BIOACTIVE METABOLITES OF SELECTED KENYAN PLANTS USED AS BIOPESTICIDES AGAINST *ACANTHOSCELIDES OBTECTUS* IN BUNGOMA DISTRICT, KENYA

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(Chemistry)

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2008

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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**.**

2008

DECLARATION

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

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DEDICATION

This work is dedicated to my beloveth father and mother, Mr and Mrs Mining who inspired me to be what Iam.

ACKNOWLEDGEMENTS

I am grateful to the almighty God for giving me life and strength to overcome challenges. I appreciate the cordial assistance rendered by my supervisors, Professor Mabel Imbuga, department of Biochemistry, Jomo Kenya University of Agriculture and Technology (JKUAT), Prof. Teresa Akeng'a, department of chemistry, Kigali Institute of Science and Technology (KIST) and Prof. Paul Tarus of Chemistry department, Kenyatta University (KU) for their kind and keen guidance, supervision and persistent support throughout the course of research and writing up of the thesis.

I would like to vehemently express my sincere gratitude to Dr Tsanuo, M.K, the Chairman Chemistry department, JKUAT for his guidance in data analysis and write up of this thesis, Mr Mawili Makusa, the Chief Technician for allowing me to use their laboratory facilities for extraction and fractionation of crude extracts, isolation and purification of pure compounds.

I am most grateful to VicRes (Sida /Sarec) for funding much of my work.

I would also like to thank Mr. Muthanga, (Botany department of JKUAT) and Mrs. Gladys Nyaga, Zoology department, JKUAT for their guidance and assitance in carrying out the antibacterial activity and insecticidal tests, respectively. I appreciate the cooperation of my fellow postgraduate students: Rose Ogara, Phyllis Kamotho, Harrison Wanyika, and Mungai Delide.

My sincere thank are forwarded to all friends and colleagues who have contributed morally and materially for the successful completion of my thesis, they include Mr.Polycarp Lutta Kweyu who gave me his computer to write my thesis he also assisted me in the N.M.R data acquisition of the organic compounds in Botswana,

my brother Philip Kirui who supported me with money for my up keep and my friend Samwel Mirie for his constant encouragement during my laboratory work.

I am greatly indebted to my Fiancée, Ruth Sum, for the social and psychological support she accorded me through out the entire period of the research work. Finally, but not lastly, I am grateful to my brothers and sisters in the Lord who have unceasingly been praying for me as I struggled with the research work. To all who have contributed in anyway to my success, I say, may God give you perfect peace and long life.

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ABBREVIATIONS

AAGR	Average Annual Growth Rate
^{13}C NMR	Carbon-13 nuclear magnetic resonance
CC	Column chromatography
J	Coupling constant
$CDCl_3$	Dueterated chloroform
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloromethane
DEPT	Distortionless Enhancement Polarisation Transfer.
DMSO	Dimethyl sulphoxide
d	Doublet
dd	Doublet of doublet
EIMS	Electron Impact Ionization Mass Spectroscopy
EPA	Environmental protection agency
EtOAc	Ethyl acetate
FPP	Farnesyl pyrophosphate
FQPA	Food quality protection act
FT-IR	Fourier-transform infrared
Hz	Hertz
HPLC	High Performance Liquid Chromatography
IPM	Integrated Pest Management
LD_{50}	Lethal dose at 50 %
m	Multiplet

MeOH	Methanol
mp	Melting point
ms	Mass spectrometry
m/z	Mass to charge ratio
1-D	One dimensional
ppm	Parts per million
PTLC	Preparative thin layer chromatography
^{1}H NMR	Proton nuclear magnetic resonance
s	Singlet
SAM	S-adenosyl methionine
t	Triplet
TLC	Thin layer chromatography
TMS	Tetramethylsilane
2-D-NMR	Two dimensional Nuclear Magnetic Resonance
UV	Ultraviolet
VLC	Vacuum liquid chromatography

ABSTRACT

The post-harvest pests are known to cause severe losses of beans in particular the bean weevil (*Acanthoscelide obtectus*) necessitating immediate and long-term measures. Convectional pesticides have been used to protect stored grain but social and environmental repercussions associated with their uses and pests resistance have aroused researchers to search for newer, potent, eco-friendly, biodegradable and more convenient to use pesticides. The use of natural products derived from the metabolic activity of plants may constitute a new avenue of pest control. This thesis describes phytochemical and biological investigations of leaves, root, and stem bark crude extracts and isolated compounds of selected Kenyan plants used to control the bean weevil (*Acanthoscelide obtectus)*. The plants studied were: *Senna didymobotrya* (L.) Irwin and Barneby, *Euclea divinorum* Hiern*, and Ziziphus abyssinica* (A) Rich. The powdered plant materials were sequentially extracted with solvents of increasing polarity starting with hexane, dichloromethane, ethyl acetate, and methanol. The extracts were concentrated using a rotary evaporator at 45^0C in vacuo and the extracts stored in low temperatures. Isolation of biologically active compounds was done through Column Chromatography (CC) in Silica gel and Sephadex LH20, Vacuum Liquid Chromatography (VLC) and preparative thin layer chromatography monitored by Thin Layer Chromatography (TLC). Bioassay tests of the compounds were done against brine shrimp and against bean weevil to establish their insecticidal activity. Hexane and dichloromethane, crude extracts of *Senna didymobotrya*, *Euclea divinorum* and *Ziziphus* *abyssinica* showed lethality against brine shrimp with LD$_{50}$ of 345.1 and 195.0; 952.0 and 689.9; 699 and 871.5 ppm, respectively. Dichloromethane extract of *Senna didymobotrya* exhibited the highest toxicity and therefore, was the most bioactive extract. Dichloromethane extract of *Senna didymobotyra* showed the highest adulticidal activity of 95±0.06 and 100±0.0 at 750 and 1000 ppm respectively and is a potential source for novel bean protectant against bean weevil, *Euclea divinorum, and Ziziphus abyssinica* extracts had moderate adulticidal activity. Anti-bacterial activity tests for the hexane, crude extracts of *Senna didymobotrya* pods showed an inhibition zone of 10.3, 12.3, 6.0, 10.3, and 23.6 mm against *Candida albicans, Bacillus subtilis*, *Pseudomonas aureginosa, and Escherichia coli* and *Staphylococcus aureus*, respectively, while dichloromethane extract showed an inhibition zone of 9.3, 9.3, 6.0, 7.6, and 10.3 mm on the same bacteria, respectively. Both extracts showed mild to low activity towards both gram-negative bacteria and gram-positive bacteria. Structure elucidation of the isolated compounds was done using various spectroscopic techniques; Infrared (IR), Ultraviolet (UV), Mass Spectroscopy (MS) and Nuclear Magnetic Resonance (NMR). Four compounds were isolated; compound **52** (fraction 5) and **53** (fraction 7) were isolated from hexane extract of *S. didymobotrya* root bark and compounds **54** (fraction 8) and **55** (fraction 10) were isolated from DCM extract of *S. didymobotrya*. The isolated compounds [**52**] and [**53**] exhibited low activity (≤ 8mm) towards *P. aureus* and *S. aureus*, while compounds [**54**] and physcion [**55**] were active towards *S. aureus* but inactive towards *P. aureginosa*. The result presented in this study demonstrates a

possible scientific rationale for the incorporation of the root bark of *Senna didymobotrya* into traditional medicine and methods of grain protection in Bungoma district, and are likely to have been selected after empirical demonstration of their efficacy over long period of time.

CHAPTER ONE

1.0 INTRODUCTION

1.1 General introduction

Food quality and security pose a big challenge to man. Although a variety of solutions to this problem exist, one of the more important aspect is to improve efficiency in food production and post harvest practices to ensure that food loses are minimized if not eliminated and food produced is of good quality and safe for human consumption. Kenya, like other tropical countries, suffers crop losses due to pests. This is partly attributed to conducive conditions for insect survival (Perthunen, 1972). Besides other causes of food losses, like crop diseases and weeds, both pre and post harvest pests consume up to 40% of Africa's grain. In addition, post harvest losses incurred yearly on stored durable agricultural food products in Kenya have been estimated to be between 10% and 30% (Golob, 1985; Muhihu and Kubata, 1985).

The common legumes cultivated in Africa include, common beans (*Phaseolus vulgaris*), pigeon pea (*Cajanus cajan*), cowpeas (*Vigna unguiculata*), groundnuts (*Arachis hypodaea*). Grain legumes form a very important part of the diet of people who live in tropical and subtropical countries, contributing to 33% of man's protein intake. Legume protein is of high quality and inexpensive (Kim *et al.*, 2003) and the grains can be consumed while green or dry and also serve as a source of oil (Minja, 1997). The legume residues also provide high quality livestock feed. Beans are the most important source of protein for the majority of vegetarians and people who cannot afford animal-based proteins.

Although a lot of effort and resources are invested in production of these grain legumes in the field, a lot of the grain is lost later in the granaries due to storage pests. The main storage pests being *Prostephanus truncates, Sitophilus zeamais, Sitotroga cerealella, Acanthoscelide obtectus* and *Callosobruchus maculates*. Among the bean weevil pest is the *A. obtectus*. The infestation of *A. obtectus* may start in the field and continues to feed and multiply in grain during storage. *Acanthoscelides* sp. are commonly found on cowpeas and common beans (Abate *et al*., 2000; Olubayo, 1993).

More than 200 species of insects are known to live and feed on legumes, in turn attacking foliage, flowers, pods and roots (Ranga Rao and Shanower, 1999). Insect pests are among the main biotic constraints to legume production because they feed on the plants from seedling stage to storage, although the most economic damage occurs during the reproductive phase to storage, starting from flower buds (Reed *et al*., 1989).

Synthetic insecticides have effectively been used against pests (Muhihu and Kibata, 1985; Giles, 1994), however negative human and environmental repercussions like human poisoning, insect resistance, and ecological imbalance associated with the use of these insecticides have been observed. Some chemicals like Pirimophos-Methyl (Actellic supper), which is used by most farmers in Kenya, may penetrate into the stored grain and may be toxic (Silim-Nahdy, 1995).

The need for safe and environmentally friendly insect control measures has become a key area in scientific research in which the use of botanicals or plant products in management of storage pest is widespread in Africa (Jillian *et al*., 1988). Biological control, development of pest resistant crops, non-chemical alternatives such as cultural changes in farming systems and development and discovery of new insecticides are some of the alternatives of pest control. One of the areas being explored is the use of biorationals or plant materials derived from the metabolic activity of plants (Delobel *et al*., 1987). This approach has advantages in pest control because natural products are renewable, biodegradable and may be transformed into products that are not harmful to the environment, are more selective and less resistant to biological degradation (Alkofahi *et al*., 1989).

The use of biorationals has been exploited by various communities worldwide for a long time. It has been shown that *Tephrosia vogelii* Hook f. (Fabaceae) affected adult survival of *Caryedon serratus* (ground nut seed beetle) through fumigation or direct contact against the adult insects in laboratory tests (Stoll, 1986). Extracts of fresh or dry plants are normally more effective than powders especially on adult beetles either as repellants or as toxicants (Boeke *et al*., 2001).

Plant extracts are organic in nature and are easily biodegradable or photo degraded and thus their field application greatly reduces the problem of environmental contamination (Agona *et al*., 2001). Some of the indigenous methods of controlling the bruchid beetle are use of; wood ash, plant materials like *T. vogelii, Tagetes minuta* L. (Asteraceae) *Nicotiana tabacum* L. (Magnolopsida) (Theodore *et al*., 2001).

This study was aimed at finding ways of reducing or eliminating post harvest losses from storage pest namely, the bruchid beetle (*Acanthoscelides obtectus*) in stored legume grains (*Phaseolus vulgaris*) by use of natural products present in some selected plants.

1.2 JUSTIFICATION

Demand for beans is increasing due to ever increasing population and due to the diverse use of the grain. Although a lot of resources are invested on the production of this food grain much is lost during storage due to pests. Good storage practices are important to retain the quality and quantity of grains from the fields. Improving grain storage would mean less hunger, improved nutrition for the individuals leading to higher standard of living and a sounder economy for the nation.

Prophylactic methods have not constrained the pests to acceptable levels. Synthetic pesticides may be in use but due to persistence, resistance, and high cost, availability of these convectional insecticide and potential health hazard both to consumers and environment have necessitated continued use of local plant products. In spite of the usage of these plants they remain uninvestigated for efficacy, safety and their active principles.

Kenya is endowed with rich diversity of plants with a variety of metabolites some of them being used traditionally as natural pesticides. These metabolites from plants are normally environmentally friendly. These compounds represent a large reservoir of biopesticides, which is largely untapped.

This study aims at validating the efficacy, safety, and active principles of a few selected botanicals used in pest control for potential application by farmers in Bungoma district of Kenya as a food security strategy.

1.3 Hypothesis

1) The stem and root bark of *Senna didymobotrya*, leaves of *Euclea divinorum* and the stem bark of *Ziziphus abyssinica* contain active components against stored pests *Acanthoscelides obtectus* and microbes that can be isolated.
2) The isolated compounds have insecticidal properties and are active against bacteria and fungi.

1.4 OBJECTIVE

1.4.1 General objective

The general objective of the study was to evaluate selected Kenyan plants used by small-scale farmers as a source of biopesticides for storage legume pest control in Bungoma District in Western Province, Kenya.

1.4.2 Specific objectives

(i) To carry out sequential extraction of air-dried leaves and stem barks of *Senna didymobotrya*, *Euclea divinorum* and *Ziziphus abyssinica*.

(ii) To carry out insecticidal activity bioassay of crude extracts against the bean weevil.

(iii) To carry out bioassay guided fractionation and isolation of active constituents from the crude extracts of the selected plants by chromatographic techniques.

(iv) To carry out bioassay test of the isolated compounds against bean weevil (*Acanthoscelide obtectus*) and toxicity test on the purified compound using the brine shrimp lethality test and antimicrobial test on selected microorganism.

(v) To characterize the structures of the isolated compounds using various spectroscopic techniques (IR, UV, MS and ^{1}H and ^{13}C-NMR and 2 D- NMR)

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Bean storage insect pests

The main pests of stored legumes are the bean weevils which include; *Acanthoscelides* sp, *Callosobruchus* sp and *Zabrotes* sp.). These insect pests cause the greatest loss to stored produce and this has been attributed to their high rate of multiplication and short development period. Bruchid beetle (*Acanthoscelides obtectus*) is the main legume pest found in Africa and is a major concern especially in Sub-Sahara Africa (Labeyrie, 1981) and for this reason it was the pest selected for biocontrol in this study.

2.1.1 Bean weevil – (*Acanthoscelides obtectus*)

Bruchid beetle *Acanthoscelides obtectus* belongs to the class Insecta and Order and Family Coleoptera and Bruchidae, respectively. In this family there are three genera *Acanthoscelides* sp, *Callosobruchus* sp and *Zabrotes* sp. Their common features are prolonged rostrum to form the snout, elbowed antennae, elytra fully cover the abdomen and approximately 3 to 4 mm long.

Plate 1. A photograph of *Acanthoscelides obtectus*

The distribution of *Acanthoscelides obtectus* ranges from tropical to temperate zones. It is the primary pest of stored grain through out the warmer parts of the world. *A. obtectus* main hosts are beans with alternative hosts such as sorghum and other cereals. Infestation by bean weevil starts in the field when the crop has attained physiological maturity and is drying (De Lima, 1974). The larvae bore thin tunnel from the surface towards the inside of the grain kernels (Hill and Walter, 1990). The damage caused by larvae and adult bruchids on beans may be extremely high, upto 18% weight loss has been observed when beans were kept at 70% relative humidity for a period of 37 days (Adams, 1976). Nutrition value is also affected through the organoleptic changes. The deterioration of the grains by contaminating them with their excretions, eggs, dead or broken parts of their bodies also occurs (Adams, 1976).

The only effective control measure for the bean weevil is a range of synthetic pesticides in the form of gaseous fumigants and residual insecticides. These synthetic pesticides pose a substantial risk to the environment and non-target organisms.

2.2 Pesticides

Pesticides, as defined by the Colorado Organic Certification Act, 2004 (COCA), are substances or organisms that prevent, destroy, repel or mitigate a pest. Pesticides are classified according to the type of pests they act upon and are divided into herbicides (weed killers), rodenticides, fungicides, insecticides, avicides (bird killers), molluscicides, acaricides (mite killers), nematicides and bactericides (Seena *et al.,* 2004; Kumar, 1984). Pests and diseases globally cause a 30 - 40% loss in available crop production (Oakland, 1981; Dennis, 1983). Development of modern pesticides took a quantum leap forwards after the First World War and the developments advanced even faster after the Second World War. In a rapidly changing World, people realize the importance of safer agricultural practices and in particular, the responsible use of pesticides. The question however is, how we achieve effective food production while minimizing the impacts of pesticide on the broader environment. In the olden days man used various botanicals and mechanical methods to combat insect pests to protect his food supply. It is reported that before man discovered and developed the modern synthetic insecticides, insects and other arthropods pests were generally treated with a variety of inorganic compounds (Bitran *et al*., 1983).

