potato dextrose agar spread in a Petri dish for 24 hours. This procedure was similar to the anti-bacterial test above. Paper discs dipped into the samples of different concentrations (100 - 1000) ppm were placed on the Petri dish containing the potato dextrose agar. A control experiment where a paper disc was dipped into distilled water was also set. The experiment was monitored for 48 hours after which the inhibition diameters were measured.

3.5 Chromatographic Isolation of Bioactive Compounds

The *n*-hexane crude extract was packed in a vacuum liquid chromatography column (VLC) and chromatographed using silica gel (Kiesel gel 60 Art 77 29 MERCK) by gradient elution and a number of chromatographic fractions were collected. These fractions were fractionated further using silica gel 60 (230 - 400) mesh to get pure products.

3.5.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) was performed on aluminium (Al) sheets precoated with Silica gel 60 F_{254} (MERCK) with a 0.2 mm layer thickness. TLC plates were used throughout the present study as a scouting technique in the isolation and purification process to monitor the separation process. The separated compounds on the chromatograms were examined under UV light at 254 nm and 366 nm using a hand lamp and visualized by spraying with 95% methanol and 5% concentrated sulphuric acid, then heating the TLC plate at 110 ^{o}C for five minutes or with Dragendorff and allowed to dry at room temperature.

Preparative TLC was performed using normal phase Silica gel (F_{254} Merek) precoated on aluminium plate (20 cm by 20 cm) and a layer of thickness of 0.25 mm. This was carried out where applicable to determine the purity of compounds.

3.5.2 Vacuum Liquid Chromatography (VLC)

VLC column was packed with thin layer chromatography silica gel 60 (6 - 35 microns mesh) Known masses of crude samples were loaded into the column and eluted with appropriate solvent system to fractionate the crude into fractions containing compounds according to polarity.

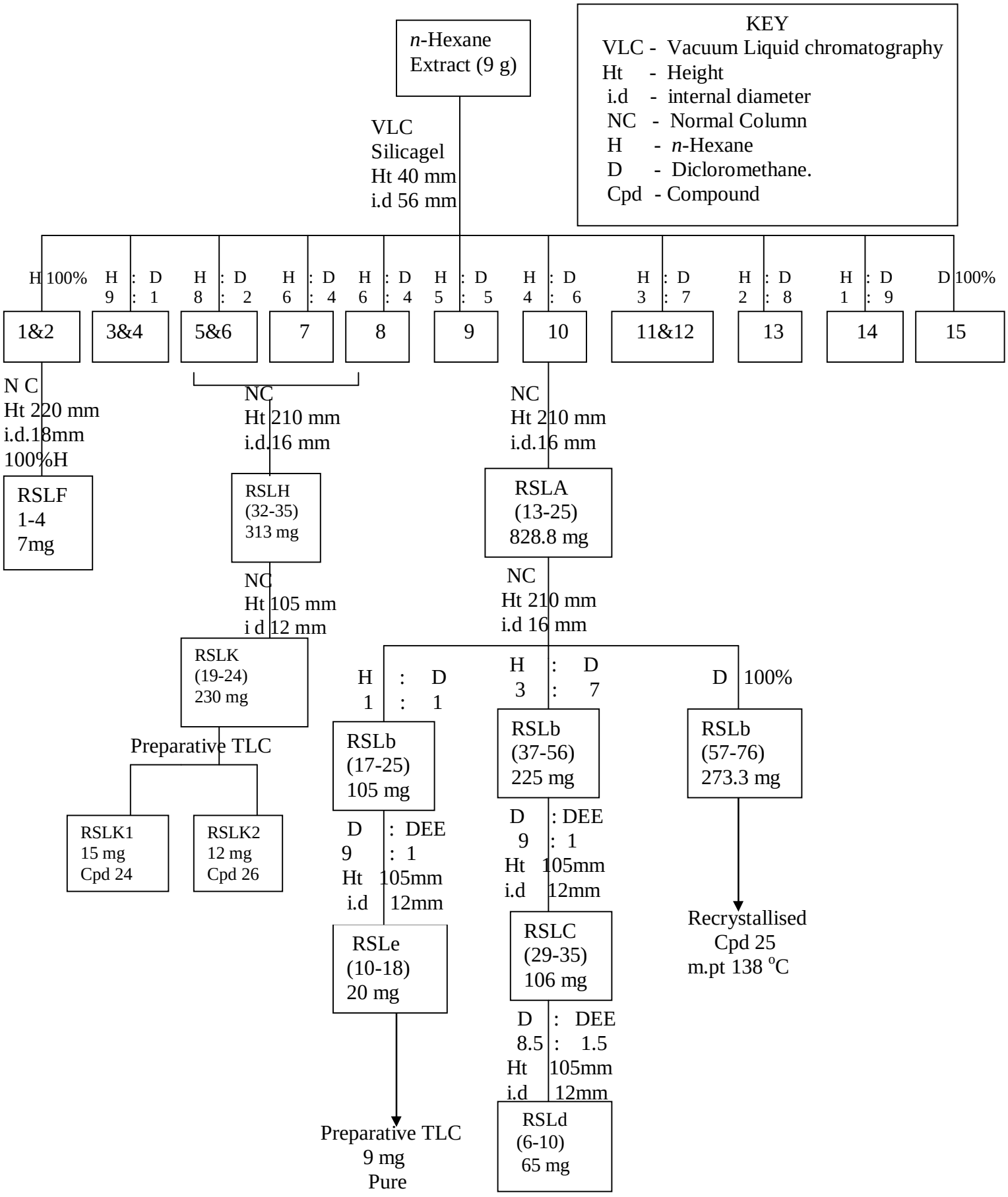
3.5.3 Column Chromatography (CC)

Column chromatography was carried out using glass column of internal diameter, which varied between 1.5 - 5.5 cm, and of length 12 to 55 cm. Silica gel 60 (0.040 - 0.063 mm, 230 - 400 mesh, MERCK) was used and slurry-packing method was employed.

3.5.4 Dragendorff Spraying Reagent

Dragendorff spraying reagent for detection of alkaloids was prepared by mixing 0.85 g of basic bismuth nitrate with prepared mixture of 40 ml of water, 10 ml of acetic acid and 8 g of potassium iodide (KI) dissolved in 10 ml of water to give solution A. Solution B was prepared by dissolving 20 ml of acetic acid in 100 ml of water. The spray reagent was prepared immediately before use, by taking 1 ml of solution A and adding to 10 ml of solution B. Larger quantities were obtained by increasing the ratios of solutions A and B accordingly (Harborne, 1982).

Scheme 2: *n*-Hexane Extract Fractionation



3.6 NMR Spectroscopy

Some suspected pure compounds from fractions were subjected to NMR spectroscopy. A 400 MHz NMR spectrometer was used. The spectra were recorded in $CDCl_3$ as the internal standard. The chemical shifts reported in δ (ppm) units relative to TMS signal and coupling constant (J) in Hz.

3.7 Fourier Transform-Infra Red (FT-IR) Spectroscopy

Some IR spectra were taken in dichloromethane solution and recorded on a Shimazdu (model FT-IR -8400 CE) with absorption given in wave numbers (cm^{-1}). The spectrum was recorded after background correction in the range 4000 – 400 cm^{-1}.

3.8 Melting points determination

Melting points (Mpt) were determined on electro thermal (Gallenkamp) melting point apparatus and expressed in degree centigrade (^oC) and were uncorrected.