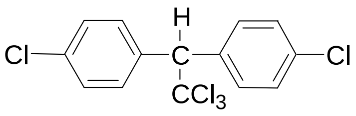
The first major commercial pesticides were arsenical compounds introduced in 1867 to control insects (Ware, 1983; Kumar, 1983) and copper-based preparation Bordeaux mixture such as $Cu_4(CH_3CO_2)_2(AsO_2)_2$, was used to control fungi attacking grapes (Ainsworth, 1981). Other pesticides introduced early were lime sulfur and burning sulphur in 1890, lead arsenate in 1892 and nicotine sulfate in 1904 (Ware, 1983). All the above inorganic compounds were non-ideal pesticides, as they were non-specific, had high mammalian toxicity, non-biodegradable and very persistent in soils (Matoksy *et al*., 1988).

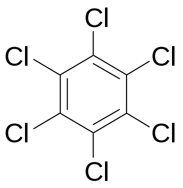
Later on, these inorganic pesticides were replaced by various organic pesticides, which include organic thiocynates that were marketed in the USA as livestock and household insecticides. However, in 1939, Paul Muller of Switzerland discovered the insecticidal properties of DDT (dichlorodiphenyltrichloroethane) (**1**) and the era of modern synthetic organic insecticide chemicals was introduced in full force (Whitehead and Bowers, 1983; Alloways and Ayres, 1994).

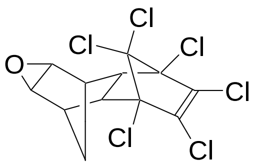
2.2.1 Organochlorines pesticides

Organochlorine compounds are broad-spectrum insecticides and are a very persistent group, which usually kill both by contact and as stomach poisons. They have been very effective in insect control, but due to their persistence in the environment and accumulation in the food chain, their use has been banned in most countries worldwide (Alloways and Ayres, 1994).

It has also been observed that the major pests have already become resistant to the organochloride compounds and so their utilization has diminished greatly. The most prominent organochlorine pesticides compounds include dichlorodiphenyl trichloromethane (DDT) (**1**), lindane (**2**), and dieldrin (**3**)



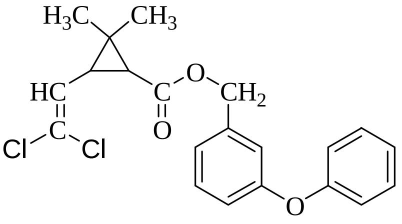




1 **2** **3**

2.2.2 Organophosphates

Organophosphates replaced the organochlorine insecticides when it became apparent that the latter had detrimental health and environmental effects. Currently, many organophosphates insecticides used in agriculture and urban pest management systems are being reviewed under the Environmental Protection Agency (EPA) interpretation of the 1996 on Food Quality Protection Act (FQPA) (FAO, 1996). The one commonly used organophosphate insecticide to arrest the post harvest losses of bean grain caused by *Acanthoscelides obtectus* is Actelic Super (0.3 permethrin (**4**) and 1.6 pirimiphos-methyl (**5**) (Arthur, 2002). It has very low mammalian toxicity and acts very quickly both as a fumigant and by contact action (Alloways and Ayres, 1994).



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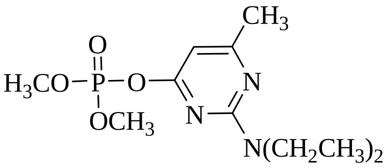

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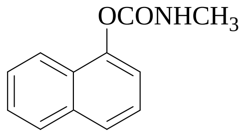
2.2.3 Carbamates

Carbamates are a class of pesticides containing the esters of N-methyl or occasionally N, N dimethyl carbamic acid. The relative toxicity of a particular carbamate varies widely depending on its chemical structure. The carbamate work as acetylcholinerase inhibitors for insect control, however, enzyme inhibition is more easily reversed and insect can recover if too low dose is given. They have a broad spectrum of activity and usually act by topical contact or stomach action (Luke, 1981). This class of insecticide includes Carbaryl (**6**) and aldicarb (**7**).



6

$CH_3SC(CH_3)_2CHNO_2CONHCH_3$

7

Several pressures have accelerated the search for more environmentally and toxicologically safe, more selective and efficacious pesticides. The increasing incidence of resistance is also fuelling the need for new pesticides. Furthermore, most synthetic chemicals pose substantial societal and environmental risk thus natural compounds have increasingly become the focus of those interested in discovery of pesticides.

2.2.4 Biopesticides from plants

There has been a worldwide effort aimed at screening plant species for bioactivity against pest species. Plants have been screened for repellency and protectants against the bean weevil (Balandrin and Lee, 1985).

Throughout history, plants have been successfully exploited as insecticides, insect repellents and insect antifeedants (Nakanishi, 1977). Protection of stored products generally involves mixing grain with protectants made up of plant materials with or without minerals (Agona *et al.*, 2001). The precise strategy used by different communities varies from place to place and appears to depend partly on the type and efficiency of suitable flora available in different locations. The organic chemistry extraction and isolation techniques have significantly improved in the recent past. As a result it has made it possible to isolate and identify the main biologically active chemicals from plants (Huang, 1992).

Natural products from plants, animals and those from microbial organisms are a vast source of bioactive substances, which have been exploited only to a limited extent as models in the synthesis of practical insecticides. The advent of powerful analytical tools such as NMR, MS, HPLC and related techniques has had a great impact in the research work. It has facilitated identification of important bioactive principles isolated from natural product extracts at an increasing pace. The use of bio pesticides needs to be encouraged since they are readily available. A major drawback in the use of biopesticides is the lack of standardization of formulation, dosage determination and shelf–life data. The knowledge of adverse effects of synthetic pesticides is rapidly shifting the worldwide attention to non – synthetic safer pesticides of plant origin.

According to an updated technical market research report on biopesticides market from Business Communications Company, (www.bccresearch.com), the total global market for synthetic pesticides was valued at $26.7 billion in 2005, and is expected to decline at an average annual growth rate (AAGR) of 1.1%, to $25.3 billion in 2010 (www.bccresearch.com). This decline is attributed to the detrimental effect the synthetic pesticide have on the environment (Table 1).

As the overall market for pesticides is showing a decline, the biopesticides market is growing rapidly, increasing from $672 million in 2005 and is projected to be over $1 billion in 2010, at an AAGR of 9.9%. Biopesticides currently have 2.5% of the overall pesticides market, but its share of the market will increase to over 4.2% by 2010.

Table1. Global biopesticides and synthetic pesticides market, 2003 through 2005, and 2010 ($ millions (www.bccresearch.com).

Type	2003	2004	2005	2010	AAGR%(2005-2010)
Biopesticide	468	562	672	1075	9.9
Synthetic pesticides	27,144	26,600	26,076	24,205	-1.5
Total	27,612	27,162	26,748	25,280	-1.1

Key**: AAGR**-Average Annual Growth Rate.

Biopesticides are biodegradable, environmentally friendly and readily available. Kenya has wide and diverse ecological zones, which are well endowed with viable botanicals.

For a developing country like Kenya, the importation of chemical pesticides depletes the foreign exchange available. Since the country produces large volumes of pyrethrum flowers (http://www.new-agri.co.uk) and has the capacity to produce *Azadirachta indica A.* Juss and other potential plants, there is need to use them in pest management. Other examples of such plants includes *Mimosa tenniflora* and *Mexican marigold* (Akeng'a *et al.,* 2004).

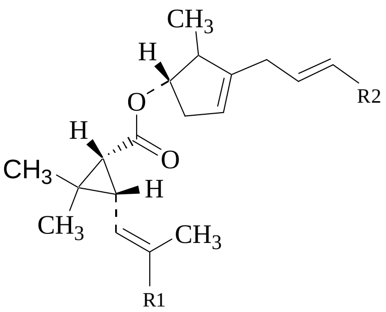
2.2.5 Plants derived pesticides

Several useful pesticides have been isolated and characterized. Probably the most successful use of plant product, as an insecticide is that of pyrethrins, obtained from pyrethrum plant (*Chrysanthenum cinerariaefolium*).

Plate 2. A photograph of *Chrysanthenum cinerariaefolium* flowers

The chemical structures of the six-terpernoid esters (pyrethrins) responsible for the insecticidal activity have been elucidated**.** The pyrethrins have the advantage of low mammalian toxicity and a low environmental hazard.

The major active ingredients have been identified as pyrethrin **I** and **II** (**8, 9**) and cinerins **I** and **II** (**10, 11)** and jambolin **I** and **II** (**12, 13)** pyrethrin **I** **(8)** is the most insecticidal component, with pyrethin **II (9)** providing much of the knock-down (paralyzing) effect (Crombie, 1980**).**



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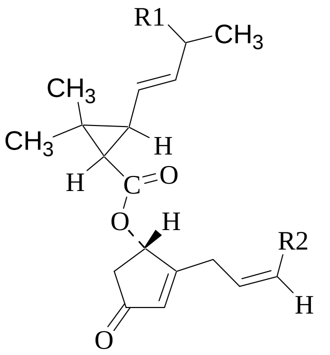
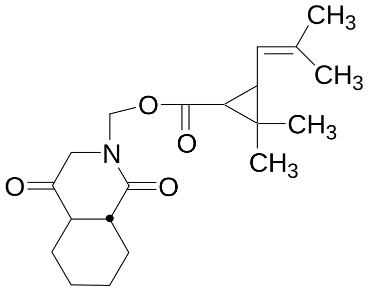


II

8:	R1: CH_3	R2: $CH=CH_2$	**9**:	R1: $COOCH_3$	R2: $CH_2=CH$
10:	R1: CH_3	R2: CH_3	**11**:	R1: $COOCH_3$	R2: CH_3
12:	R1: CH_3	R2: CH_3	**13**:	R1: $COOCH_3$	R2: CH_3

Synthetic pyrethroids have been made and have better photo stability and are generally more active than their natural counterparts, they include; tetramethrin (**14**), cypemethrin (**15**) and tefluthrin (**16**) (Eliot and Janes, 1978).



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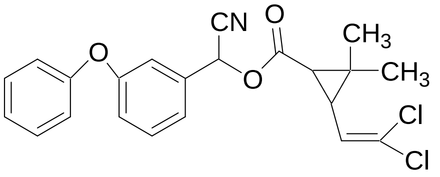

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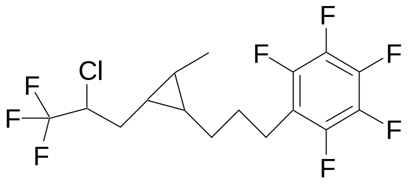

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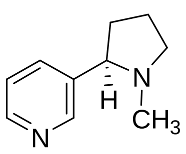
Plant terpenoids have also been shown to have insecticidal or other insect-inhibiting activities; for instance, azadirachtin and other terpenoids of the limonoid group from the families Meliaceae and Rutaceae are potent growth inhibitors of several insect species (Duke, 1985).

Nicotine (**17**) and nornicotine (**18)** components of several members of the genus *Nicotiana* (tobacco) have been used commercially as insecticides. *Nicotiana rustica* is the chief commercial source. Other natural analogues of nicotine have been shown to have significant insecticidal properties such as neonicotine (**19**) produced from the shrub *Anabasis aphyllain*. Due to high mammalian toxicity and environmental liability, its development was dropped with the advent of the newer synthetic insecticides (Whitehead and Bowers, 1983).



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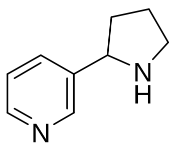

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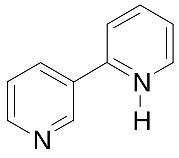

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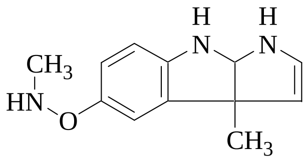
Physostimine (**20**), an alkaloid from *Physostigma venenosum* was the compound upon which carbamate insecticides were designed (Matoksy *et al.*, 1988). Furoquinoline and beta-carboline alkaloids such as dictamine (**21**) and harmaline (**22**), respectively, are potent photosensitizing compounds that are highly toxic to insect larvae in sunlight.



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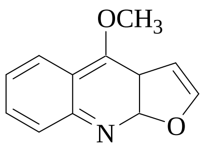

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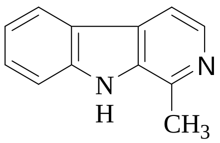

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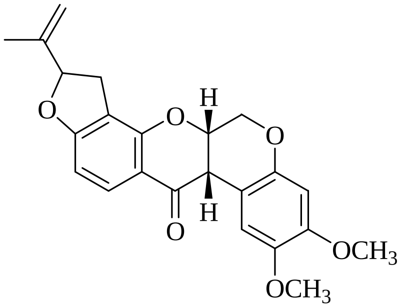
Rotenone (**23**) is among the oldest natural insecticides to be extracted from the roots of *Derris elliptica* Wallich. Benth (Leguminosae), *Lonchocarpus nicoa* Aublet DC (Rosidae) and *Tephrosia vogelii*. Rotenone is a flavonoid derivative that strongly inhibits mitochondrial respiration (Duke, 1990).



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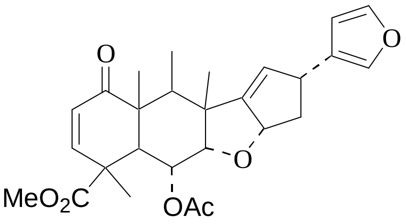
Insects (weevils) develop resistance to insecticides such as Actellic super hence need arose from continuous search for other potent compounds to this end (Barnes *et al*., 1996).

Researchers are screening for bioactive compounds against destructive pests. Several plants have already been worked on, this include the neem tree *(Azadiracta indica*) which seems to hold some potential as lead source for new insecticides (Mayunga, 2002).

About 24 biologically active compounds have been extracted from the neem tree, however, only four of the compounds have shown to be highly effective in their activity as pesticides, they include; nimbin **(24),** azandirachtin **(25),** salannin (**26**) and 6-desacetylnimbin (**27**) ((Jacobson, 1990).



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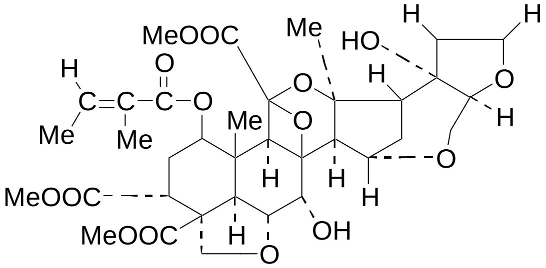

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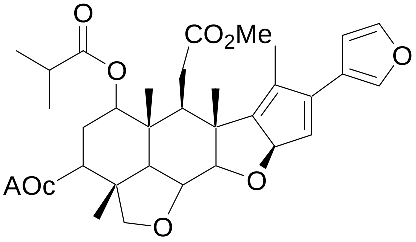

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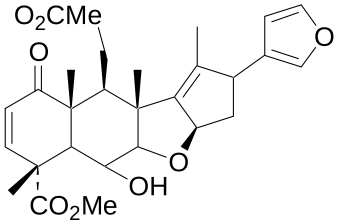

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According to research carried out, a mixture of different types of flavonoids or flavonoids combined with terpenoids can be advantageous because their combined effects may be more stable and more effective against insects than single compounds (Jerry *et al*., 1998**).**

Until recently most pesticides including botanicals pesticides have had to have knockdown activity in order to attract attention of users. This is now slowly changing with realization of the importance of Intergrated Pest Management (IPM). Botanical pesticides are now being seen not as a total replacement of any particular pesticide but rather as an important input of the IPM package to reduce dependence on synthetic chemical (Van Wyke *et al*., 2002).

2.2.6 Biological Control

Apart from higher plants, ferns, fungi and bacteria may have insecticidal components. An example is the case of the *Bacillus thuringiensis* bacterium commonly known as BT, affects stored product moth larvae and is available commercially for use on grains. Various predators, parasitols and microbial pathogens of stored pest have considerable potential for providing needed pest management alternatives for post harvest practices. However, bean weevil was not susceptible to the mentioned alternatives, but several isolates show promise for control of other beetles (www.entomology wisc.edu).

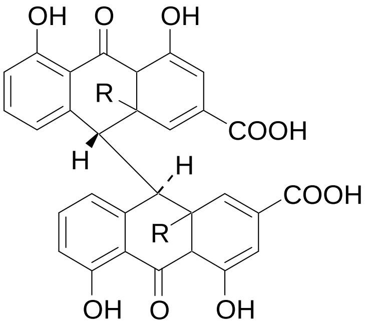
2.2.7 Bioactivity

Brine shrimp test is a method for testing bioactive compounds. It is a simple, cheap, rapid and convenient as an in-house general bioassay tool. It is an indication of cytotoxicity, pesticidal effect and various pharmacological actions. Bioactive compounds are almost toxic at high doses, and could be considered pharmacologically active at low dose. This method gives the concentration at which 50% of the napulii die (LD_{50}) of the tested sample. It is used to monitor fractionation of cytotoxic and active extracts in bioactive-guided isolations (Meyer *et al*., 1982).

2.2.8 Family Caesalpiniaceae

The family Caesalpiniaceae comprises about 150 genera and 2200 species, which are mostly tropical and subtropical trees and shrubs. The leaves are stipulate, alternate and mostly pinnately compound but may be bipinnate. The family is important for both medicinal and pesticides use. There are about 23 genera of this family in Kenya, the most common genera include *Cassia, Senna* and *Caesalpinia.* (Beentje, 1994).

In the Casalpiniaceae family, a variety of organic compounds have been isolated. The active constituents in both senna leaf and fruit are dianthrone glycosides, principally sennosides A **(28)** and B **(29)**. Minor constituents include sennosides C (**30**) and D (**31**). (Seigler, 1985).



R-Glc = Sennoside A

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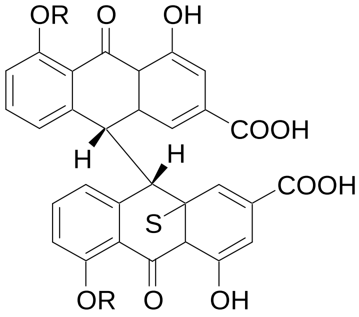

R-Glc= Sennoside B

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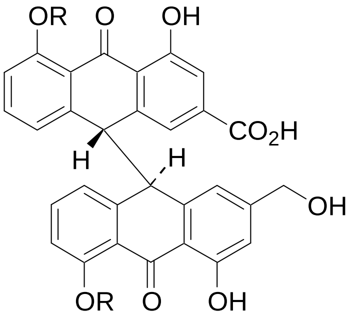

R= Glc, Sennoside C

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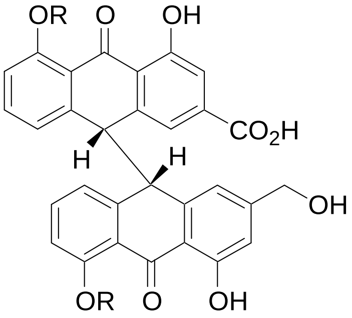

R= Glc, Sennoside D

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2.2.8.1 The genus *Senna*

Senna belongs to the family Caesalpiniaceae and comprises of 10 species that grow in most parts of Kenya. Some species are cultivated as ornamental, but some are used as herbal remedies. The Kenyan species are some of the commonest trees of the dry bush land found around Coast, Central and Western provinces. Among the common members of this genus include *S. obtusifolia* (L.) Irwin & Barneby, *S. singuena* (Del) Lock, *S. petersiana* (Bolle) Lock, *S. baccarini* (Chiov.) Lock (Beentje, 1994).

The dried leaves of *S. anguistifolia* Vahl are used as a purgative, anti-malaria medicine, and is commonly used as a stupefacient for fishing and also used as a purgative. The bark is boiled in water to make a decoction used to treat malaria, mumps, pneumonia and other diseases. *In vitro* cultures of *S. didymobotrya* has compounds that can be converted into low-energy sweeteners and insecticides.

The root of *S. singueana* is used for stomach ache medicine. Many anthraquinones and flavonoids have been isolated from the pods, the anthraquinones were found to be active against *Escherichia coli* and *Staphylococcus aureus* (Gizachew, 2004).

2.2.8.2 The species *Senna didymobotrya*

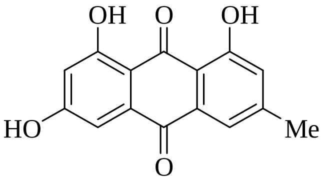
Senna didymobotyra is a shrub or tree of between 1-7.5 m tall and has leaves with 8-18 pairs of leaflets, leaflets elliptic apex rounded or obtuse, with a mucro 1-3 mm long, 2-6.5 by 2.5 mm more or less pubescent. Flowers are yellow and petals are between 1.8-2.7 by 1-1.6 cm. The fruit is flattened oblong 8-12 by 1.5-2.5 cm.

In Kenya, the plant is found in forests in the Kakamega district (Western province). They grow along the rivers, by the lakeside and at forest edges (in damp sites) (Beentje, 1994).

Plate 3. A photograph of *Senna didymobotrya*.

Senna didymobotrya bark contains tannins. The leaf infusion is used as emetic against malaria by the Kipsigis. Stems are used against ringworm by the Luyha (Beentje, 1994). The plant is also used as a biopesticide by the Luyha community and they use it in form of ash where they mix with the grain in the store.

Phytochemical studies carried out have shown that *S. didymobotrya* contain the anthraquinones, among them are emodin (**32**) (Ojewole, 2000).



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Larvicidal assays of aqueous extracts of the leaves stem and root barks of *S. didymobotrya* against malaria vector showed that larval stages are prone to the lethal effects of the plant extracts and the root bark extract possessed the strongest larvicidal activity (Ojewole, 2000). The survey carried out in Bungoma district indicates that local farmers have been using the plant in the control of bean weevil. Going by the findings stated above, further chemical and biological investigation on this plant would be of great importance.

2.2.9 Family Ebenaceae

The family Ebenaceae has 14 genera and 530 species, which are characterized by alternate leaves without stipules. Inflorescence usually cymose, flowers unisexual, 3-8 merous, corolla with a tube, it has three stamens but at a times they can be many. The fruit is usually a berry. In this family, there are two main genera that grow in Kenya, they include; *Diospyros* and *Euclea*.

Plants of Ebenaceae family are widely distributed in Central and Western Kenya. The Luo uses a decoction of the roots of *Euclea divinorum* as purgative, the Kipsigis use the bark infusion of *E. divinorum* as an appetizer. The bark infusion of *Diopyros scabra* is employed against womens' stomach troubles by the Samburu, and the Pokot use it as a ceremonial tree (Beentje, 1994).

2.2.9.1 The genus *Euclea*

There are about 25 species of evergreen shrubs or small trees up to about 6 m tall, often branching from the base or sometimes with a single stem. It is a species common in bush, dry forest margins, thorn shrub, and open woodland. There are around three species of this genus that grow in Kenya; they include *E. natalensis* ADC. F.White, *E. divinorum* Hiern and *E. racemosa* Murr (Beentje, 1994).

The fruits of *E. divinorum* are edible in East Africa. Several species of the genus *Euclea* is rich in naphthoquinone and triterpenoid (Nkunya, 1992). Phytochemical studies on

Euclea sp. have resulted in the isolation of several naphthoquinones, which have a wide range of activities, including antiplasmodic and antibacterial effects (Karlina *et al*., 2006).

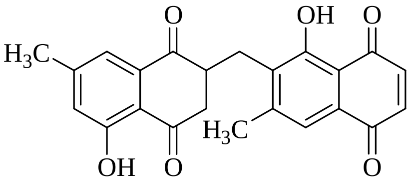
2.2.9.2 The species *Euclea divinorum*

Euclea divinorum is a shrub or tree of between 1.8 -10 m tall and bark is smooth, green, leaves alternate, without stipules. Inflorescence usually cymose, flowers unisexual, 3-8 merous, corolla with a tube, stamens are three or more. It grows well in dry forest or bushland, often a weed of pastures, due to its phenomenal power of coppicing and root suckering.

Plate 4. A photograph of *Euclea divinorum*

The plant is known by different names among the locals; the Tugen call it Uswet, the Luhya- Kumuchanjasi and the Marakwet-Jeptuiya. A decoction of the roots of *Euclea divinorum* is used as purgative by the Luos and the Kipsigis use a bark infusion as an appetizer. It is widely distributed in the Western part of Kenya and Rift Valley (Beentje, 1994).The Luyha community employ this plant as a source of grain protectant and the farmers normally mix it with other plants and this shows that when blending of the plant material is carried out the activity of the plant material is improved.

Euclea unguilata roots contain two naphthoquinones, diospyrin **(33**), and 7-methyljuglone **(34)** as major constituents (Costa *et al.,* 2001).



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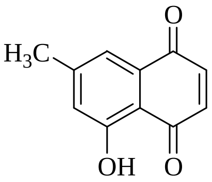

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Aqueous extract from the plant *Euclea divinorum* inhibited proteolytic activities of *Bacteroides* sp. by 50% at concentrations of 200 µg/ml. The extracts from *Euclea* also possess inhibitory components that interfere with the virulence and growth of periodontopathic bacteria *in vivo* (Costa *et al*., 2001).

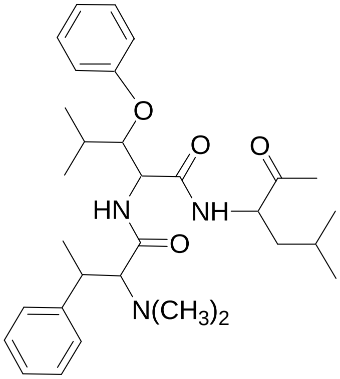
2.2.10 The Family Rhamnaceae

The family Rhamnaceae comprises of about 20 genera and 100 species of deciduous or evergreen trees and shrubs distributed in the tropical and subtropical regions of the world (Johnson, 1963). The family is as important as medicinal plants. In this family, there are 10 genera which occur on nearly every continent. Whereas other species like *Ziziphus mauritania* Lam, *Z. mucronata* Willd, *Z. Spina-christi* (L.) Desf. var. *microphylla* are restricted in their distribution to distinct areas notably Coastal regions, Western province and Lake Turkana in Kenya, respectively. They are also found in Ethiopia and South Africa (Van Wyke *et al*., 2002).

2.2.10.1 The genus *Ziziphus*

The genus *Ziziphus* has been known to contain some chemical compounds that possess pharmacological activity. The bark infusions are used as emetics in cough and chest problems, hot infusions are popular as a remedy for dysentery. Decoctions of roots are applied externally to boils, sores and glandular swellings, not only to promote healing but also for pain relief (Beentje, 1994).

Several species of the genus *Ziziphus* contain alkaloids, commonly referred to as peptide alkaloids. Structurally related alkaloids from this genus have been isolated. The strong sedative effects of *Z. vulgaris* and *Z. jujuba* are due to the presence of frangufoline **(35).** From the roots, stem bark and leaves of *Z. mucronata* the compound mucronine D **(36)** has been isolated (Van Wyke *et al*., 2002).



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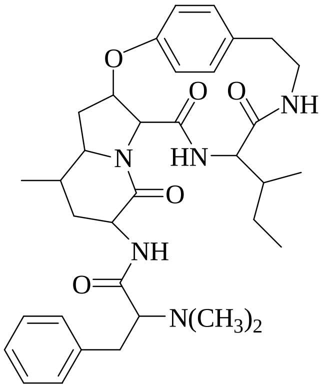

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2.2.10.2 The species *Ziziphus abyssinica* A. Rich.

Ziziphus abyssinica is a shrub or trees, often with spiny stipules, the branches often zigzag. The leaves are alternate or with three opposite veins from the base with serrulate margins. The flowers are merous; usually axillary in cymes or thyrses, the fruit is one seeded drupe. The plant is known by different names by the locals, the Borana call it:-

K'urkuna, the Kamba call it Muae, the Meru, Thilarii and the Luyha call it Kumkomboti. The fruits are edible and the wood is hard and durable (Beentje, 1994).

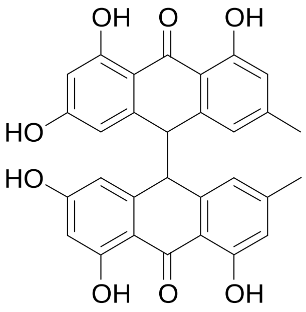
Plate 5. A photograph of *Ziziphus abyssinica*

2.3 Biosynthesis of secondary metabolites.

2.3.1 Anthraquinones

Anthraquinone are organic bases found mainly in plants, over 200 of these compounds are known from flowering plants, and many others are produced by lichens and fungi. Many anthraquinones occur as glycosides within the plant, but are converted into the agylcones by β-glycosidase or oxidative processes. These compounds are partly responsible for the colors of several tropical woods. In basic solution, most anthraquinones produce a deep red or blue color (Inouye and Leister, 1988).

As is true for the naphathoquinones, anthraquinones are synthesized by a variety of routes in plants and fungi (Packteer, 1980). Most are derived from either acetate-malonate pathways. Emodin (**32**) and hypericin (**37)** are derived from acetate-malonate precursors, which on the other hand appears to result from folding of the polyketide chain in one arrangement (Ellis, 1988).



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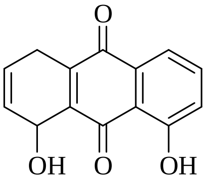
Anthraquinone derived from the acetate-malonate pathways are particularly common in fungi and lichens, but are often in higher plants as well. Acetate–malonate derived anthraquinones usually can be distinguished by their structures because they possess substituents in both benzenoid rings of the anthraquinone nucleus (Inouye *et al*., 1988).

Anthraquinones are involved frequently in plant-insect interactions. For example, one anthraquinone; dianthrol (**38**) and the related anthrones have been found in the larvae of the elm leaf chrysomelid. Two anthraquinones rhein **(39)** and chrysazin **(40)** are both potent microbial growth inhibitors (Cudlin *et al*., 1976).



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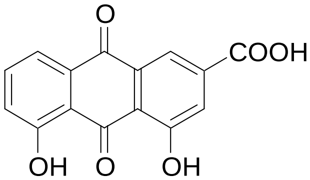

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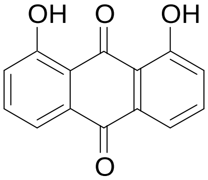

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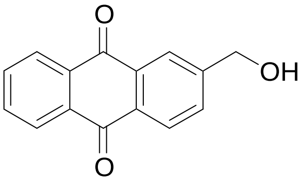
Several types of woods that contain anthraquinones and related compounds are highly resistant to attack by marine borers (Southwell and Bultman, 1971). On the other hand, 2-hydroxymethylanthraquinone **(41)** and 2, 3- dihydroxymethyl- 9, 10-anthraquinone (**42**) in the heartwood of teak (*Tectona grandis*, Verbenaceae) is effective in inhibiting termite activity (Rudman and Gay, 1961; Seigler, 1983).



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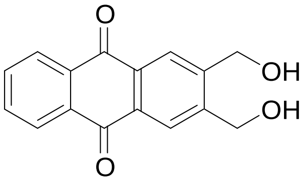

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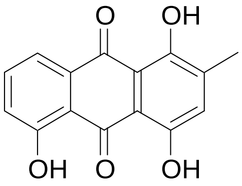
A number of natural anthraquinone derivatives are also excellent examples of acetate-derived structures**.** Endocrocin (Fig 1) found in species of *Penicillium* and *Aspergillus* fungi is formed by folding a polyketide containing eight C_2 units to form the periphery of the carbon skeleton. Three aldol-type condensations would give a hypothetical intermediate 1, except for a crucial carbonyl oxygen in the centre ring, endocrocin results by enolization reactions, one of which involves the vinylogous enolization -CH_2-CH=CH-CO- → -CH=CH-CH=C(OH)-.

The additional carbonyl oxygen must be introduced at some stage during the biosynthesis by oxidative process, for which we have little information. Emodin a metabolite of some *Penicillium* species, but also found in higher plants, for example *Rhamnus* and *Rumex* species, would appear to be formed from endocrocin by a simple decarboxylation reaction. This is facilitated by the adjacent phenol function. *O*-methylation of emodin [**32**] would then lead to physcion [**55**]. Islandicin [**43**] is another anthraquinone pigment produced by *Penicillium islandicum,* and differs from emodin [**32**] in two ways, one hydroxyl is missing, and a new hydroxyl has been incorporated adjacent to the methyl. Without any evidence for the sequence of such reactions, the structure of intermediate 2 shows the result of three aldol condensations and reduction of a carbonyl. A dehydration reaction, two oxidations, and a decarboxylation are necessary to attain the islandicin [**43**] structure



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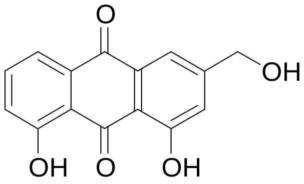
In aloe-emodin (**44**) and rhein [**39**], the same oxygen function is lost by reduction as in islandicin [**43**], and decarboxylation occurs. The three compounds are interrelated by sequential oxidation of the methyl in chrysophanol to a hydroxymethyl in aloe-emodin, and a carboxyl in rhein [**39**].