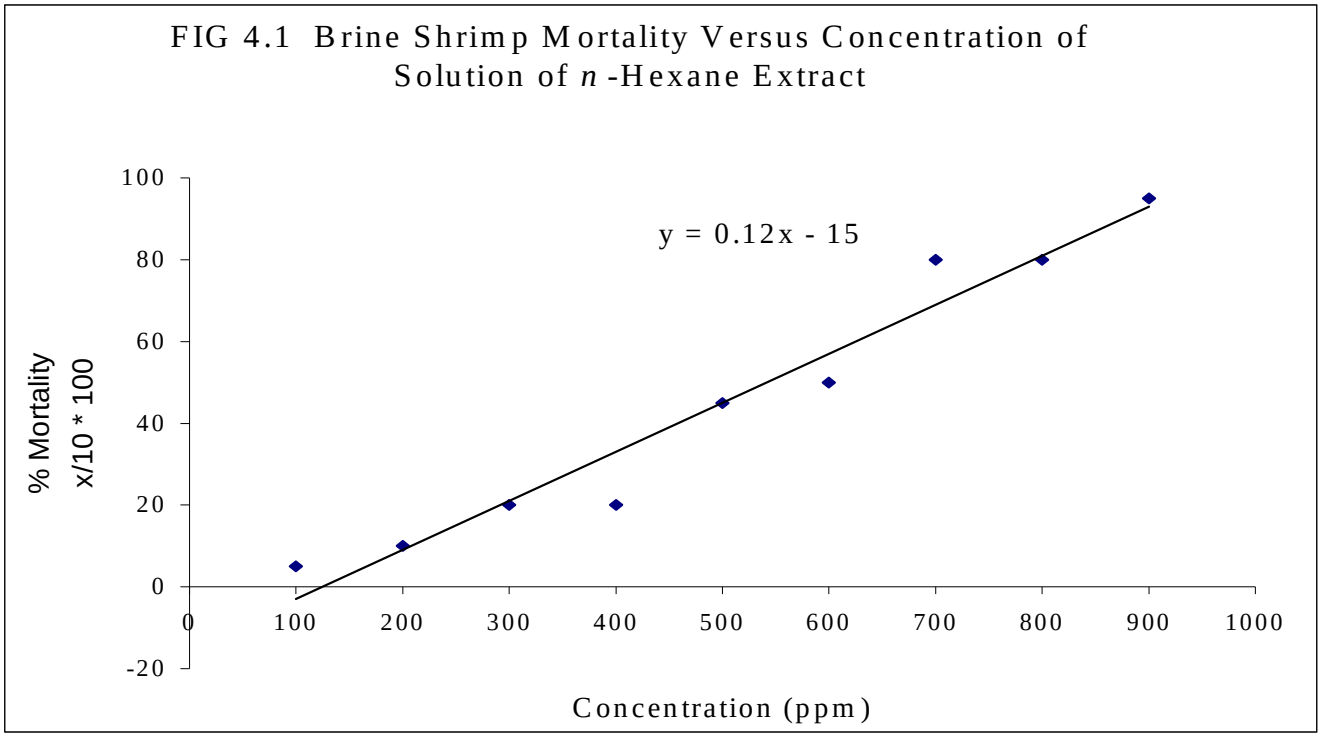
CHAPTER FOUR

RESULTS AND DISCUSSION

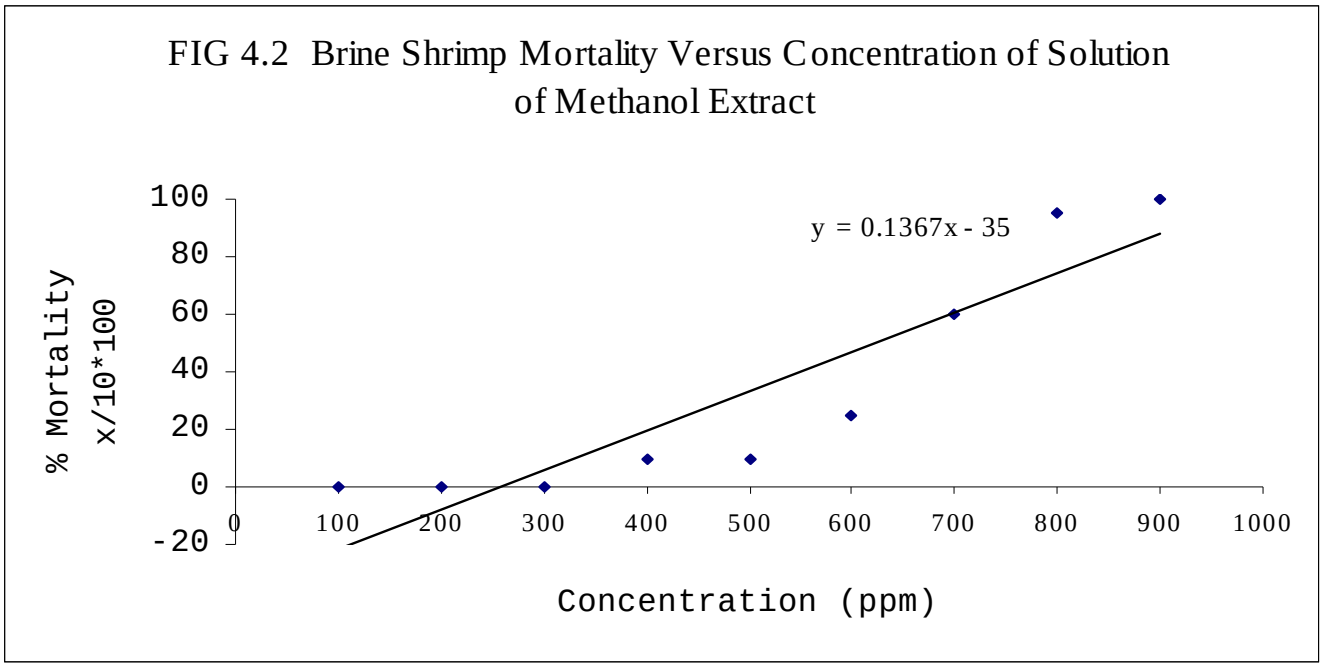
4.1 Brine Shrimp Data

The lethality test to evaluate the bioactivity of crude extracts was done using brine shrimp *(Artemia salina*) larvae as the test organism. Since every assay had ten brine shrimps the living shrimps were counted and the dead shrimps were determined by subtracting from ten. Every concentration was done in triplicate and the average number of dead brine shrimp was determined. Percentage mortality was calculated by taking the averaged number of dead shrimps divided by ten and multiplying by 100.