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The structural modifications undergone by the basic polyketide are according to the timing of the steps in the synthetic sequence. Thus 'missing' oxygen functions appear to be reduced out well before the folded and cyclized polyketide is detached from the enzyme and are mediated by a reductase component of the enzyme complex during chain elongation before the cyclization reaction (Leistner, 1961).

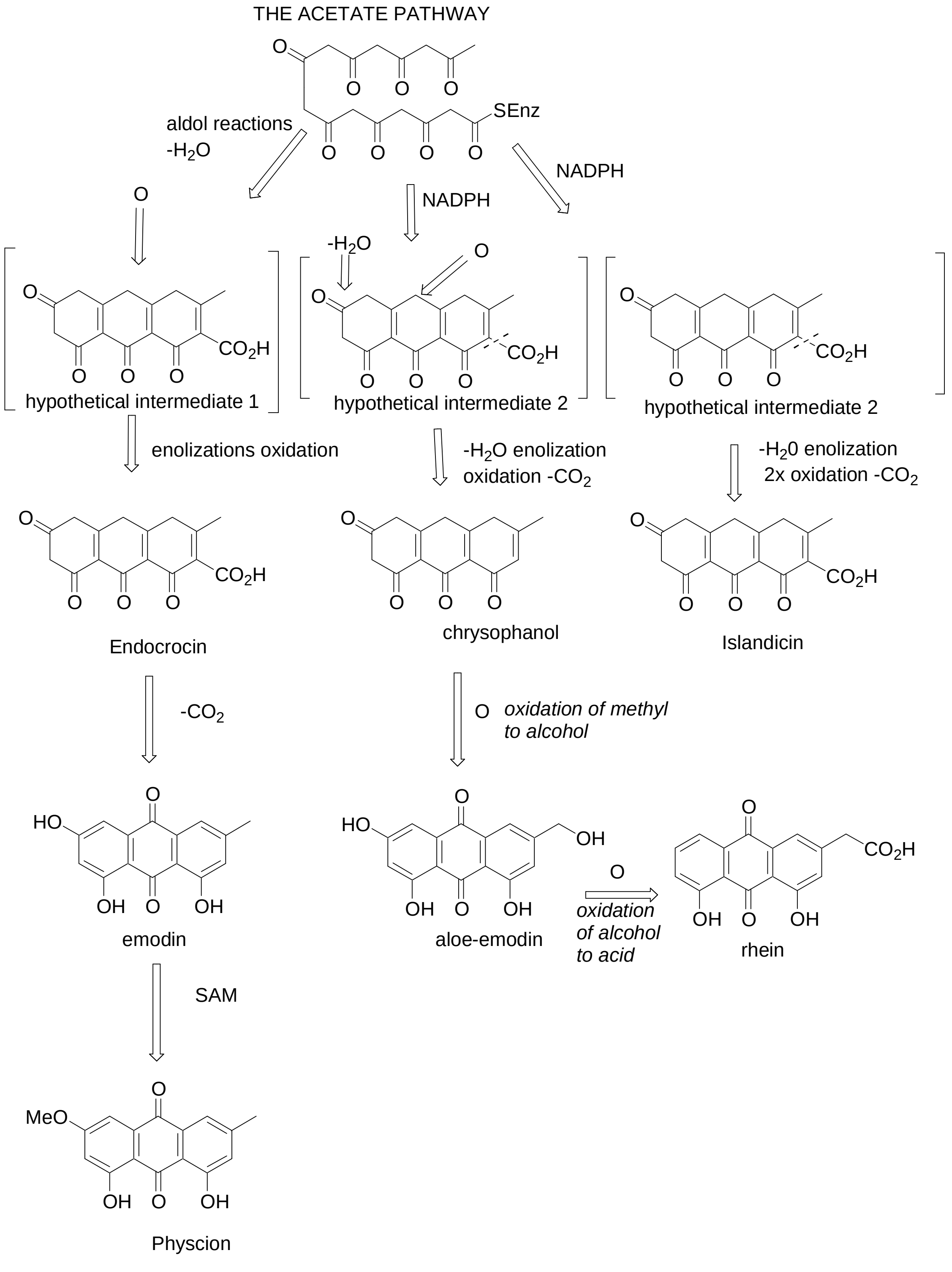
On the other hand, reactions like the decarboxylation, O-methylation, and sequential oxidation of a methyl to a carboxyl are representative of transformations occurring after the cyclization reaction.

The quinine system in anthraquinone is built up by an oxidation of the central cyclohexadienone ring, again at a nucleophilic centre activated by the enone system. Methyls on an aromatic ring are also activated towards oxidation, facilitating the chrysophanol → aloe-emodin oxidation, for example decarboxylation. Endocrocin conversion to emodin is readily achieved in the presence of an *ortho* phenol function, though a *para* phenol can also facilitate this (Leister, 1985).

Note that many other natural anthraquinone structures are not formed via acetate pathway, but by a more elaborate sequence involving shikimate and an isoprene unit. Such structures do not contain the characteristic *meta* oxygenation pattern, and often have oxygenation in only one aromatic ring (Leister, 1985).

Emodin [**32**], aloe-emodin [**44**], and rhein [**39**] form the basis of a range of purgative anthraquinone derivatives found in long established laxatives such as *Senna* sp, Cascara, Frangula, *Rhuem acuminatum* and *aloe arborescens*. The free anthraquinones themselves have little therapeutic activity and need to be in the form of water-soluble glycosides to exert their action (Leister, 1985).

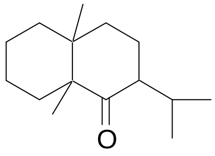
Figure 1 Biosynthesis of Anthraquinones (Leister, 1985)



2.3.2 Terpenoids

Terpenoids are ubiquitous secondary metabolites of terrestrial and marine flora and fauna, occurring in the free form as well as in the forms of ether, esters and glycoside. Terpenoids may be classified according to the number of isoprenoid units incorporated, hemiterpenes, monoterpenes and the variants irregular monoterpenes and iridoids, sesterpenes, triterpenes, tetraterpenes, and higher terpenoids representing groups with increasing numbers of isoprene units. Chemically, terpenoids are generally lipid-soluble and are located in the cytoplasm of the plant cell (Seigler, 1985).

Although medicinal uses of this class of compounds have been limited, considerable recent work strongly indicates their great potential. Medicinal agents include volatile oils, pyrethrins (**8**, **9**, **10**, **11**, **12** and **13**) valerian (**45**), artemisinin (**46**), vitamin A (**47**) and D (**48**), progesterone (**49**), cholesterol (**51**), progesterone drugs, estrogen drugs and androgen drugs (Mahato and Kundu, 1994).



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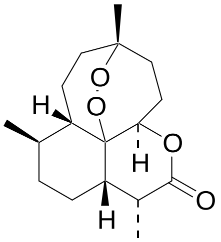

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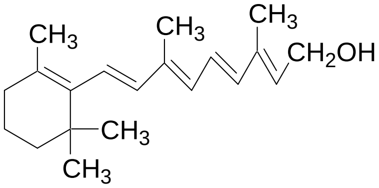

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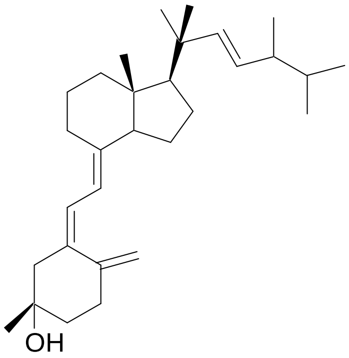

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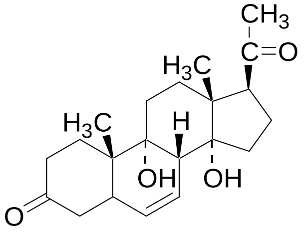

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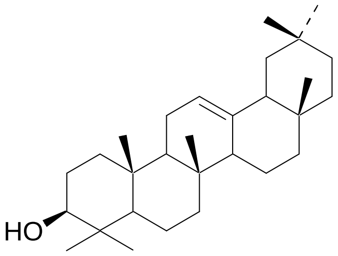
There are two pathways leading to terpenoids, the mevalonate pathway and mevalonate-independent pathway via deoxyxylulose phosphate. Animals appear to lack the deoxyxylulose phosphate pathway, and thus utilizes mevalonate pathway exclusively. Plants are equipped to employ both pathways often concurrently. Mevanolate pathway enzymes are localized in the cystosol, whereas deoxyxylulose phosphate pathway enzymes are found in the chloroplasts. The mevalonate pathway forms triterpenoids and steroids, whilst most other terpenoids are formed in the chloroplasts and are deoxyxylulose phosphate derived (Poulter, 1990).

2.3.1 Biosynthesis of triterpenoids

Triterpenes are formed when two molecules of farnesyl diphosphate (FPP) and joined tail to tail to yield the hydrocarbon squalene (Fig 2). The FPP forms allylic cation in presence of squalene snythase. This allylic cation joins another FPP molecule leading to the formation of presqualene PP as attack of the 2,3-double bond of FPP on the farnesyl cation, analogous to the chain extension using isopentyl diphosphate (IPP). The resultant tertiary cation is discharged by loss of proton and formation of the cyclopropane ring, giving presqualene PP. Loss of diphosphate from presqualene PP would give the unfavorable primary cation, which via Wagner-Meerwein rearrangement can generate a tertiary carbon and achieve the required C-1 – C-1′ bond. Breaking the original but redundant C-1 – C-2′ bond can give an allylic cation, and the generation of squalene is completed by supply of hydride from NADPH (Poulter, 1990).

Cyclization of squalene via the intermediate squalene-2,3-oxide, produced in a reaction catalysed by a flavoprotein requiring oxygen and NADPH co-factor. If squalene is suitably positioned and folded on the enzyme surface (cyclase enzyme) chair-chair boat conformation then the transient dammarenyl cation is formed. The dammarenyl cation undergoes further carbocation promoted cyclization. Wagner-Meerwein 1,2-alkyl shifts occurs giving baccharenyl cation, which relieves some ring strain by creating a 6-membered ring despite sacrificing a tertiary carboncation for secondary one (Popjak and Agnew, 1975).

A pentacyclic ring system can then form by cyclization on the double bond giving a new 5-membered ring and tertiary lupenyl cation. Loss of a proton from the lupenyl cation. gives the lupeol. Ring expansion on the lupenyl cation by bond migration gives the oleanyl system (Fig 2), this ion is discharged by hydride migration and loss of a proton giving β-amyrin (**50**). Formation of the isomeric α-amyrin involves first the migration of a methyl in the oleanyl cation, then discharge of the new taraxasteryl cation by 3 hydride migration and loss of a proton (Banthorpe and Charlwood, 1980).



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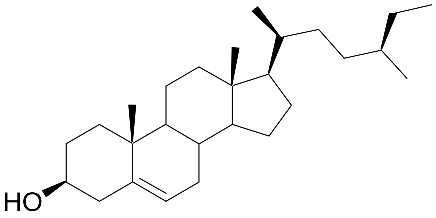
Many triterpenes have antiherbivore activity, although phytosterols are required for insect and fungal growth, tritepenoid compounds may interfere with this process and exhibit anti-herbivore or anti-fungal effects. In general, those that are highly oxygenated seem to be more active against herbivores and fungus (Croteau and Johnson, 1985).

2.4 Phytosterols

Sterols play a major role in membrane of plant cells. The main sterol in plants, fungi and algae are characterized by one-carbon or two-carbon substituent on the side chain, attached at C-24 in the cholesterol **(51).** In plants, sterols arise through the intermediacy of cycloartenol and squalene 2, 3-oxide (Figure 2). In animals sterols arise from lanosterol and squalene 2,3-oxide utilizing lanosterol cyclase. In most plants, campesterol and stigmasterol are the most abundant phytosterols.



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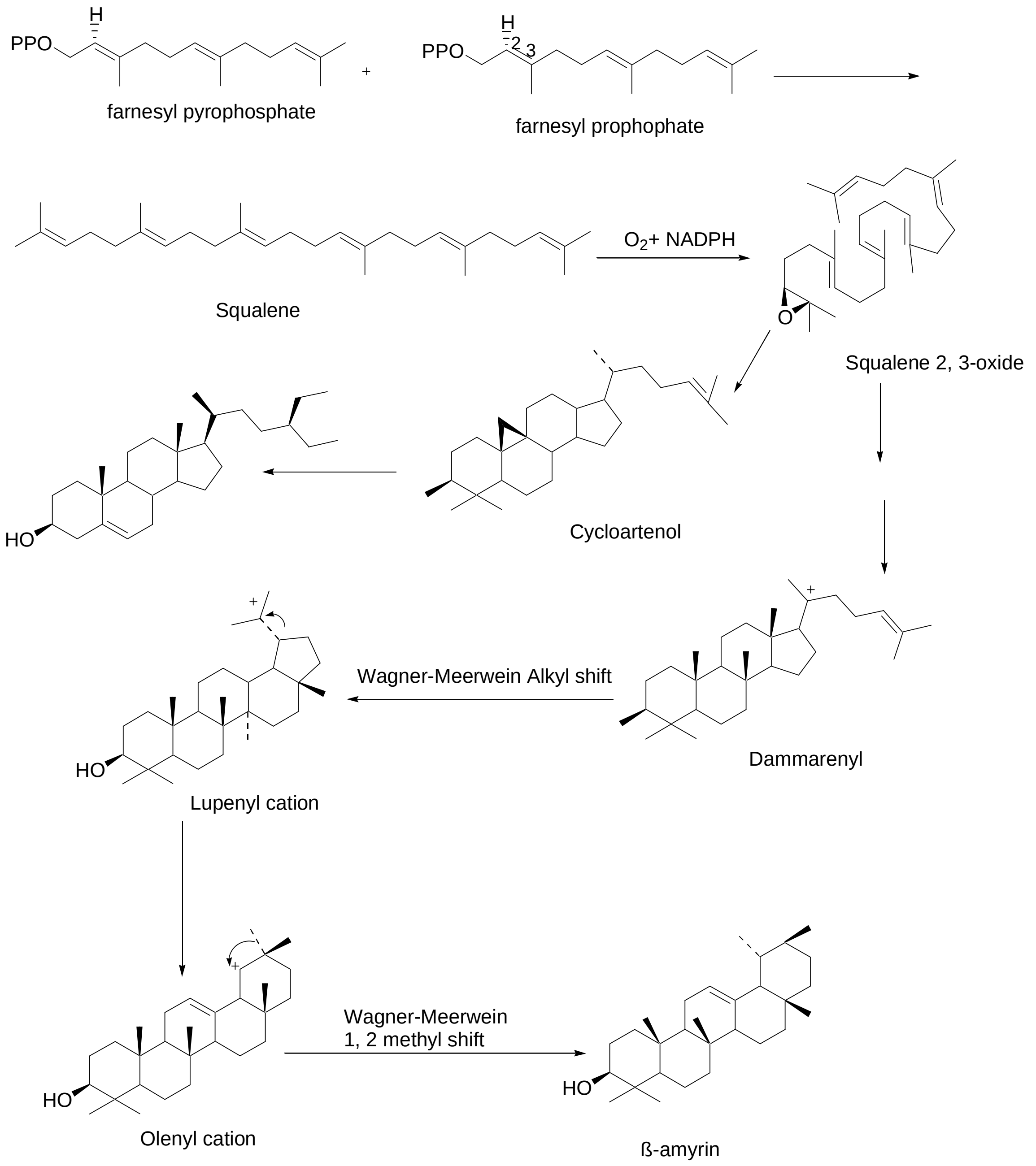
These compounds arise from cycloartenal by a series of reactions including modification of the side chain. Alkylation of the side chain may occur at C-24 with successive methyl groups derived from S-adenosyl methionine (SAM). The first addition is catalyzed by sterol-S-adenosylmethionine methyl transferase (Goodwin, 1980).

A number of plant sterols have been reported to possess growth-regulating activity and developmental modification properties in plants. For example, β-sitosterol initiates flower buds in *Chrysanthemum* species. Phytosterols are of particular importance to insects, nematodes and certain crustaceans, because they cannot synthesis cholesterol *de novo*. These organisms degrade dietary phytosterol C-29 and C-28 to C-27 sterols (Mandava, 1979).

Fig 2: Biosynthesis of triterpenes (**Leistner, 1985)**



CHAPTER THREE

3.0 EXPERIMENTAL

3.1 Plant material

The root bark of *Senna didymobotrya*, leaves of *Euclea divinorum* and stem bark of *Ziziphus abyssinica* were collected from Bokoli area, Bungoma district in October, 2006. After identification by a plant taxonomist, reference specimens were deposited at Jomo Kenyatta University Herbarium at the Department of Botany. The voucher specimens of *Senna didymobotrya, Euclea divinorum and Ziziphus abyssininca* were recorded as JMB/4/06/01, JMB4/06/03 and JMB06/03, respectively.

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°C. The solvents (methanol, ethyl acetate, dichloromethane and n-hexane) used in present study were general purpose reagents obtained from Kobian (K) Ltd, Nairobi and were freshly distilled before use.

3.3 Extraction

The root and stem bark of *Senna didymobotrya*, leaves of *Euclea divinorum* and stem bark of *Ziziphus abyssinica* were air dried in a well-ventilated room and then ground to powder. The amounts of the plant material used were as shown below:

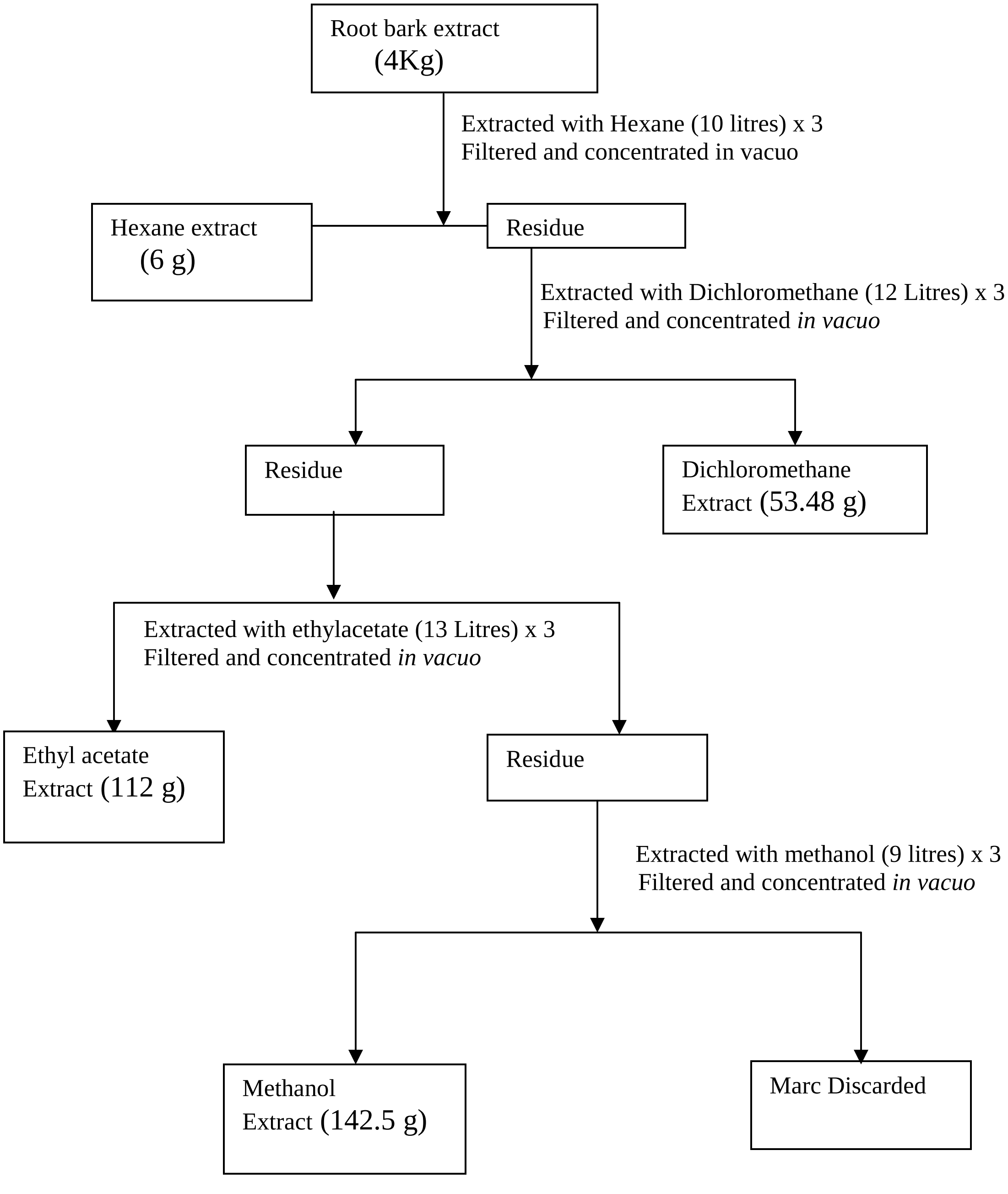
Table 2.0: Plant material and the yields**.**

Plant genus and species	**Plant part**	**Yields**
Senna didymobotrya	Root bark	4.0 Kg
Senna didymobotrya	Stem bark	4.0 Kg
Ziziphus abyssinica	Stem bark	5.5 Kg.
Euclea divinorum	Leaves	2.6 Kg.

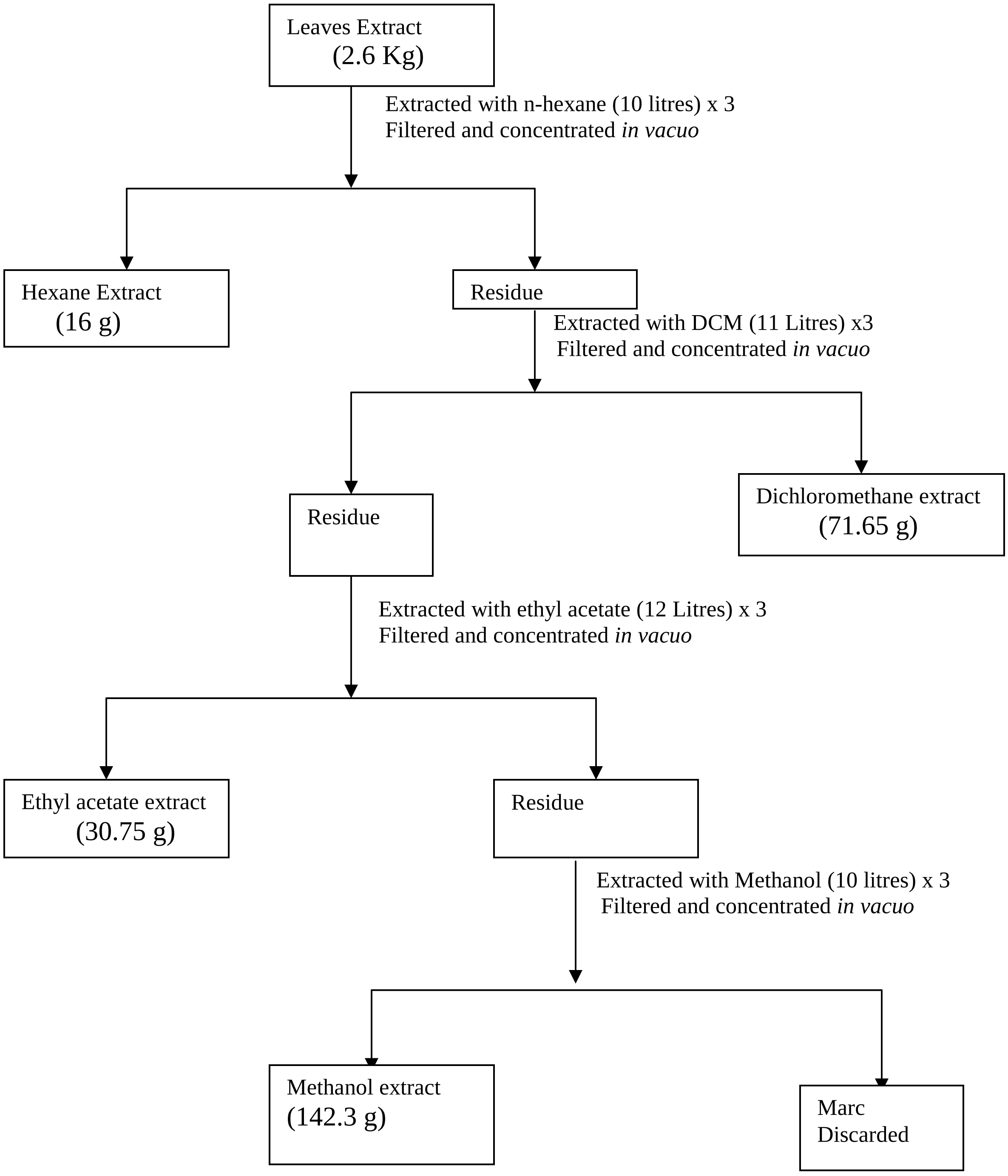
The plant material powders were sequentially extracted with hexane (10 Litres), DCM (12 Litres), EtOAc (13 Litres) and MeOH (9 Litres), respectively in order of increasing polarity at room temperature for three days and the process repeated three times with occasional shaking. The schematic extraction is as shown in the schemes 1-3.

Filtration followed and the filtrates from each solvent were combined and concentrated in vacuo and later stored in the fridge at low temperatures. This yielded 6 g and 53.48 g of hexane and DCM extracts of *Senna didymobotrya* stem bark, respectively, and 16 g and 71.65 g of hexane and DCM extracts of *Euclea divinorum*, respectively, and 5 g and 25.34 g of hexane and DCM extracts of *Ziziphus abyssinica*, respectively. Extraction processes are summarized in Scheme 1, 2 and 3.

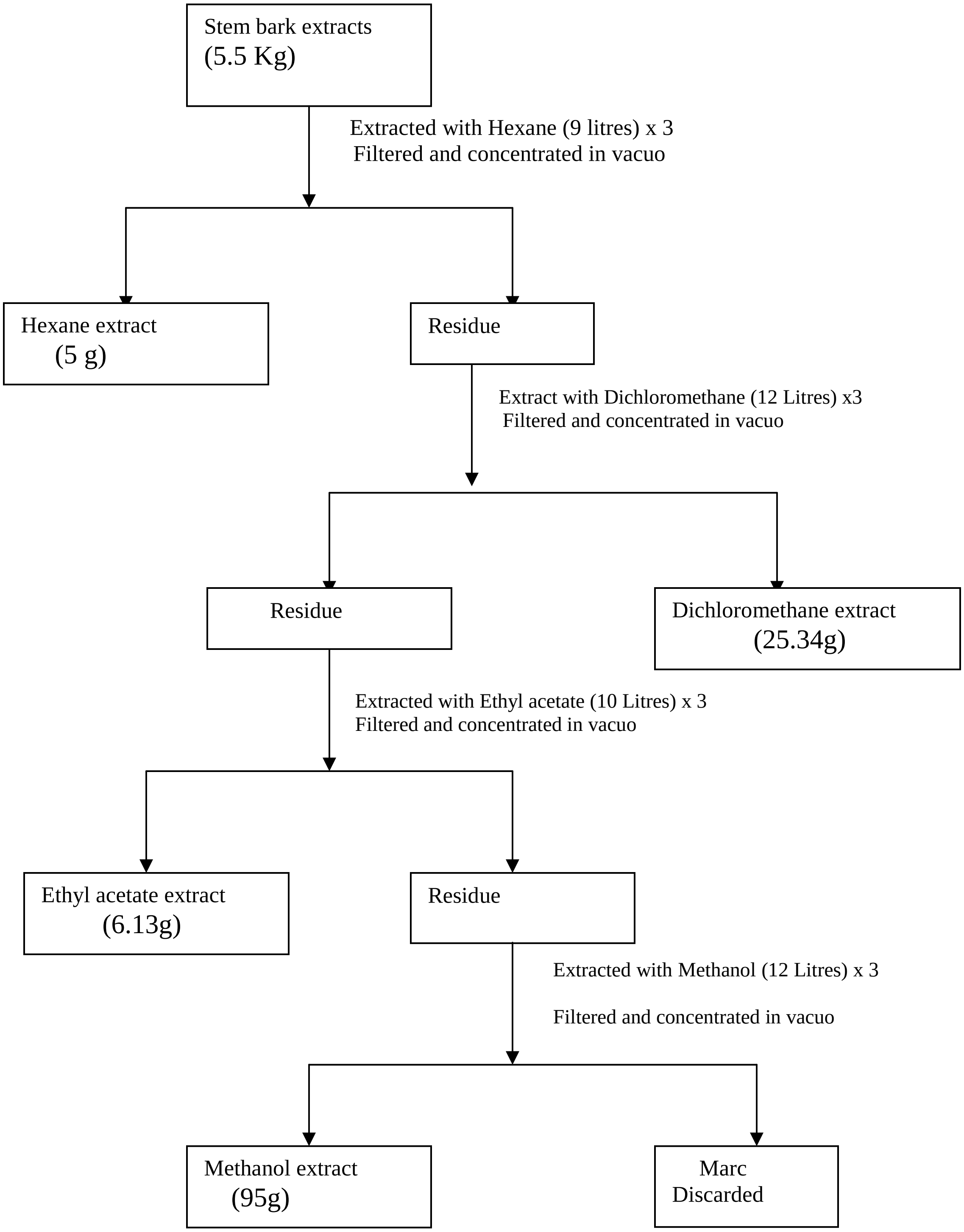
Scheme 1: Flow diagram for the solvent extraction of the powdered root bark of *Senna didymobotrya*



Scheme 2: Flow diagram for solvent extraction of the powdered leaves of *Euclea divinorum.*



Scheme 3: Flow diagram for solvent extraction of the powdered stem bark of *Ziziphus abyssinica*



3.4 Biological activity test

Before embarking on fractionation of extracts, toxicity test, antibacterial and adulticidal tests were performed.

3.4.1 Brine shrimp bioassay test

The brine shrimp test to evaluate the bioactivity of crude extracts and pure compounds was carried out using brine shrimp (*Artemia salina*) larvae as the test organism. Artificial seawater prepared by dissolving artificial sea salt (Sigma Chemicals Co. UK) in distilled water (ca 38 gl^{-1}) was filled into incubation tank (25 cm in diameter and 3.5 cm in height) divided into two unequal compartments separated by a perforated polystyrene wall at room temperature. Brine shrimp eggs (0.5 g) were sprinkled into the larger compartment, which was darkened by covering with hard paper while the smaller compartment was illuminated with an electrical bulb. After 48 hours of hatching the phototropic nauplii were collected from the illuminated compartment by using a micropipette (Meyer *et al*., 1982).

Solutions of 1000, 800, 600, 400 and 200 μg/ml were prepared by dissolving 1 g of each crude extract in 1 ml of dimethyl sulphoxide (DMSO). Serial dilution for the three plants extracts in different vials each with a volume of 10 ml were made by adding artificial seawater and then 10 brine shrimps were transferred into each of the three vials. The experimental vials were maintained under illumination conditions. Controls were placed in a mixture of artificial seawater and 1 ml of DMSO. The nauplii were counted microscopically in the stem of the pipette against a lit background. Each assay was repeated three times and the average number of survived larvae after 24 hours was recorded.

Lethality concentrations (LC_{50} values) for each assay were calculated by taking average of the four experiments and subjecting data to probit analysis, using SAS program version 8.2.

3.4.2 Antimicrobial activity

The potential of natural products or any other agents for use as antiseptic, disinfectant or antibiotic can be assessed *in-vitro* using microbial test organisms. In this study, two assays were carried out: (a) antibacterial assay and (b) antifungal assay.

3.4.2.1 Microbial test cultures and growth conditions

Escherichia coli (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853) gram-negative bacteria and *Staphylococcus aureus* (ATCC 22923), *Staphylococcus aureus* gram-positive bacteria bacterial strains were used. *Candida albicans* (ATCC 90028) was used as fungal test microorganisms. Bacterial strains were maintained on Nutrient Agar (NA) Petri dishes at 4^0C while fungi were maintained on Sabourand Dextrose Agar (SDA) in petri dishes. Antifungal and antibacterial *in-vitro* assays were done using disc diffusion (Hutchinson, 1986). All the procedures were done according to Clinical laboratory standard Institute Standards procedures and quality control. 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. The inhibition zones measured in millimeters as described by (Chhabra and Usio, 1990).

3.4.2.2 Media preparation

Mueller Hinton Agar (MHA) (OXOID, UK) was used for bacteria bioassay while SDA (OXOID, UK) was used for fungi as solid media; MHA was prepared by dissolving 19 g in 500 ml of distilled water and brought to boil to completely dissolve. Sterilization was achieved by autoclaving at 121^{o}C for 15 minutes.