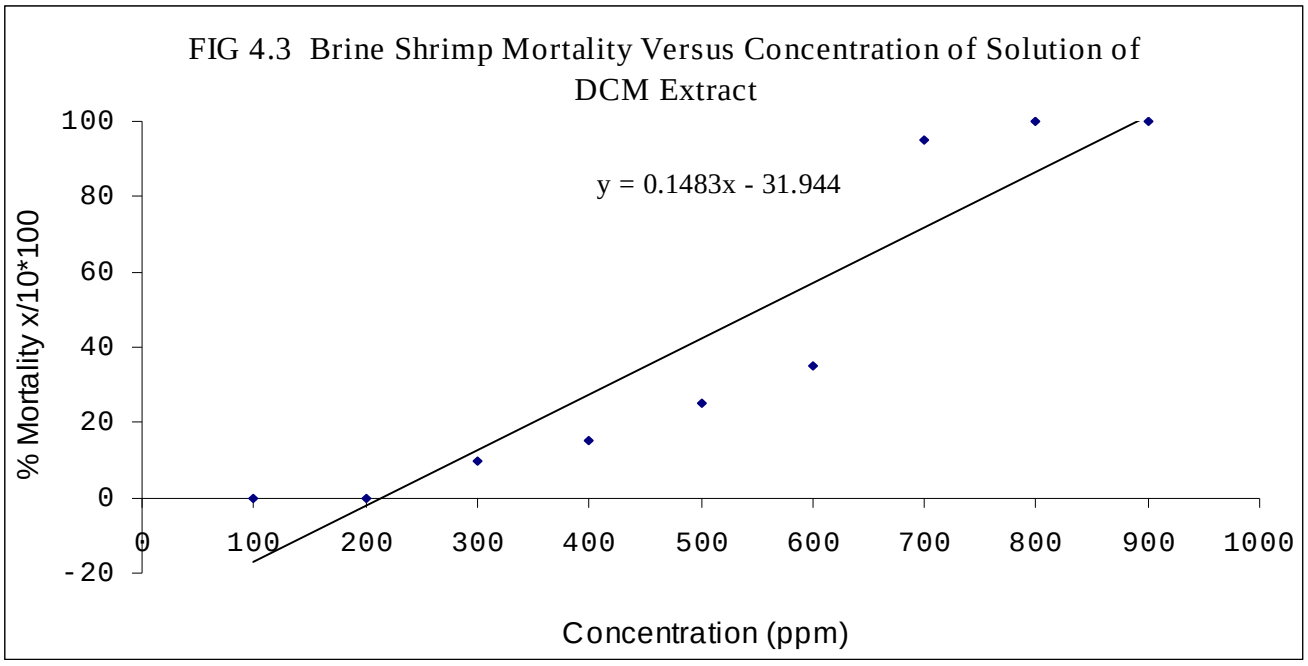
Graphs of concentration of extracts against percentage mortality of brine shrimp were drawn.



The n-hexane extract was active against brine shrimp. It was evident that the mortality of brine shrimp was directly proportional to the concentration.



The methanol extract was also active against the brine shrimp. Lethality was observed only after a concentration of 300 ppm, and increased gradually as the concentration increased.



The DCM extract was active against brine shrimp. Lethality was observed from a concentration of 200 ppm and increased gradually with concentration.

4.1.1 Brine Shrimp Assay Test Analysis

Further analysis of the brine shrimp data was done using SAS program and indicated that the extracts were not toxic to brine shrimp. A crude extract is considered toxic up to a concentration of 240 ppm (Meyer *et al*., 1982).

Chem. 1 MeOH extract

LD_{50} 639.44 ppm

Chem. 2 DCM extract

LD_{50} 553.21 ppm

Chem. 3 n-Hexane extract

LD_{50} 506.11 ppm

Table 1 Brine Shrimp Data Analysis

Chem	100	200	300	400	500	600	700	800	900
1	0 ± 0^{Ac}	0 ± 0^{Ac}	0 ± 0^{Ac}	10 ± 0^{Ac}	10 ± 0^{Ac}	20 ± 5^{Ac}	60 ± 0^{Ab}	95 ± 5^{Aa}	100 ± 0^{Aa}
2	0 ± 0^{Ad}	0 ± 0^{Ad}	10 ± 0^{Acd}	15 ± 5^{Acd}	25 ± 5^{Ado}	35 ± 5^{Ab}	95 ± 5^{Aa}	100 ± 0^{Aa}	100 ± 0^{Aa}
3	5 ± 0^{Ac}	10 ± 0^{Acb}	20 ± 0^{Acb}	20 ± 0^{Acb}	45 ± 5^{Acab}	50 ± 0^{Acab}	80 ± 0^{Aab}	80 ± 0^{Aab}	95 ± 5^{Aa}

Means with the same capital letters within the same column are not significantly different at 95% confidence limit.

Means with the same letters within the same row are not significantly different at 95% confidence limit.

The effect of toxicity however increased with increase in concentration and significant effect was observed above concentration of 600 ppm for the three crude extracts. The LD_{50} of the extracts were above 240 ppm, therefore the extracts were not toxic to brine shrimp. It therefore indicated that any compounds from the extracts could be used for pesticidal, fungicidal and pharmacological effects if active against any microorganisms.

4.2 Anti-Bacteria and Anti-Fungi Assay Test Data

The crude extracts and some fractions were subjected to anti-bacterial and anti-fungal test against *P. aeruginosa, E. coli, S. aureus, B. subtilis* and fungi *C. albicans*. The agar diffusion assay method was used, where 100 µg/disc was loaded on a sterile paper disc of 6 mm diameter and incubated for 24 hr. The inhibition zones of the circular regions around each disc were measured using a ruler in millimeters.

Graphs of concentration of extract and fractions against inhibition diameters of the various microorganisms were drawn using data from the tables. Inhibition diameters of above 6 mm meant that the sample was bioactive against the microorganism. The greater the inhibition diameter the better the sample was in inhibiting growth of respective micro-organism.

Another important factor was the consistency with increase in concentration against inhibition diameter. Samples that showed this consistency were better because efficacy could be adjusted by varying the concentration.

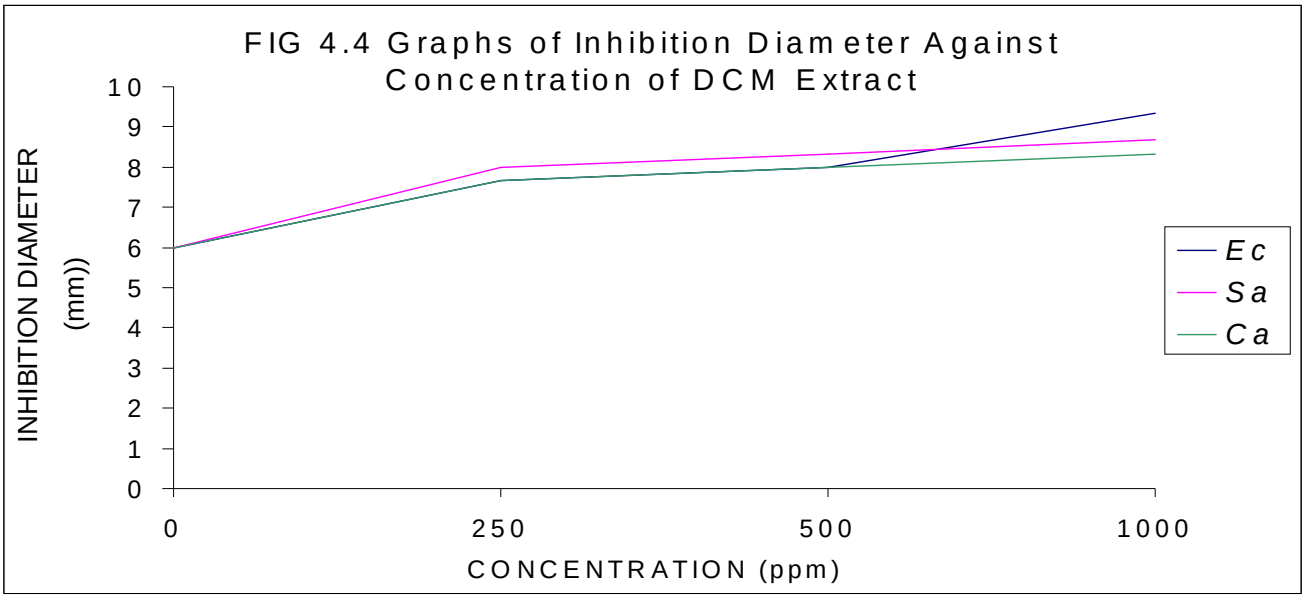
The following bacteria and fungi were used.

Code		Organism
Ec	–	*Escherichia coli*
Sa	_	*Staphylococcus aureus*
Ca	_	*Candida albicans*
Bs	_	*Bacillus subtilis*
Pa	_	*Pseudomonas aeruginosa*

The DCM extract exhibited anti-bacterial and anti-fungal activity against Gram positive *S. aureus*, Gram negative *E. coli* and fungi *C. albicans*. DCM extract had greater inhibiting effect to *E. coli* than both *S. aureus* and *C. albicans.*

Table 2 Inhibition diameter (mm) against concentration (ppm) for DCM extract

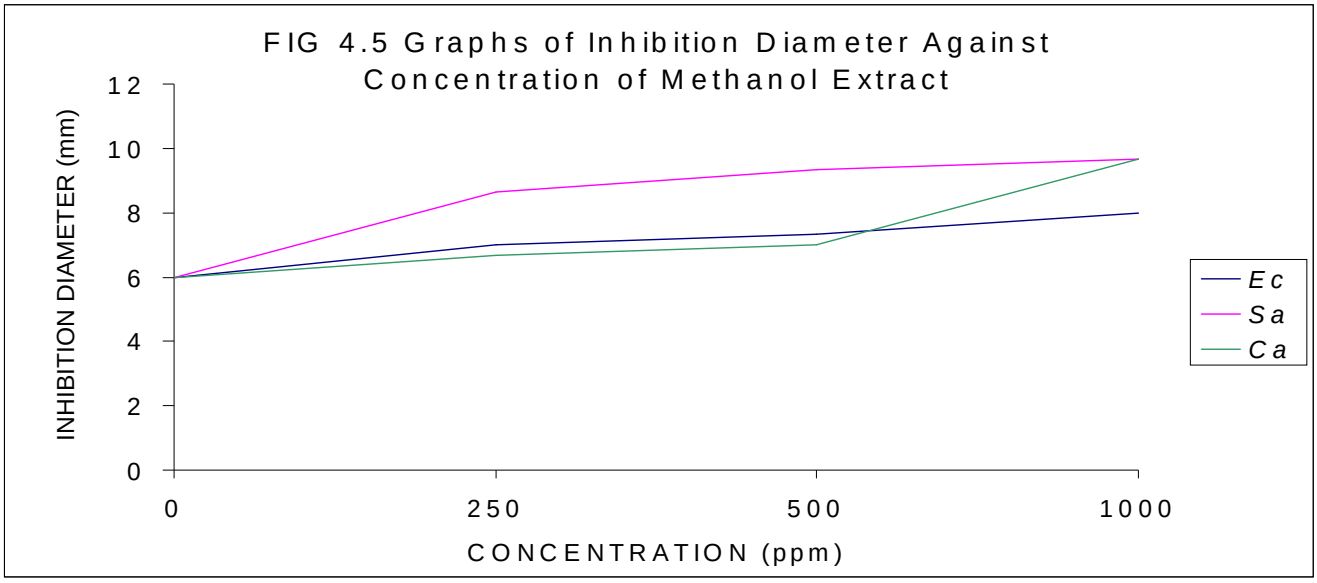
	0	250	500	1000
Ec	6.00	7.67	8.00	9.33
Sa	6.00	8.00	8.33	8.67
Ca	6.00	7.66	8.00	8.33



Methanol extract also exhibited low anti-bacterial and anti-fungal activity against the three organisms. However there was more potent in inhibition to *S. aureus* than both *C. albicans* and *E. coli.*

Table 3 Inhibition diameter (mm) against concentration (ppm) for methanol extract

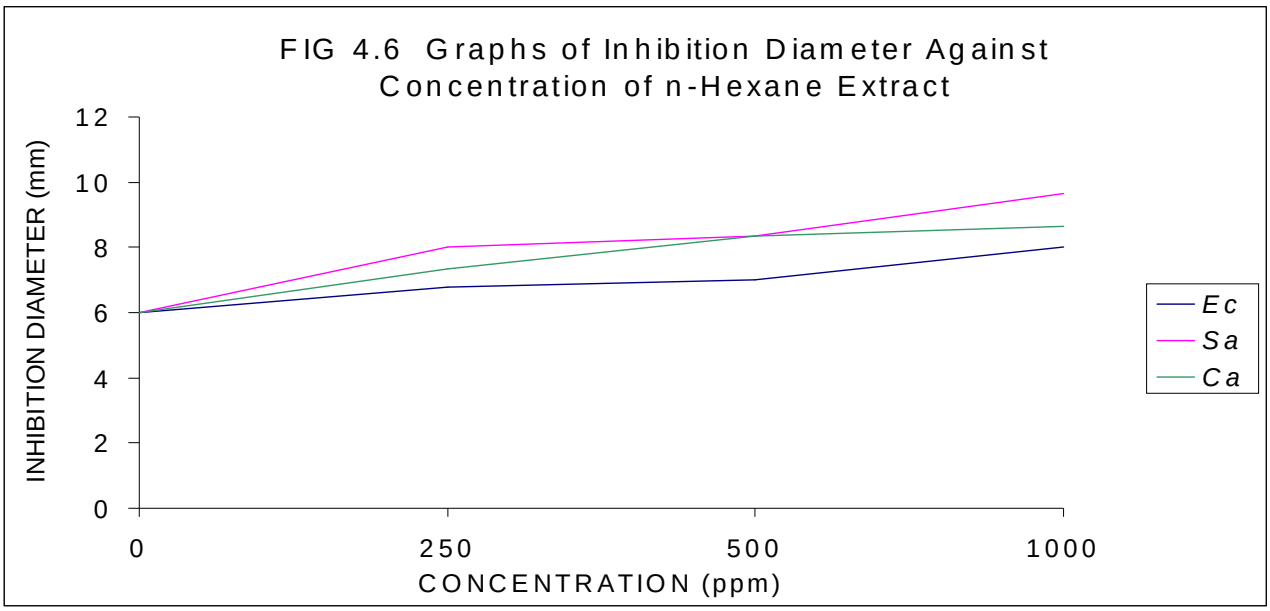
	0	250	500	1000
Ec	6.00	7.00	7.33	8.00
Sa	6.00	8.66	9.33	9.66
Ca	6.00	6.66	7.00	9.66



The n-hexane extract too exhibited low anti-bacterial and anti-fungal activity, but there was improved consistency of increased inhibition with rise of concentration. The extract had more potent in inhibition in *S. aureus* than both *E. coli* and *C. albicans.*

Table 4 Inhibition diameter (mm) against concentration (ppm) for *n*-hexane extract