SDA was prepared by dissolving 65 g in 1 litre of distilled sterilized water, brought to boil to ensure complete dissolution. Autoclaved at 121^{o}C for 15 minutes to ensured sterilization of the media. The media (20 ml) was dispensed onto the pre-sterilized Petri dishes yielding uniform depths of 4 mm. They were then covered and allowed to cool and harden at room temperature. Filter paper discs (6 mm diameter) were prepared and sterilized by autoclaving.

3.4.2.3 Antimicrobial activity assay

Disc diffusion method was employed in the preliminary antimicrobial screening of both the crude organic extracts and selected isolated purified compounds. Test strains suspensions of 0.5ml McFarland were prepared from fresh cultures using normal saline. The plates were aseptically streaked with the test microorganism using a sterile swab and allowed to dry for a few minutes. Test extracts (100 mg) were dissolved in 1ml of DMSO.

Sterile 6 mm diameter filter paper discs were impregnated with 100 mg/ml of the crude extract material and using sterile forceps the discs placed aseptically on the inoculated agar plates. The plates were then incubated for 24 hours at 37^oC for bacteria and at 28^oC for fungi. The experiments were carried out in triplicates. Presence of a clear circular zone around the sample impregnated with test sample was used as an inhibition indicator of activity.

The results (mean values, n = 3) were recorded by measuring these zones diameter using a ruler and are uncorrected Disc impregnated with the solvent (DMSO) was included as controls. For comparative purposes standard drug gentamycim (10 µg/disc) was the positive control in the assay for antibacterial and antifungal assays.

3.4.3 Adulticidal testing against bean weevil

3.4.3.1 Culturing of bean weevil

Bean weevils contaminated grains used for the study were obtained locally from KARI Kisii and Bungoma district in Western Province. Dried grains were sieved to remove dirt, fine dust and broken or shriveled kernels. One hundred 6 weeks old *Acanthoscelides obtectus* insects, obtained from a laboratory colony reared under ambient conditions with natural photoperiods on untreated (insecticide-free) bean, *A. obtectus* were reared in a 1-litre jar containing 50 g uninfected whole bean grain as described by (Haines, 1991).

The jar was covered, labeled, and left at 20^0 C. It was moved into the rearing area for one week for adults to develop. The adult weevils were removed from the medium using 2.0

mm and 0.7 mm opening sieves and discarded. The medium was then returned to the culture jar, covered and allowed to stand for four weeks for adult weevil to emerge. The rearing of test insects was done in the laboratory at room temperature. All equipments used were thoroughly cleaned and sterilized using ethanol.

3.4.3.2 Adulticidal assay

This bioassay test was carried in the laboratory to determine the efficacy of botanicals under different dosage levels. For the crude plant extracts four concentrations (250ppm, 500 ppm, 750 ppm, and 1000 ppm.) were admixed with 50 g of disinfected bean. An untreated sample and Actellic super (pirimophos-methyl) at the recommended rate of 0.5 % (w/w) were used as negative and positive controls, respectively.

The crude extracts were prepared by dissolving 1 g of the test sample in 1ml of DCM and then mixed with 5 grams of Talc ($Mg_3Si_4O_{10}(OH)_2$) and thoroughly mixed and DCM evaporated in the fume cupboard. These concentrations were admixed with 50 g of beans held in jam jags covered with ventilated lids. The grain was then swirled within the jam until a proper admixture was realized. Each treatment was replicated three times.

The walls of holding trays used for handling the colony were coated with a thin film of fluorocarbon resin (Teflon) to prevent individuals from escaping. Twenty randomly selected adults of *Acanthoscelides obtectus* of mixed sexes and age were starved for 24 hours before being introduced into each jam jag. After seven days, the number of dead and live *A. obtectus* in each treatment was recorded from which the mean average mortalities and percentage mean mortality were calculated (Bekele *et al*., 1996).

The percentage mean mortality result was then subjected to SAS version 8.2 a computer program for analysis to obtain LD_{50}

3.5 Separations and purification of constituents

3.5.1 Chromatographic techniques

The following Chromatographic methods were employed in this study.

3.5.1.1 Thin Layer Chromatography (TLC).

Thin layer chromatography (TLC) was perforated on aluminium sheets precoated with silica gel 60 F_{254} (Merck) with a 0.25 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 (Model UV GL-58 mineral light lamp of 254 and 366 nm). Visualization was carried out by spraying with anisaldehyde or dragendorff reagents, then the TLC plate was allowed to dry at room temperature before heating at 110^{0}C for five minutes.

Preparative TLC was done using normal phase silica gel (F254 Merck) precoated on aluminium plate (20 x 20 cm, thickness of 0.25 mm).

3.5.1.2 Column chromatography (CC)

Column chromatography was carried out using glass column (1.5-5.5 cm in diameter-12-55 cm in length). Silica gel 60 (0.40 - 0.0630, 230-400 mesh, Merck) was used and slurry-packing method was employed.

3.5.1.3 Vacuum liquid chromatography(VLC)

VLC column were packed with thin layer chromatography silica gel 60 (6-35 microns mesh, ASTM). Known masses of crude samples were loaded into the column (4 cm wide x 30 cm long) and eluted with appropriate solvent system to fractionate the crude into fractions containing compounds according to polarity.

3.5.1.4 Gel chromatography

Separation and purification were carried out using Sephadex ® LH-20 (Pharmacia), eluted with a mixture of MeOH and DCM in the ratio of 1:1. Recrystallization of solid compounds in suitable solvents were carried out where applicable.

3.6 Fractionation and isolation

3.6.1 Hexane extract of *Senna didymobotrya*

The hexane crude extract was subjected to TLC and eluted with the several solvent systems, the best solvent system was found to be hexane: DCM 3:7 and was used as a guide for column chromatography was carried out. A column 4.0 cm diameter and 50 cm was packed with 150 g of silica gel 60 (70-230 mesh, ASTM, Merck). Hexane extract (26

g) was thoroughly mixed with equal amount of silica gel 60 (70-230 mesh, ASTM, Merck).

The dried mixture was loaded onto the column then eluted with solvent system starting from 100% hexane (500 mls), 60% hexane in DCM (500 mls), 40% hexane in DCM (500 mls), 20% of hexane in DCM (500 mls) and finally 100% DCM (500 mls) then followed by 1, 2.5, 10, 20 and 30% MeOH in DCM (500 mls). One hundred and ten fractions of 20 mls each were collected and concentrated. The fractions that showed similar components were pooled together and in total eleven fractions were obtained and concentrated as follows Fraction 1 (9 mg), fraction 2 (13 mg) fraction 3 (15 mg), fraction 4 (17) fraction 5 (18.9 mg), fraction 6 (15 mg) fraction 7 (13 mg) fraction 8 (12 mg), fraction 9 (16 mg), fraction 10 (8 mg) and fraction 11 (20 mg). Recrystallization of the fraction 5 (21 mg) with MeOH gave compound **52**.

3.6.2 Dichloromethane (DCM) extracts of *Senna didymobotrya*

The *Senna didymobotrya* root bark dichloromethane extract (62 g) was mixed thoroughly with some dichloromethane (300 mls) and 10 g of silica gel, 60 (0.04-0.063 mm, ASTM, Merck) and allowed to dry overnight in the fume cupboard. Vacuum Liquid Chromatography (VLC) column was dry packed with 300 g of silica gel 60 (6-35 micron, ASTM, Merck).

The mixture was loaded onto the column ((1.5 cm and 35 cm) then eluted with solvent starting from 100% hexane (300 mls) and increasing polarity sequentially to 100% DCM

(300 mls), and later up to MeOH: DCM (3:7) (300 mls). Eighty-one fractions (20 mls each) were collected and concentrated in vacuo. From TLC analysis similar fractions were pooled together. The UV active spots on TLC were considered for further separation.

From VLC of the DCM extract, fractions 21-28 (1.03 g) was loaded on Sephadex column (4 cm x 30 cm) and eluted with DCM and MeOH in the ratio of 1:1 to give 28 sub fractions. Sub fractions 1-20 were mixed and subjected to column chromatography and eluted with EtOAc and DCM in the ratio of 1:3, this afforded compound **52** (21 mg).

Fraction 56-63 from VLC was subjected to column chromatography and eluted with EtOAc:DCM in the ratio of 1:9. The sub fraction 15-27 was loaded onto Sephadex column and eluted with DCM and MeOH in the ratio 1:1 to give 17 sub-fractions. Sub fraction 5-9 was crystallized with MeOH and this afforded compound **53** (14 mg).

3.6.3 Dichloromethane extract of *Ziziphus abyssinica*

The *Ziziphus abyssinica* DCM extract (22.58 g) was subjected to vacuum liquid chromatography (VLC) separation on silica gel 60 (6-35 micron, ASTM, Merck) and eluted with hexane 100 % (400 mls) with increasing amount of DCM (400 mls) and later increasing the amount of MeOH in DCM up to 10 percent, 48 fractions (10 mls) were collected. The fractions were subjected to TLC and then monitored with the UV lamp at the two wavelengths of 254 and 366 nm, respectively. The crude extracts obtained from the plant in question were used in the bioassay on weevil, and the results obtained showed low activity of 40±0.0 at 750 ppm as compared with the Actellic super which showed an activity of 95±0.0 at 750 ppm and hence no further separation and purification was done on this crude extracts.

3.7 Spraying reagents

3.7.1 Anisaldehyde

Anisaldehyde-sulphuric acid spray reagent was prepared by mixing anisaldehyde, acetic acid, MeOH and concentrated sulphuric acid in the ratio 0.5:10:85:5, respectively (Merck and Darmstadt, 1980).

3.7.2 Dragendorff reagent

Dragendorff reagent for detection of alkaloids was prepared by mixing 0.85 g basic bismuth nitrate with 40 ml of water. Solution A was prepared by mixing 10 ml of acetic acid and 8 g of potassium iodide dissolved in 10 ml of water. 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 mixing solution A and solution B in ratio 1:10, respectively.

3.8 Spectroscopic methods used in structural elucidation

In structural elucidation using spectroscopic methods, there is no established procedure that is to be followed (Silverstein *et al*., 1981).Generally, one tries to establish a structure from as many different spectra as possible.

3.8.1 Melting points analysis

Melting points (mp) were determined on an electro thermal (Gallenkamp) melting point apparatus and expressed in degree centigrade (^{0}C) and were uncorrected.

3.8.2 Ultraviolet –Visible spectroscopy

Ultraviolet-visible spectra mainly gives information about the extent of multiple bond or aromatic conjugation within molecules (Scheiman, 1974). Energy absorbed in the uv-visible region produces changes in electronic energy in the molecule resulting from transition of an electron from an occupied molecular orbital (usually a nonbonding p-orbital or Π bonding – orbital) to the next higher energy orbital (an antibonding Π* or σ* orbital). (Silverstein *et al*., 1981). Absorption of uv radiation is therefore dependent upon the electronic structure of the compound. Ultraviolet-visible spectroscopy can differentiate between a variety of chromophoric systems s well as between similar systems of different structures.

In general, intense absorption in the range 200-300 nm (ε_{max} 10000-20000) normally signifies the conjugation of at least two chromophores. Presence of medium-intensity bands at 210-300(ε_{max} 5000-16000) commonly indicates aromatic derivatives with polar substituents (Dyke *et al*., 1978).

3.8.3 Fourier Transform-Infra Red (FT-IR)

Absorption of infrared radiation cause changes in vibrational and rotational transitions in molecules. Radiation involved is usually in the range of 4000-200 cm^{-1} . Absorptions due to combinations or overtone mode may occur between 10000 cm^{-1} and 4000 cm^{-1} but are of very limited use (Straughan, 1976). The group frequency region (4000-135 cm^{-1}) is the most reliable region from spectra-structure correlation because absorptions in this region come from substituent groups and are localized (Dyke *et al*., 1978). Another important area for examination of an IR spectrum is the region 909-650 cm^{-1}. Absence of a strong absorption band in this region generally indicates lack of an aromatic structure.

This can further be confirmed by checking for lack of strong skeletal bands, C=C stretch, in the 1600-1300 cm^{-1} region of the spectrum. The region 1300-909 cm^{-1} is termed the 'finger- print' region. The region is useful in the identification of an unknown by comparing the spectrum of the unknown with that of a known compound. The IR spectra recorded on a Shimazdu (Model FR-IR-8400 CE) with absorption given in wave numbers (cm^{-1}) in chloroform solution or potassium bromide (KBr) discs was used in my study. The spectrum was recorded after background correction in the range 4000-400 cm^{-1}

3.8.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance deals with the interaction between the magnetic moments of atomic nuclei and magnetic fields. The magnetic moment of the nucleus is associated with the nuclear spin S. Only those nuclei for which S= 0 have a magnetic moment. The nuclei H, C, N and P have isotopes with spin 0.5 and are of great use for organic chemistry because they have magnetic moments which can interact with the magnetic fields.(Kessler *et al.*, 1988).

In ^{13}C NMR spectra, the chemical shifts are the most useful parameters. The range of shifts for the common classes of organic compounds is 0-240 ppm downfield from the reference signal, usually tetramethylsilane (TMS). Many of the important organic compounds functional groups are directly observable. For example carbonyl carbon nuclei of aliphatic ketones are the most highly deshielded of all carbonyls (Harbone, 1998).

The ^{1}H NMR gives record of difference in the magnetic properties of hydrogen nuclei which absorbs electromagnetic radiation in the radiofrequency region, 60-500 MHz (Silverstein *et al*., 1978). The ^{1}H NMR spectra appears predominantly in the region 0-10 ppm downfield from the reference signal, usually tetramethylsilane (TMS).

^{1}H and ^{13}C NMR spectra were recorded at room temperature on a Bruker DPX-400 NMR spectrometer. ^{1}H NMR was recorded at 300 MHz and ^{13}C NMR was observed at 75 MHz. The spectra were recorded in $CDCl_3$ as the solvent and TMS as the internal standard. The chemical shifts reported in δ (ppm) units relative to TMS signal and the peaks multiplicities were recorded (*m, t*, *d* and *s*) in coupling constant (J) in Hz.

3.8.5 Mass Spectrometry

A mass spectrum is a presentation of positively charged fragments, including the molecular ion, versus their relative concentrations. The most intense peak, the base peak, is assigned a value of 100% and the intensities of other peaks are reported as a percentage of the base peak (Silverstein *et al*., 1981).

Several methods can be used to produce ions. Some of these methods used for ion production are by electron bombardment of the sample vapour, thermal evaporation of the sample from a metallic surface and irradiation of the sample vapour with ultraviolet radiation of short wavelength (Dyke *et al*., 1978).Mass spectroscopy has two principle uses; (a) to measure molecular weights from which exact molecular formulae can be deduced, and (b) to detect within a molecule the places at which it prefers to fragment. From the fragmentation pattern, the presence of recognized groups within the molecule can be deduced. The presence of an M-15 peak (loss of CH_3), or M-18 peak (loss of H_2O) etc are taken as confirmation of molecular ion peak (Silverstein *et al*., 1978).

3.8.6 Distortionless enhancement polarization transfer (DEPT).

Information on the number of protons attached to a carbon in a compound can be obtained from a ^{13}C-NMR spectra by carrying out Attached Proton Test (APT). APT makes a ^{13}C nuclei bearing a single proton, two protons, three protons and no proton display doublet, triplet, quartet and singlet respectively. The same APT information can be obtained by preparing Distortioness Enhancement by Polarization Transfer (DEPT) spectra in which the following three separate measurements are made; (a) broadband ^{1}H decoupled spectrum (b) CH_3 and CH signals positive, CH_2 signals negative spectra, and (c) CH signal spectrum only (Duddeck and Dietrich, 1989).

3.8.7 Physical and spectroscopic data of isolated compounds

Compound **52**

White crystalline solid; m.p. 129˚C, $R_{f,}$ 0.51 (Hexane:DCM, 1:4 v/v), IR $\bar{\nu}_{max}$ cm^{-1} (KBr disc,) 3422, 2920, 1640, 1463, 1380, 1048, 1021, 723.

^{1}H-NMR ($CDCl_{3,}$ 300 MHz) δ, ppm 0.68 (3H, *s*, H-18), 0.83 (3H, *s* H-21), 0.85 (3H, *s*, H-26), 1.00 (3H, *s*, H-19), 1.25 (3H, s, H-29), 1.57 (3H, *s*, H-27), 1.85(H, *d*, J= 3.3Hz, H-2) 2.00 (2H, *d*, J = 3.3, Hz, H-7), 2.27 (2H, *d*, J = 1.8), 3.54 (H, *m*, H-3), 5.35 (2H, *d* J = 5.3, Hz, H-5 and H-6) and 5.35 (H, *d*, 5.1, Hz, H-22 and H-23). ^{13}C-NMR, (Table 9).