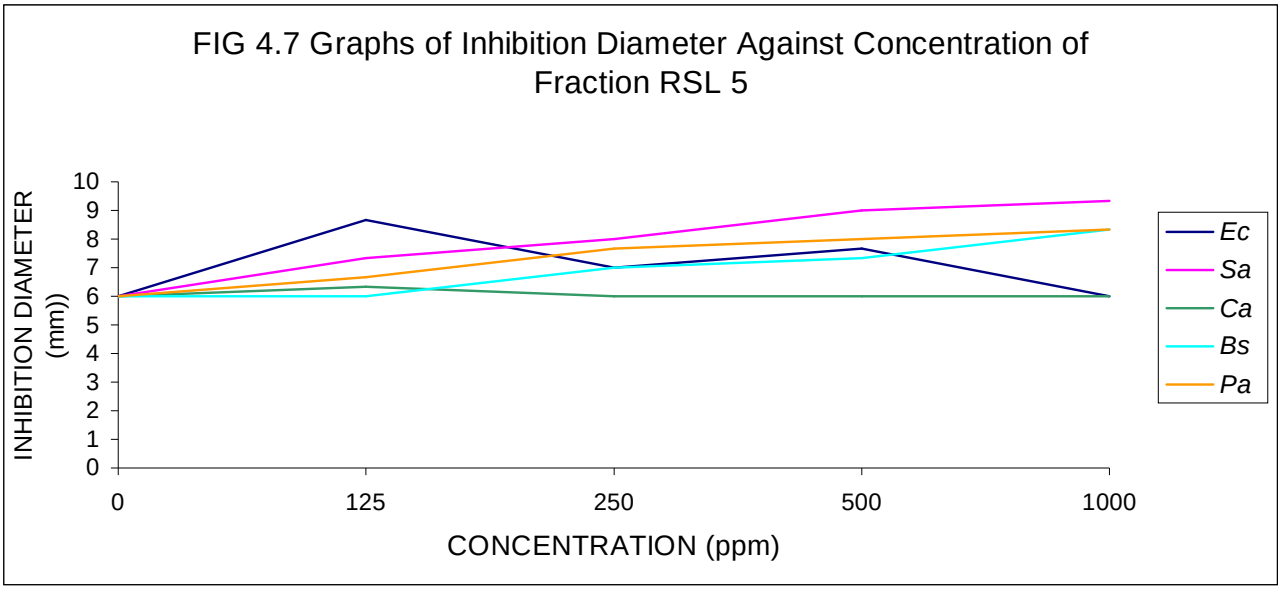
	0	250	500	1000
Ec	6.00	6.80	7.00	8.00
Sa	6.00	8.00	8.33	9.66
Ca	6.00	7.33	8.33	8.66



Fraction RSL 5 had great inhibiting effect to *S. aureus* followed by *P. aeruginosa, B. subtilis* and *C. albicans*. However, *E. coli* did not show concentration consistency.

Table 5 Inhibition diameter (mm) against concentration (ppm) for Fraction RSL5 extract

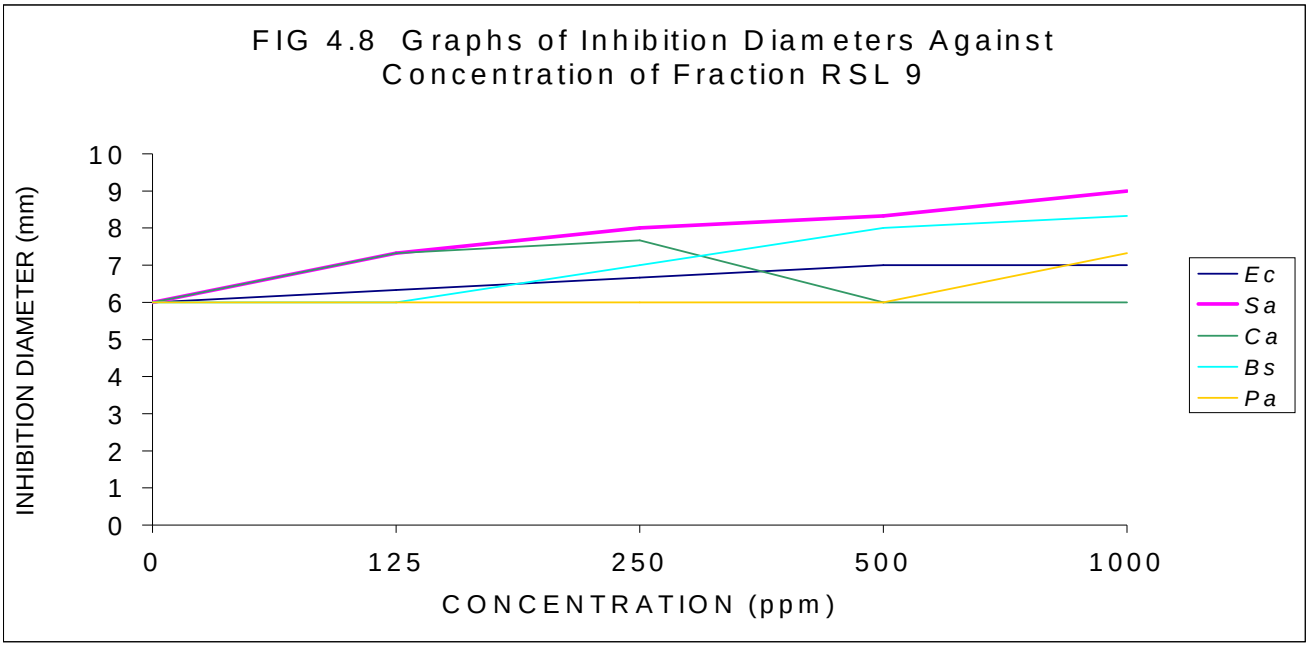
	0	125	250	500	1000
Ec	6.00	8.67	7.00	7.67	6.00
Sa	6.00	7.33	8.00	9.00	9.33
Ca	6.00	6.33	6.00	6.00	6.00
Bs	6.00	6.00	7.00	7.33	8.33
Pa	6.00	6.67	7.67	8.00	8.33



Fraction RSL 9 was highly potent against *S. aureus*, followed by *B. subtilis*, *E. coli* and *P. aeruginosa* in that order. However, *C. albicans* did not indicate concentration consistency.

Table 6 Inhibition diameter(mm) against concentration(ppm) for Fraction RSL9 extract

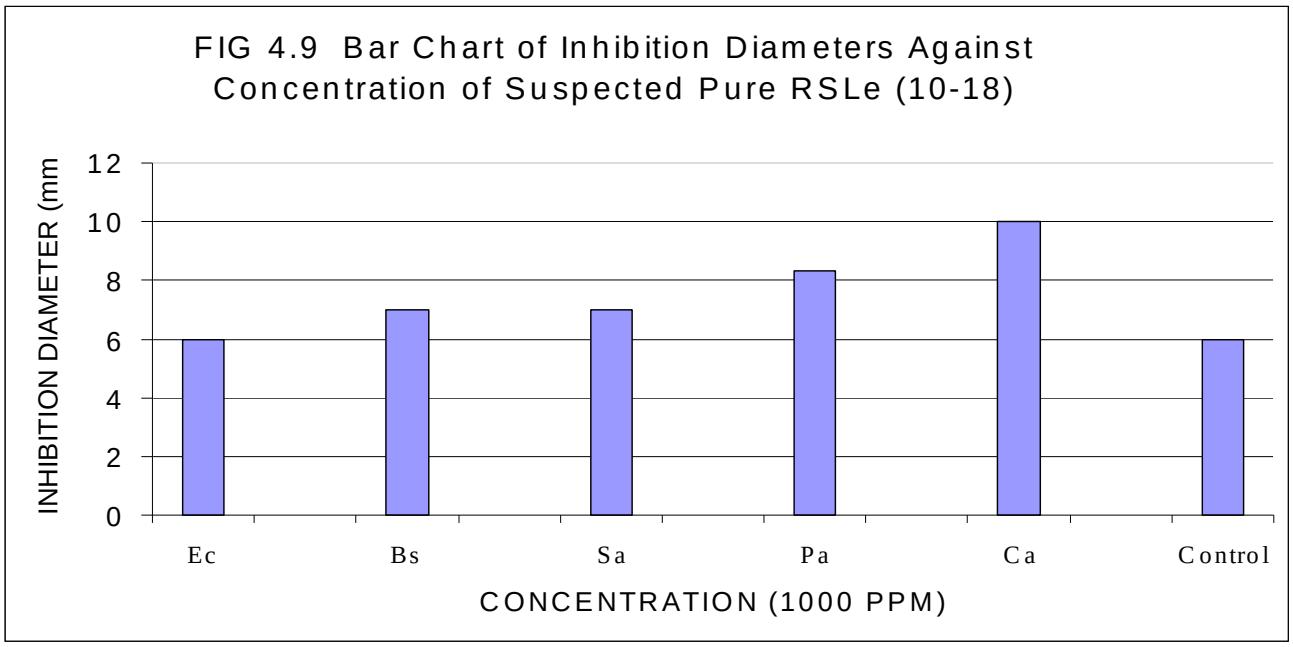
	0	125	250	500	1000
Ec	6.00	6.33	6.67	7.00	7.00
Sa	6.00	7.33	8.00	8.33	9.00
Ca	6.00	7.33	7.67	6.00	6.00
Bs	6.00	6.00	7.00	8.00	8.33
Pa	6.00	6.00	6.00	6.00	7.33



Suspected pure compound RSLe (10 - 18) showed greatest inhibition to *C. albicans* and *P. aeruginosa* at a concentration of 1000 ppm. It had little effect on *S. aureus* and *B. sabtilis* but no effect on *E. coli*.

Table 7 Inhibition diameters (mm) against various organisms at a concentration of 1000 (ppm) of RSLe (10 - 18)

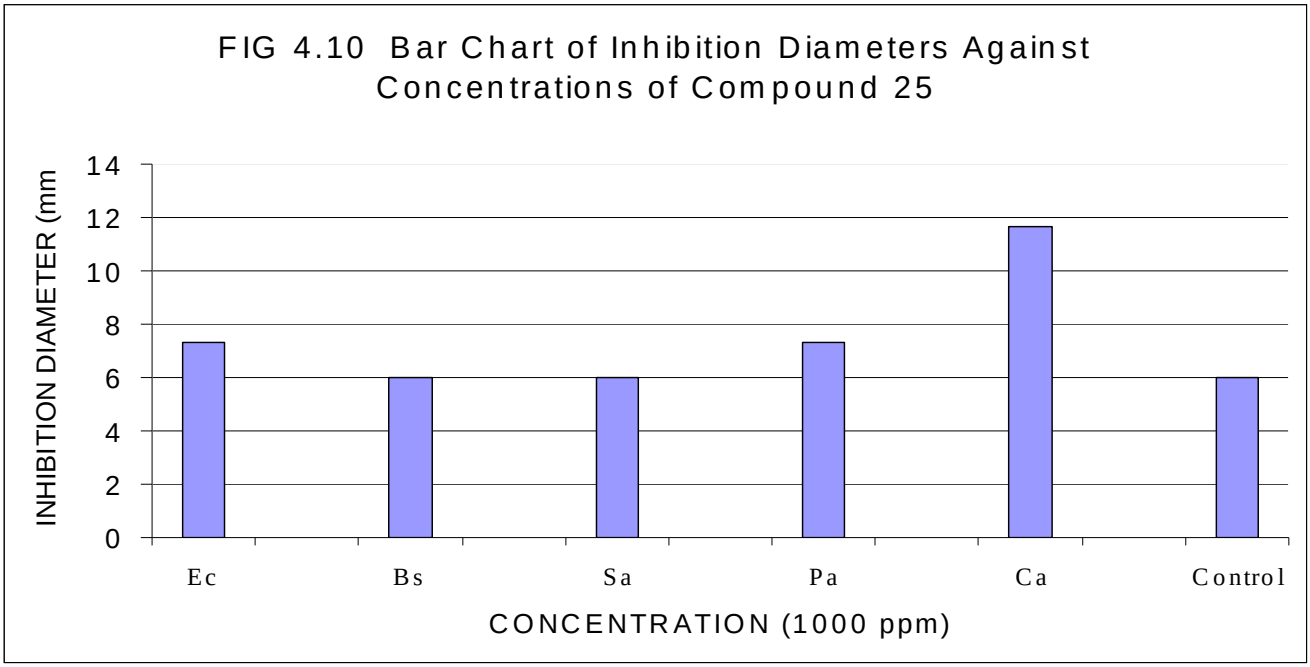
Micro-organism	Ec	Bs	Sa	Pa	Ca	Control
Average (mm)	6.00	7.00	7.00	8.33	10.00	6.00



Compound **25** later identified as β-sitosterol was most potent on *C. albicans*. It had no effect on *S. aureus* and *B. subtilis, P. aeruginosa* and *E. coli*.

Table 8 Inhibition diameters (mm) against various organisms at a concentration of 1000 (ppm) of compound 25.

Micro-organism	Ec	Bs	Sa	Pa	Ca	Control
Average (mm)	7.33	6.00	6.00	7.33	11.67	6.00



Crude *n*-hexane extract 1000 ppm had inhibition diameter of 9.6 mm against *S. aureus* (*Sa*), 8.6 mm against *C. albicans (Ca*) and 8.0 mm against *E. coli* (*Ec*), respectively. It implied that the *n*-hexane extract had potential as an anti-biotic and anti-fungi effect against those organisms.

Fraction RSL5 had even enhanced inhibition against *S. aureus* of 9.3 mm at 1000 ppm but reduced activity to the other organisms. Fraction RSL9 too had activity against *S. aureus* of inhibition diameter 8.3 mm at 1000 ppm and again reduced activity to the other organisms.

Suspected pure RSLe (10 - 18) had reduced activity against *S. aureus* of 7.0 mm at 1000 ppm. Also pure compound RSLb (57 - 75) had no activity against *S. aureus* but had inhibited *P. aeruginosa (Pa*) by 7.3 mm at 1000 ppm.

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Pure compound RSLb (57 - 75) and suspected pure compound RSLe (10 - 18) had moderate activity against *C. albicans* of 12 mm and 11.6 mm, respectively at 1000 ppm each.

To test for pyrolizidine alkaloids, the extracts were spotted on TLC plates and after spraying with dragendorff did not show yellow or orange spots and hence pyrolizidine alkaloids were deduced to be absent.

4.3 Physical and Spectroscopic Data of Isolated Compounds

Compound **24:** White crystals, Mpt.162 - 164 ^{0}C, Rf, 0.5 (EtOAc/Hexane) (1:9), IR ν_{max} 3357, 1643 1458, 1381, 1036, 995, ^{13}C NMR (400 MHz, $CDCl_3$) δ 38.6 (C-1), 27.3 (C-2), 79.4 (C-3), 38.6 (C-4), 55.6 (C-5),18.8 (C-6), 32.9 (C-7), 38.6 (C-8), 48.0 (C-9), 37.6 (C-10), 23.9 (C-11), 122.14 (C-12), 145.6 (C-13), 42.1 (C-14), 26.6 (C-15), 27.4 (C-16), 32.9 (C-17), 47.7 (C-18), 47.2 (C-19), 30.8 (C-20), 33.7 (C-21), 37.3 (C-22), 28.4 (C-23), 15.8 (C-24), 15.9(C-25), 17.2 (C-26), 26.4 (C-27), 28.5 (C-28), 33.0 (C-29), 24.0 (C-30).