Compound **53**

White crystalline solid; m.p.158-159˚C, $R_{f,}$ 0.49 (Hex:DCM, 3:7 v/v), IR $\bar{\nu}_{max}$ cm^{-1} (KBr disc,) 3422, 2920, 1640, 1463, 1380, 1048, 1021, 723.

^{1}H-NMR (CDCl$_{3,}$ 300 MHz) δ, in ppm, 0.68 (3H, *s*, H-18), 0.83 (3H, *s* H-21, 0.85 (3H, *s*, H-26), 1.00 (3H, *s*, H-19), 1.25 (3H, *s*, H-29), 1.57 (3H, s, H-27), 1.85 (H $_{eq,}$ *d*, = 3 Hz, H-2) 2.00 (2H, *d*, = 3.3 Hz, H-7), 3.35 (H, *m*, H-3) and 5.37 (1H, *d*, =5.3 Hz, H-5 and H-6), 1.26 (H, *s*, H-22 and H-23) ^{13}C-NMR, (Table 10).

Compound **54**

Orange crystals of m.p, 194-195˚C, $R_{f,}$ 0.52 (Hex:DCM, 2:4 v/v), IR $\bar{\nu}_{max}$ cm^{-1} (KBr disc,) 3425, 1627, 1479, 1272 and 752.

^{1}H-NMR (CDCl$_{3,}$ δ, in ppm, 300 MHz) (table 11) and ^{13}C-NMR (75 MHz), (Table 12).

EIMS m/z (relative intensity): 254 [M] (100), 226 (20), 149(18), 129(21).

Compound **55**

Orange crystals; m.p, 206-207˚C, R_f, 0.52 (Hex:DCM, 2:4 v/v), IR $\bar{\nu}_{max}$ cm^{-1} (KBr disc) 3425, 1627, 1479, 1272 and 752.

^{1}H-NMR (CDCl$_{3,}$ δ, in ppm, 300 MHz) (table 13) and ^{13}C-NMR (75 MHz), (Table 14).

EIMS m/z (relative intensity): 284 $[M]^+$ (50), 256 [M-CO] (24), 254 (100), 226(20) 198 (10).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Introduction

Powdered root bark and leaves of *Senna didymobotrya* and *Euclea divinorum* respectively, were extracted by soaking in hexane, dichloromethane, ethyl acetate and methanol to get the crude extracts. The yields of these extracts and the percentage yields are presented in table 3.

Table 3: Plant material with percentage yields of extracts.

Plant	*Senna didymobotrya*			*Ziziphus abyssinica*			*Euclea divinorum*		
Extract	Hex.	DCM	EtOAc	Hex.	DCM	EtOAc	Hex.	DCM	EtOAc
Yields (g)	6	53.45	85.63	5	25.34	6.13	16	71.65	30..75
% Yields	0.1	0.89	1.42	0.09	0.46	0.11	0.61	2.75	1.18

The percentage yields of hexane extracts was lower for the three plant materials than the extracts of the other solvents. The DCM yields for the *Euclea divinorum* was the highest followed by the ethyl acetate yields for *Senna didymobotrya.* This extracts were subjected to various bioassay which included brine shrimp lethality test, antibacterial, antifungal test and adulticidal test against bean weevil were performed before embarking on fractionation of the crude extracts.

4.2 Brine shrimp assay results

The crude extracts (Hexane and DCM) of *Senna didmobotrya*, *Ziziphus abyssinica* and *Euclea divinorum*) were tested for toxicity against the brine shrimp lethality assay. The mortality results expressed as percentage were subjected to SAS and data analyzed to give the LD_{50}± S.D. The results are shown in Table 4.

Table 4: The mean lethality values ± S.D (n=3) for plant extracts screened against brine shrimp (*Artemia salina*, Leach).

Plant extracts	Concentration in ppm				
	200	400	600	800	1000
*Sd*BH	13±5.7	23±5.7	36±5.7	53±5.7	63±5.7
*Sd*BD	23±5.7	36±5.7	53±5.7	63±5.7	83±5.7
*Ed*LH	10±10	16±5.7	26±5.7	46±5.7	56±5.7
*Ed*LD	16±5.7	16±5.7	33±5.7	46±5.7	53±5.7
*Za*BH	13±5.7	10±0	23±5.7	40±0	53±5.7
*Za*BD	23±5.7	36±5.7	43±5.7	56±5.7	63±5.7

Table 5: The mean LD_{50} values ± S.D for plant extracts screened against brine shrimp (*Artemia salina*, Leach).

Plants Extract	*SdBH*	*SdBD*	*ZaBH*	*ZaBD*	*EdLH*	*EdLD*
LD_{50}± S.D.(μg/ml)	345.1± 0.8	195.0± 0.7	699 ± 1.0	871.5±0.6	952 ± 0.5	689.9± 0.3

*Sd*BH=*Senna didymobotrya* root bark, hexane extract; *Sd*BD=*Senna didymobotrya* root bark, DCM extract
ZaBH=*Z. abbyssinica* stem bark, hexane extract; ZaBD=*Z. abyssinica* stem bark, DCM extract
*Ed*LH= *Euclea divinorum* leaves, hexane extract, *Ed*LD= *Euclea divinorum* dichloromethane extract.

A Crude extract is considered active up to a concentration of 240 μg/ml (Meyer *et al*,. 1982). Therefore, the dichloromethane extracts of *S. didymobotrya* root bark with LD_{50} values of 195.0 μg/ml was considered active, while the DCM extract of *S. didymobotrya* root bark showed mild toxicity against brine shrimp with LD_{50} values of 345.1 μg/ml.

The hexane and DCM extracts of *Z. abyssinica and E. divinorum* showed low toxicity against brine shrimp with LD_{50} values of 699.0 and 871.5 μg/ml 952 and 689 μg/ml, respectively. Since brine shrimp is an indicator of toxicity, various pharmacological actions, and pesticidal effects (Meyer *et al*., 1982), it was deduced that the DCM extracts of *Senna didymobotrya* had the better bioactivity than the extracts of *E. divinorum* and *Z. abbyssinica*, respectively against brine shrimp.

4.3 Adulticidal results

The crude extracts of Z*iziphus abyssinica*, *Senna didymobotrya* and *Euclea divinorum* were further subjected to adulticidal test against bean weevil. The effects doses of hexane and DCM extracts on bean weevil after seven days were determined and mean percentage values computed and the results are summarized in Table 6.

Table 6: Mean percentage (%) adulticidal (± S.D n=3) of hexane and DCM extract from *Senna didymobotrya, Ziziphus abyssinica and Euclea divinorum* against the bean weevil (*Acanthoscelide obtectus*).

Plant extract	250ppm	500ppm	750ppm	1000ppm
*Sd*BH	25.0 ± 0.5c	45.0 ± 0.3c	50.0 ± 1.6c	80.0 ± 1.6b
*Sd*BD	25.0 ± 0.5c	75.0 ± 0.3b	95.0 ± 0.0b	100.0 ± 0a
*Za*BH	15.0 ± 0.3e	20.0 ± 0.3g	30.0 ± 0.3e	40.0 ± 0.3e
*Za*BD	20.0 ± 0.3d	30.0 ± 0.3e	40.0 ± 0.0d	40.0 ± 0.0d
*Ed*LH	15.0 ± 0.3e	25.0 ± 0.0f	30.0 ± 0.3e	35.0 ± 0.3f
*Ed*LD	30.0 ± 0.6b	40.0 ± 0.0d	50.0 ± 0.5c	60.0 ± 0.3c
Actellic super	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a	100.0 ± 0.0a
Negative control	0.0	0.0	0.0	0.0

Key: *Sd*BH= *Senna .didymobotrya*, root bark, hexane extract;
*Sd*RD = *Senna didymotrya* root bark, DCM extract
*Za*BH= *Ziziphus abbyssinica* stem bark, hexane extract;
ZaBD= *Ziziphus abyssinica* stem bark, DCM extract.
*Ed*LH= *E. divinorum* leaves, hexane extract;
*Ed*LD= *Euclea divinorum* leaves, DCM extract

Mean values with the same letters within the same column are not significantly different at 95% confidence level (Tukey's studentized test).

From the results in the table 6, it is evident that adulticidal activities are dose dependent. Out of the three plant extracts, *Senna didymobotrya* root bark had the highest mean adulticidal activity at almost all doses, the hexane and DCM extracts showed 100% and 95% adulticidal at 750 and 1000 ppm, respectively. The results were comparable to the positive control, Actellic super, a synthetic pesticide at the recommended rate of 0.5%.

Ziziphus abyssinica extracts (*Za*BH and *Za*BD) displayed lower adulticidal activity, with activity between 20-30% comparable to the negative control. However, as the concentration increases the activity of the extracts increased significantly ($p < 0.05$).

The fact that, the crude extracts at high concentration had a noteworthy mean percentage adulticidal activity against the bean weevil is encouraging and this justifies the use of this plant material as grain protectants against destructive pests. It is evident that the crude plant extract could offer suitable and sustainable alternative to synthetic pesticide, and thus represents an attractive candidature for field evaluation as post-harvest protection of grain.

These natural plant products may improve efficiency in post harvest practices as a strategy of providing people with sufficient and healthy food in an ecologically sustainable way. Being natural, protectants from plant materials would be easily degraded by biological factors, whereby cases of pollution and poisoning would be reduced. Improving grain storage would mean less hunger, improved nutrition for individual, a higher standard of living and a sounder economy for the nation.

The fact that the other two plant extracts showed a mild to low activity could be because the bioactive metabolites can be active when they are mixed with those of other plants, this is because farmers use a mixture of plants for crop protection, and this mixture could be active due to synergistic effects.

The brine shrimp and adulticidal activity results for the crude extracts in table 5 and 6, respectively shows that the extracts of *S. didymobotrya* had higher toxicity as well as adulticidal activity against bean weevil. The extracts of both *Z. abyssinica and E. divinorum* showed low brine shrimp and adulticidal activity. It was evident that toxicity against brine shrimp may be a basis of deducing an active adulticidal activity.

4.4 Results of *in-vitro* antimicrobial test of crude extracts

Table 7: Antibacterial and antifungal activity of hexane extracts of *S. didymobotrya* root bark, pods and *E. divinorum* leaves.

Microorganism	Activity expressed in mm of inhibition zones diameter (mean values n = 3)			
	*Sd*RH	*Sd*PH	*Ed*LH	Control
Escherichia coli	15.6±0.7	10.3±0.6	11.0±1.2	6.0±0.0
Staphylococcus aureus	18.6±0.6	23.6±0.5	18.0±1.0	6.0±0.0
Pseudomonas aeruginosa	6.0±1.0	6.0±0.7	6.0±0.8	6.0±0.0
Bacillus subtilis	12.3±0.5	12.6±1.2	13.0±0.1	6.0±0.0
Candida albicans	15.3±0.7	10.3±0.8	7.0±0.2	6.0±0.0

Table 8: Antibacterial and antifungal activity of dichloromethane (DCM) of *S. didymobotrya* roots, pods and *Euclea divinorum* leaves.

Microorganism	Activity expressed in mm of inhibition zones diameter (mean values n = 3)			
	SdRD	*SdPD*	*EdLD*	Control
Escherichia coli	7.6±0.5	7.6±0.7	17.6±1.0	6.0±0.0
Staphylococcus aureus	10.3±0.6	10.3±0.8	10.6±1.5	6.0±0.0
Pseudomonas aeruginosa	6.0±1.3	6.0±0.7	6.0±0.9	6.0±0.0
Bacillus subtilis	10.3±1.2	9.3±1.0	12.6±0.8	6.0±0.0
Candida albicans	12.3±0.5	9.3±0.9	6.6±1.2	6.0±0.0

Key: *Sd*RD=*Senna didymobotrya* root bark DCM extract.
*Sd*PD= *Senna didymobotrya* pods, DCM extract
*Sd*RH=*Senna didymobotrya* root bark hexane extract,
*Ed*LD=*Euclea divinorum* leaves, DCM extract

4.4.1 Antibacterial tests

It can be clearly noted that among the crude extracts, majority of them showed low to moderate antibacterial and antifungal activity against the microorganisms (table 7 and 8). The hexane extracts of *S. didymobotrya* and *E. divinorum* showed good activity against *S. aureus* and *B. subtillis* with zones of inhibition averaging 23 mm, 12 mm, 18 mm and 13 mm, respectively. The rest showed mild activity against the test organism. The results obtained were an indication that the Gram-positive bacterium (*S. aereus* and *B. subtillis*) are more susceptible to antibiotics than the Gram-negative bacterium (*P. aeruginosa* and *E. coli*) which are known to be more difficult to inhibit. The results obtained were in agreement with the previous report for *Teclea trichocarpa*, in this report the hexane and DCM extracts showed good activities towards the Gram- positive bacterium *S. aereus* and *B. subtilis* with inhibition zones averaging 22 mm, 13mm, 16 mm and 12 mm, respectively (Phillipson *et al*., 1990).

Among the dichloromethane extracts, the trend was also observed where the DCM extracts of *S. didymobotrya* and *Euclea divinorum* exhibited moderate activity against the Gram-positive bacterium (*S. aureus* and *B. suntilis*) with inhibition zones of 10 mm, 10 mm and 10 mm, 12 mm, respectively. It was also noted that the *Euclea divinorum* DCM extract had a high activity of 17.6 mm against the Gram-negative bacterium E. *coli*. The S. *didymobotrya* DCM extract also registered a moderate activity of 12.3 mm against the yeast, *Candida albicans* (Chahbra *et al*., 1990)

These results are a clear indication that these plants can be used in traditional medicine against bacterial infection and can be significance in therapeutic treatments as they find use in many communities as herbal remedies.

4.5 Structure elucidation of compounds from *S. didymobotrya*

4.5.1 Compound 52

Compound **52** was obtained as white crystalline solid with a melting point of 129-130 ˚C. On the TLC, the compound had an R_f of 0.56 in 100% DCM. When the plate was sprayed with anisaldehyde-sulphuric acid the spot turned blue/purple, which later turns green suggesting that compound **52** was a terpenoid. A broad band at 3422 cm^{-1} in its IR spectrum was suggested to an alcoholic functional group (O-H stretch).

The ^{1}H-NMR spectrum (Appendix 1) displayed a multiplet centered at δ 3.53 characteristic of a proton geminal to a β-hydroxyl group at C-3 in triterpenoids. It also displayed a doublet at δ 5.35 suggesting an olefinic proton. The rest of the protons were between δ 0.68 and 2.28 with noticeable six-methyl peaks at δ 0.68 (3H, H-18), 0.79 (3H, H-26), 0.83 (3H, *s*, H-21), 0.91 (3H, *s*, H-19), 1.03 (3H, *s*, H-29) and 1.25 (3H, *s*, H-27), characteristic of plant sterols (Alams *et al*., 1996).

The stereochemistry of this compound at C-22 and C-23 is *cis*. The *trans* and *cis* stereochemistry can be differentiated by comparison of their coupling constant values; *trans* coupling (J= 11-19 Hz) are stronger than *cis* coupling (J= 5-14 Hz) (William, 1975). In the case of compound **52** it was confirmed to have a *cis* stereochemistry on C-22, C-23 because the coupling constant value of the protons on these carbons was J = 5.1 Hz.

The ^{13}C-NMR spectrum (Appendix 2) and the DEPT spectrum (Appendix 3) of compound **52** displayed twenty-nine carbon atoms, six methyls, eleven methylenes, nine methines and three quartenary carbons, strongly suggesting a modified triterpenoid. The carbon peak at δ 71.8, a methine was assigned to C-3 because of the attachment of the hydroxyl groups to this carbon in this class of compounds, the sterols (Mahato and Kundu, 1994).

It also showed four peaks at δ 140.8 (C-5), 121.6 (C-6), 138.2 (C-22) and 129.3 (C-23) suggesting presence of olefinic carbons, with the deshielded signal assigned to the quaternary at the bridge. The six-methyl groups were assigned at δ 11.8 (C-18), 19.3 (C-19), 18.7 (C-21), 19.7 (C-26), 19.0 (C-27) and 11.9 (C-29), as shown in Table 9.

On comparison of the ^{13}C-NMR spectra data of compound **52** with already published data it was concluded to be stigmasterol (Knight, 1974). From the above information the structure below was proposed for compound **52.** Complete assignment of the compound is presented in table 9. The identity of compound **52** was further confirmed by comparison with that of already published data (Simpson, 1986), they were found to be in close agreement. Thus the structure of compound **52** is suggested to be stigmasterol (Alam *et al*., 1996).