Compound **25:** White crystals Mpt. 138 - 138.2 ^{o}C Rf, 0.7 (DCM/DEE), IR ν_{max} 3423, 1462, 1377, 1053, 1021, 960 ^{13}C NMR (400 MHz, $CDCl_3$) δ 37.67 (C-1), 32.09 (C-2), 72.21 (C-3), 42.73 (C-4), 141.18 (C-5), 122.09 (C-6), 32.32 (C-7), 32.09 (C-8), 50.57 (C-9), 36.92 (C-10), 21.49 (C-11), 40.20 (C-12), 42.73 (C-13), 56.50 (C-14), 24.70 (C-15), 28.63 (C-16), 57.19 (C-17), 12.61 (C-18), 19.78 (C-19), 36.55 (C-20), 19.44 (C-21), 34.38 (C-22), 26.55 (C-23), 46.28 (C-24), 29.61 (C-25), 19.18 (C-26), 20.19 (C-27), 23.50 (C-28), 12.61 (C-29).

Compound **26:** White crystals, Mpt. 144 - 145 ^{o}C Rf 0.9 (EtOAc/Hexane) (1:9), ^{13}C NMR(400 MHz $CDCl_3$) δ 32.2 (C-1), 37.6 (C-2), 72.2 (C-3), 42.7 (C-4), 141.1 (C-5), 122.1 (C-6), 29.3 (C-7), 29.3 (C-8), 42.6 (C-9), 40.0 (C-10), 23.4 (C-11), 32.0 (C-12), 42.6 (C-13), 46.2 (C-14), 21.6 (C-15), 21.4 (C-16), 50.5 (C-17), 19.8 (C-18), 20.2 (C-19), 29.3 (C-20), 19.4 (C-21), 129.7 (C-22), 138.7 (C-23), 40.9 (C-24), 25.8 (C-25), 12.3 (C-26), 32.0 (C-27), 20.2 (C-28), 20.2 (C-29).

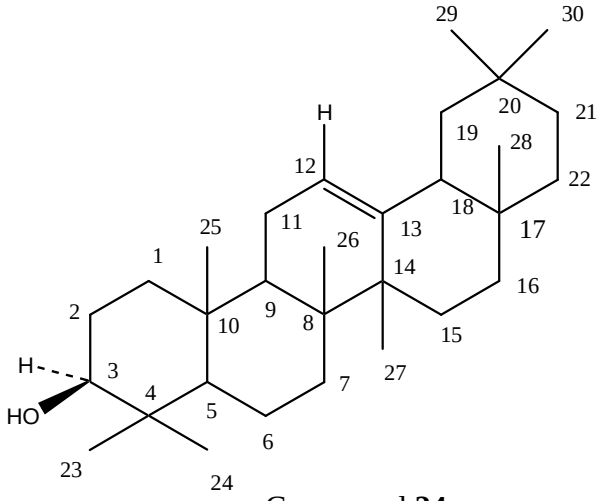
4.4 Structure Elucidation of Isolated Compounds

4.4.1 Compound 24 (Rslk 1)

Compound **24** (RSLK 1) was obtained as white crystals, with Mpt. 163 – 164 ^{0}C. On TLC the isolated compound had an Rf of 0.5 in EtOAc/Hexane (1: 9). A band at 3357 cm^{-1} in the IR spectrum suggested an OH stretch.

The ^{1}H NMR spectrum showed doublet at δ 3.2 indicated presence of a hydroxyl group (OH). Eight peaks between δ 0.7 and 1.5 indicated methyl groups, which strongly suggested a triterpenoid structure. A peak at δ 5.1 suggested an olefinic proton.

The ^{13}C NMR displayed thirty-carbon atoms, characteristic of pentacyclic triterpenoid (PCTT). The peak at δ 79.4 was attributed to hydroxylated carbon atom at position 3. Peaks at δ 122.1 and 145.6 suggested presence of double bond attached to the bridge accounting for only one olefinic proton signal at δ 5.1 in the ^{1}H NMR spectra. The ^{13}C NMR data was matched with ^{13}C NMR data of known compounds and the compound **24** was hence identified as β-amyrin (Table 9).



Compound **24**

Table 9. ^{13}C NMR spectra data for compound 24 and β-amyrin (Mahato and Kundu, 1994)

Carbon No.	β-amyrin, (δ)	Compound 24, (δ)	^{13}C and Dept
1	38.7	38.6	CH_2
2	27.3	27.3	CH_2
3	79.0	79.4	CH(OH)
4	38.8	38.6	C
5	55.3	55.6	CH
6	18.5	18.8	CH_2
7	32.8	32.9	CH_2
8	38.8	38.6	C
9	47.7	48.0	CH
10	37.6	37.6	C
11	23.6	23.9	CH_2
12	121.8	122.1	=CH
13	145.1	145.6	=C
14	41.8	42.1	C
15	26.2	26.6	CH_2
16	27.0	27.4	CH_2
17	32.5	32.9	C
18	47.4	47.7	CH
19	46.9	47.2	CH_2
20	31.1	30.8	C
21	34.8	33.7	CH_2
22	37.2	37.3	CH_2
23	28.2	28.4	CH_3
24	15.5	15.8	CH_3
25	15.6	15.9	CH_3
26	16.9	17.2	CH_3
27	26.0	26.4	CH_3
28	28.4	28.5	CH_3
29	33.3	33.0	CH_3
30	23.7	24.0	CH_3

4.4.2 Compound 25 (Rslb)

Compound **25** was obtained as a white crystalline solid with a melting point of 138 - 139^{0}C. On TLC the compound had an R_f of 0.7 in DCM/DEE, 9:1. A broad band at 3423 cm^{-1} in its IR spectrum suggested a hydroxyl function (O-H stretch).

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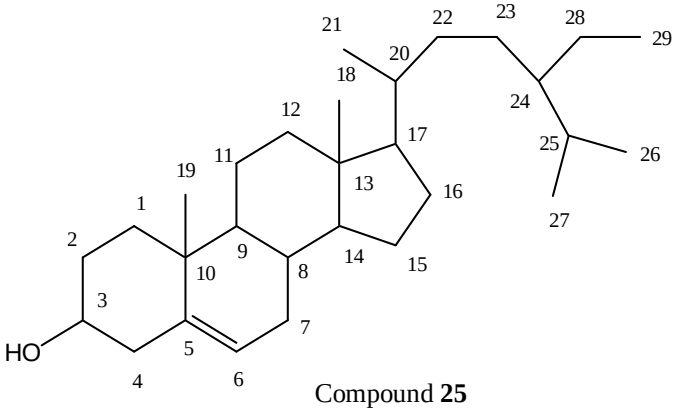
The ^{1}H NMR spectrum displayed a multiplet centered at δ 3.50 characteristic of a germinal to a β- hydroxyl group at C-3 in triterpenoids. The doublet at δ 5.3 suggested an olefinic proton. The rest of the protons were between δ 0.65 and 2.25 with noticeable six-methyl peaks protons at δ 0.65, 0.83, 0.85, 1.00, 1.25, and 1.57, characteristic of plant sterols.

The ^{13}C NMR displayed twenty-nine carbon atoms, strongly suggesting a modified triterpenoid. Carbon peak at δ 72.21, a methine was assigned to C-3 indicating an attachment of hydroxyl group to this carbon. The only quaternary alkenic carbon atom at δ 141.18 and methine alkenic carbon atom at δ 122.09 was assigned to C-5 and C-6, respectively.

This data was compared with ^{13}C NMR data of known compounds and this compound **25** was hence elucidated as β-sitosterol (Table 10). The spectra patterns were consistent with steroids skeletal structure of β-sitosterol (Chauras and Wichti, 1987). Therefore, compound **25** was elucidated as β-sitosterol. The melting point was determined as 138 ^{o}C.

Table 10. ^{13}C NMR spectra data for compound 25 and β- sitosterol (Chaurasia and Wicht, 1987)

Carbon No.	β-sitosterol, (δ)	Compound 25, (δ)	^{13}C and Dept
1	37.24	37.67	CH_2
2	31.61	32.09	CH_2
3	71.78	72.21	CH(OH)
4	42.28	42.73	CH_2
5	140.71	141.18	=C
6	121.68	122.09	=CH
7	31.89	32.32	CH_2
8	31.89	32.09	CH
9	50.11	50.57	CH
10	36.48	36.92	C
11	21.01	21.49	CH_2
12	39.77	40.20	CH_2
13	42.28	42.73	C
14	56.75	56.50	CH
15	24.28	24.70	CH_2
16	28.24	28.63	CH_2
17	56.04	57.19	CH
18	11.85	12.61	CH_3
19	19.40	19.78	CH_3
20	36.12	36.55	CH
21	19.02	19.44	CH_3
22	33.92	34.38	CH_2
23	26.02	26.55	CH_2
24	45.80	46.28	CH
25	29.08	29.61	CH
26	18.77	19.18	CH_3
27	19.80	20.19	CH_3
28	22.04	23.50	CH_2
29	11.97	12.61	CH_3



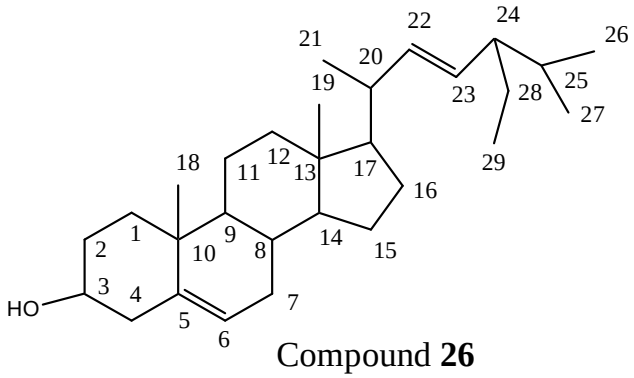
Compound **25**

4.4.3 Compound 26

Compound **26** (Rslk2) was obtained as white crystals with a melting point of 144 – 145 ^{0}C. On TLC the compound had an R_f of 0.9 in EtOAce/Hexane (1:9).

^{1}H NMR spectrum displayed a multiplet at δ 3.50 characteristic of a proton germinal to a β-hydroxyl group at C-3 in triterpenoids. It also displayed a doublet at δ 5.15 suggesting presence of an olefinic proton. The rest of the protons were between δ 0.60 and 2.20 with noticeable six methyl peaks protons at δ 0.60, 0.72, 0.80, 0.95, 1.25, and 1.62, characteristic of phytosterols.

^{13}C NMR displayed a spectrum very similar to that of stigmasterol. The peak at δ 72.2 was assigned to C-3 because of the presence of the hydroxyl group. It also displayed peaks at δ 141.2 and 122.1 suggesting the presence of olefinic carbons, with the deshielded signal assignable to the quaternary at the bridge. Six methyl groups were assigned at δ 12.23, 19.06, 19.35, 19.42, 19.83 and 29.25. Other olefinic carbons were assigned peaks at δ 129.65 and 138.74 for C-22 and C-23, respectively. This data matched very closely with that of known stigmasterol (Knight, 1974). Hence compound **26** was identified to be stigmasterol.



Compound **26**

Table 11. ^{13}C NMR spectra data for compound 26 and stigmasterol (Knight, 1974)

Carbon No.	Stigmasterol, (δ)	Compound 26 (δ)	^{13}C and Dept
1	37.24	37.6	CH_2
2	31.61	32.2	CH_2
3	71.78	72.2	CH(OH)
4	42.28	42.7	CH_2
5	140.71	141.1	=C
6	121.68	122.1	=CH
7	31.89	29.3	CH_2
8	31.89	29.3	CH
9	50.11	50.51	CH
10	36.48	37.64	C
11	21.01	21.4	CH_2
12	39.77	40.07	CH_2
13	42.28	42.61	C
14	56.75	57.15	CH
15	24.28	24.77	CH_2
16	28.24	28.66	CH_2
17	56.04	56.42	CH
18	11.85	12.23	CH_3
19	19.40	19.06	CH_3
20	36.12	36.91	CH
21	19.02	19.35	CH_3
22	129.37	129.65	=CH
23	138.28	138.74	=CH
24	45.80	40.93	CH
25	29.08	29.25	CH
26	18.77	19.42	CH_3
27	19.80	19.83	CH_3
28	22.04	22.45	CH_2
29	11.97	12.27	CH_3

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

5.1.1 The study has shown that *Senecio lyratus* contains triterpenoids. Three triterpenes were isolated, compounds, (**24),** (**25)** and (**26)**, but spectra data for one compound was not received by the time this thesis was written.

5.1.2 Compounds (**24**) and **(26**) were isolated for the first time in this plant *Senecio lyratus*. Compound (**25**) had previously been reported (Kiprono *et a*l., 2000)

5.1.3 The n-hexane extract displayed the highest toxicity against brine shrimp and was the most bioactive extract. From the three crude extracts, and the effect on micro-organisms, *S. aureus* displayed consistent and highest inhibition diameter as compared to the other micro-organisms. The suspected pure compound RsLe (10 - 18) and compound 25 showed greatest inhibition to *C. albicans*.

5.1.4 From the biological tests carried out, we observed anti-bacterial and anti-fungal effects due to various fractions. However, none of the effects could be attributed to the pure compound β-amyrin and stigmasterol directly because the amounts were too little to subject to anti-bacterial and anti-fungal tests. β-Sitosterol showed significant effect against *C. albicans* and *P. aeruginosa* (*Pa*).

5.1.5 The pure compounds did not display major inhibition to *S. aureus*, as the crude and fractions did. This indicated that there could be some other compounds not isolated that were effective against *S. aureus*, or worked synergistically with other compounds.

Although the isolated compounds are not the only compounds present in the plant material as evidenced from the TLC analysis, future work should aim at isolating other compounds not isolated in this study especially the alkaloids known to be absorbent to this species of plants and determine their bioactivity effects.

5.2 Recommendations

5.2.1 Further biological tests could be done on the pure compounds to ascertain whether they have direct impact on the anti-bacterial and anti-fungal effects that were observed in the fractions.

5.2.2 Pyrrolizidine alkaloids were revealed to be a major constituent of Senecio species but were not isolated in this research. Further research focusing on to isolation of pyrrolizidine alkaloids in *Senecio lyratus* would be quite encouraged.

5.2.3 TLC analysis showed compounds that were not isolated due to small amounts of plant materials, time, organic solvents needed and spraying reagents required. Further isolation should continue. The role played by these trace compounds in the overall biological activities of extracts should be further studied.

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