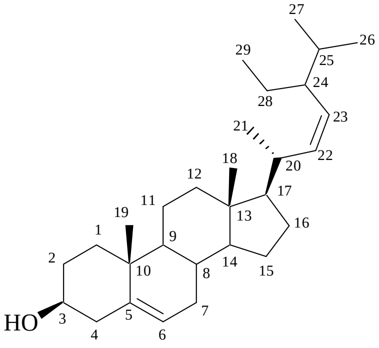


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Table 9. ^{13}C-NMR spectral data for compound **52** and stigmasterol (Alam *et al*., 1996; Wright *et al*., 1990; Simpson, 1986)

Carbon	Compound **52**	DEPT	Stigmasterol
C-1	37.2	CH_2	37.0
C-2	31.9	CH_2	31.6
C-3	71.8	CH	71.7
C-4	42.2	CH_2	41.5
C-5	140.7	C	140.2
C-6	121.6	CH	121.6
C-7	31.6	CH_2	31.7
C-8	31.9	CH	31.9
C-9	50.1	CH	50.1
C-10	36.5	C	36.7
C-11	21.0	CH_2	21.4
C-12	39.7	CH_2	39.7
C-13	42.3	C	42.3
C-14	56.8	CH	56.7
C-15	24.3	CH_2	24.3
C-16	28.2	CH_2	28.2
C-17	56.0	CH	56.0
C-18	11.8	CH_3	11.8
C-19	19.3	CH_3	19.4
C-20	36.1	CH	36.1
C-21	18.7	CH_3	18.7
C-22	138.2	CH	138.0
C-23	129.3	CH	129.1
C-24	45.9	CH	45.8
C-25	29.2	CH	29.1
C-26	19.7	CH_3	19.8
C-27	19.0	CH_3	19.0
C-28	23.1	CH_2	23.0
C-29	11.9	CH_3	11.9

4.5.2 Compound 53

Compound **53** was obtained as white crystalline solid with a melting point of 158 ˚C. On the TLC the compound had an R_f of 0.51 in hex:DCM, 2:4. When the plate was sprayed with anisaldehyde the spot turned blue/purple, which later turns green suggesting that compound **53** was a terpenoid. A broad band at 3422 cm^{-1} in its IR spectrum was suggested to an alcoholic functional group (O-H stretch).

The ^{1}H-NMR spectrum (Appendix 4) displayed a multiplet centered at δ 3.53 characteristic of a proton geminal to a β-hydroxyl group at C-3 in triterpenoids. It also displayed a doublet at δ 5.35 suggesting an olefinic proton. Several peaks between 0.68 and 2.27 catered for the rest of the proton systems, with noticeable six methyl peaks at δ 0.68 (3H, H-18), 0.83 (3H, H-21), 0.85 (3H, H-26), 1.00 (3H, H-26), 1.03 (3H, H-19) and 1.25 (3H, H-29) which strongly suggested a triterpenoid structure (Simpson, 1998).

The ^{13}C-NMR spectrum (Appendix 5) and the DEPT spectrum (Appendix 6) displayed twenty-nine carbon atoms, six methyls, eleven methylene, nine methine, and three quartenary carbon atoms (Table 10), strongly suggesting a modified triterpenoid. The peak at δ 71.8 was assigned to C-3 because of the hydroxyl groups attached to this class of compounds (Mahato and Kundu, 1994).

It also showed peaks at δ 140.7 (C-5) and 121.6 (C-6) suggesting presence of olefinic carbons, with the deshielded signal assigned to the quaternary at the bridge. The six-methyl groups were assigned at δ 11.8 (3H, C-18), 19.4 (3H, C-19), 18.7 (3H, C-21), 19.7(3H, C-26), 19.0 (3H, C-27) and 11.9 (3H, C-29). The ^{13}C-NMR spectrum of compound **53** were similar to compound **52** except that the double bond was lacking at position C-22 and C-23 (Knight, 1974).

Table 10. ^{13}C NMR spectral data for compound **53** and β-sitosterol (Knight, 1974)

Carbon	Compound **53**	DEPT	β –sitosterol
C-1	37.3	CH_2	37.3
C-2	31.9	CH_2	31.9
C-3	71.	CH	71.7
C-4	42.3	CH_2	42.2
C-5	140.7	C	140.7
C-6	121.7	CH	121.6
C-7	31.7	CH_2	31.6
C-8	31.9	CH	31.9
C-9	50.1	CH	50.1
C-10	36.5	C	36.5
C-11	21.0	CH_2	21.0
C-12	39.8	CH_2	39.7
C-13	42.3	C	42.3
C-14	56.7	CH	56.7
C-15	24.2	CH_2	24.3
C-16	28.2	CH_2	28.2
C-17	56.1	CH	56.0
C-18	11.8	CH_3	11.8
C-19	19.4	CH_3	19.4
C-20	36.1	CH	36.1
C-21	18.7	CH_3	18.7
C-22	34.0	CH_2	33.9
C-23	26.2	CH_2	26.0
C-24	45.9	CH	45.8
C-25	29.2	CH	29.1
C-26	19.7	CH_3	19.8
C-27	19.0	CH_3	19.0
C-28	23.1	CH_2	23.0
C-29	11.9	CH_3	11.9

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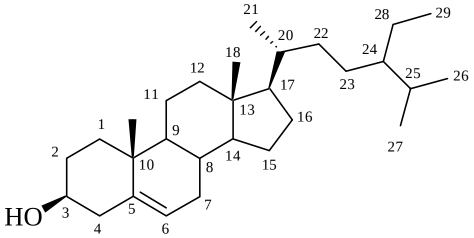
Using the ^{13}C-NMR spectral data and correlating with the published data (Knight, 1974), the peaks closely compared and compound **53** was assigned as β-sitosterol. β-sitosterol arises from the Olney cation after undergoing Wagner-Meerwein 1,2-hydride shift (Fig. 2)



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β-Sitosterol is produced commercially from Soya beans (Glycine max; Leguminosae/Fabaceae) as raw materials for semi-synthesis of medicinal steroids (Mandava, 1979).

The compound β -sitosterol is a very common chemical constituent of medicinal plants, which possesses valuable biological activity, such as antihypercholesterolaemic and estrogenic effect (Buckingham, 1998; Madan *et al*., 1981).

Recently, it was reported to show an important gastro protective activity in several experimental ulcer models in rats (Navarrete *et al*., 2002). β-sitosterol and fatty acids from *Mallotus peltatus* leaf extract were also reported to show antibacterial and anti-inflammatory activities (Chattopadhyay *et al*., 2002).

4.5.3 Compound 54

Compound **54** was isolated as orange crystals of m.p, 194-195˚C, Rf, 0.52 (hex/DCM, 2:4 v/v), [(Literature 194-197 ˚ C (Michiko *et al*., 1981)]. The mass spectrum of compound **54** (Appendix 10) gave the highest mass peak at *m/z* 254 corresponding to molecular ion [M^+] peak and [M+1] ion at *m/z* 255. This is consistent with a molecular formula at $C_{15}H_{10}O_4$ of an anthraquinone. Successive loss of CO gave peaks as *m/z* 226 and 198, respectively (Figure 3).

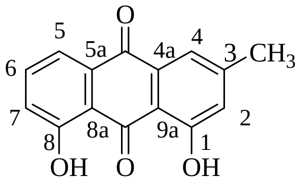
The IR (KBr disc) spectra showed absorption bands at 3425, 1627, 1479, 1272 and 752 cm^{-1} due to OH stretch, C=O stretch, –C=C- stretch, C-O stretch and C-H def, respectively (Appendix 9).

Its ^1H NMR spectrum (Appendix 7) indicated a signal at δ 2.47 (3H) this was assigned to methyl proton of substituent group on ring C position three. Further, analysis gave a signal at δ 12.12 and 12.01, these are typical of OH groups chelated to carbonyl groups, and they are attached to carbon atoms at positions one and eight in ring A and C, respectively and anthraquinone (Hilker *et al*., 1991).

The spectrum also displayed two pairs of doublets, one pair at δ 7.31 (1H, *dd*, J= 1.2, 8.4 Hz, H-7) and 7.83 (1H, *dd*, J= 1.2, 7.8, H-5), with coupling constants associated to the *meta* (J= 1.2Hz) and *ortho* (J=7.8 Hz) couplings. The spectrum showed singlet signals at δ 7.10 (1H, *s*, H-2), 7.65 (1H, *s*, H-4), and a triplet at δ 7.67 (1H, *t*, J= 8.7, H-6).

The ^{13}C NMR spectrum (Appendix 8) of compound **54** exhibited fifteen carbons; of which DEPT spectrum (Appendix 11) showed that five were methine, one methyl and nine quaternary carbon atoms. The quartenary carbons are shown to be present at δ 162.5 (C-1), 149.3 (C-3), 124.3 (C-4), 133.7 (C-4a), 162.8 (C-8), 113.1 (C-8a), 113.9 (C-9a), 192.6 (C-9, C=O), 181.8 (C-10, C=O), five methines (CH) carbons at δ 119.90 (C-2), 124.53 (C-4), 124.34 (C-5), 136.92 (C-6) and 121.33 (C-7). The signal at position three is attributed to a methyl carbon and this confirms the signal at δ 22.23 assigned to C-3 in ring C.

From the information given above the structure below was proposed for compound **54.**



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The identity of compound **54** as 1, 8-dihydroxy-3-methyl anthraquinone ($C_{15}H_{10}O_4$) was further confirmed by comparison with that of already published data (Flower, 2002). It is noted that the chemical shifts were in close agreement (Tables 11-12).

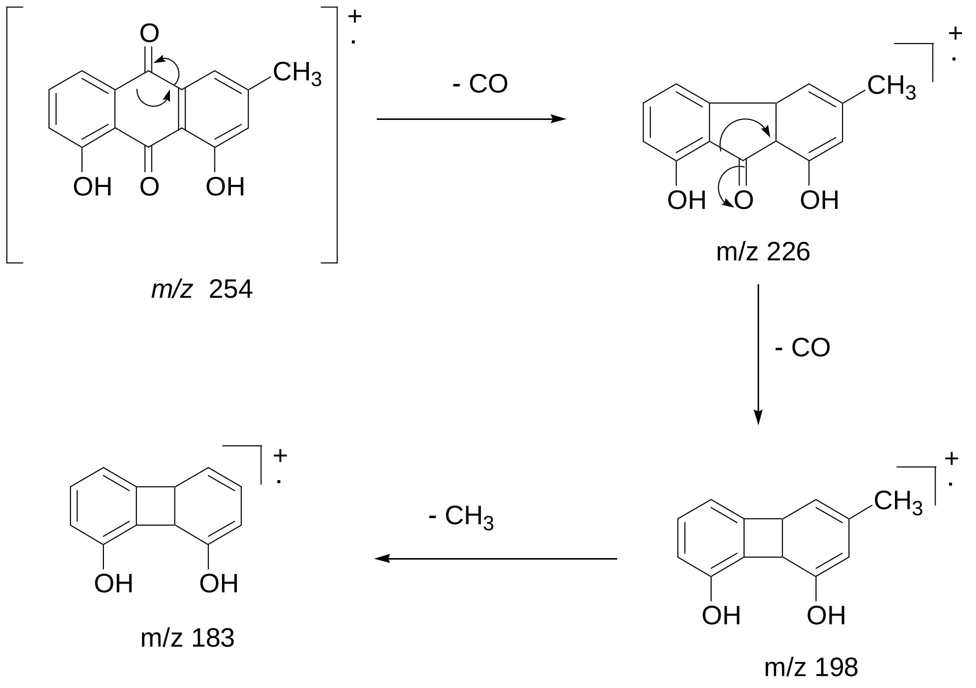
Table 11: ^{1}H-NMR (300MHz) in $CDCl_3$ of compound **54.**
(Flower, 2002; Karlina *et al.*, 2006)

Proton	Chemical shift (Multiplicity J in Hz)	Integral	Chrysophanol Acetone -d_6
1-OH	12.01 (s)	1H	11.83 (s)
H-2	7.10 (br s)	1H	7.00 (m)
3-CH_3	2.47 *s*	3H	2.45 (s)
H-7	7.31(dd 1.2, 8.4)	1H	7.17 (dd)
H-4	7.65 s	1H	7.52 (m)
H-5	7.83 (dd, 1.2)	1H	7.69 (dd)
H-6	7.67 (t, 8.7)	1H	7.61 (t)
H-8	12.12 (s)	1H	11.93 (s)

Table 12: ^{13}C-NMR (75MHz) spectral data for compound **54** $CDCl_3$ with that of authentic chrysophanol (Michiko and Koketsu, 1981).

Carbon	Compound **54**	DEPT	Chrysophanol
C-1	162.4	C	162.5
C-2	113.7	CH	113.9
C-3	149.3	C	149.3
C-4	124.5	CH	124.5
C-4a	133.6	CH	133.7
C-5	124.3	CH	124.3
C-5a	136.9	CH	136.9
C-6	133.3	C	133.2
C-7	115.8	C	113.9
C-8	162.7	C	162.8
C-8a	121.3	C	121.3
C-9	181.9	C	181.8
C-9a	119.9	C	119.9
C-10	192.5	C	192.6
3-CH_3	22.2	CH_3	22.2

Fig. 3 Proposed mass spectral fragmentation of compound 54.



4.5.4 Compound 55

Compound **55** was isolated as orange crystals of m.p, 206-207˚C, R_f, 0.52 (hex/DCM, 2:4 v/v), [(Literature 205-208 ˚C (Rasoanaivo *et al.*, 1999)].

The IR (KBr disc,) spectrum shows absorption bands at 3425, 1627, 1479, 1272 and 752 cm^{-1} due to OH stretch, C=O, due to –C=C-, C-O stretch and C-H def, respectively (Appendix 14). The region between the peaks indicating aromaticity.

The mass spectrum (Appendix 15) of compound **55** gave the highest peak at m/z 284 corresponding to a molecular ion [M^+] and [M+1] ion at m/z 285. This is consistent with a molecular formular at $C_{16} H_{12}O_5$. A peak m/z 256 due to loss of [–CO] supported the presence of the carbonyl group. The peak at m/z 226 was due to the loss of [$-OCH_3$] hence an indication of the presence of a methoxy group. The fragmentation pathway pattern of compound **55** is given in Fig**. 4.**

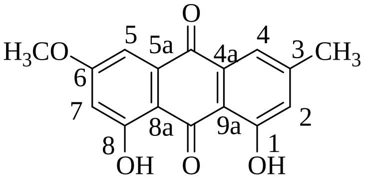
The ^{1}H NMR spectra of compound **55** (Appendix 12) indicated a peak at δ 2.47 this was assigned to methyl proton of substituent group at carbon-3. Further, analysis gave peaks at δ 12.12 and 12.32, these are typical of OH groups chelated to carbonyl moieties, thus indicating this group to be attached on different rings. The down field signal at δ 12.12 was assigned to OH on ring A and the chemical shift at δ 12.32 was assigned to OH proton on ring C. The chemical shifts at δ 7.01 (1H, s, H-2), 7.69 (1H, s, H-4), 7.37 (1H, d, J= 2.4, H-5) and 6.69 (1H, d, J= 2.4, H-7) are typical of aromatic protons in four different chemical environments as indicated in Table 13, (Melvyn 2001).

The presence of two doublets of doublet centered at δ 7.37 (H-7) and 6.70 (H-5) with coupling constant of 2.4 Hz were associated to *meta* coupling.

The ^{13}C NMR spectrum (Appendix 13) and DEPT spectrum (Appendix 16) of compound **55** exhibited sixteen carbons in which four were methine, two were methyl and ten quaternary carbon atoms. The Quaternary carbon at δ 190.80 (C-9) and 181.98 (C-10) were characteristic of C=O which confirmed the presence of carbonyl group as suggested in the IR spectrum $\bar{\nu}_{C=O}$ = 1627 cm^{-1} (Appendix 12). The signal at δ 56.05

was characteristic of a methoxy group attached to carbon 6 of an anthraquinone (Ayator and Okogun, 1982).

On the basis of the above data and comparison with the literature values, the structure below was proposed for compound **55.** The identity of compound **55** as 1, 8-dihydroxy-6-methoxy-3-methyl anthraquinone [C_{16} $H_{12}O_5$] also known as physcion was further confirmed by comparison with that of already published data in table 14 of ^{13}C NMR. It is noted that the chemical shifts and melting points [(Literature 205-208 ˚C), (Inouye and Leister, 1988; Michiko and Koketsu, 1981)] were in close agreement.



55

Fig. 4 Proposed mass spectral fragmentation of compound 55.

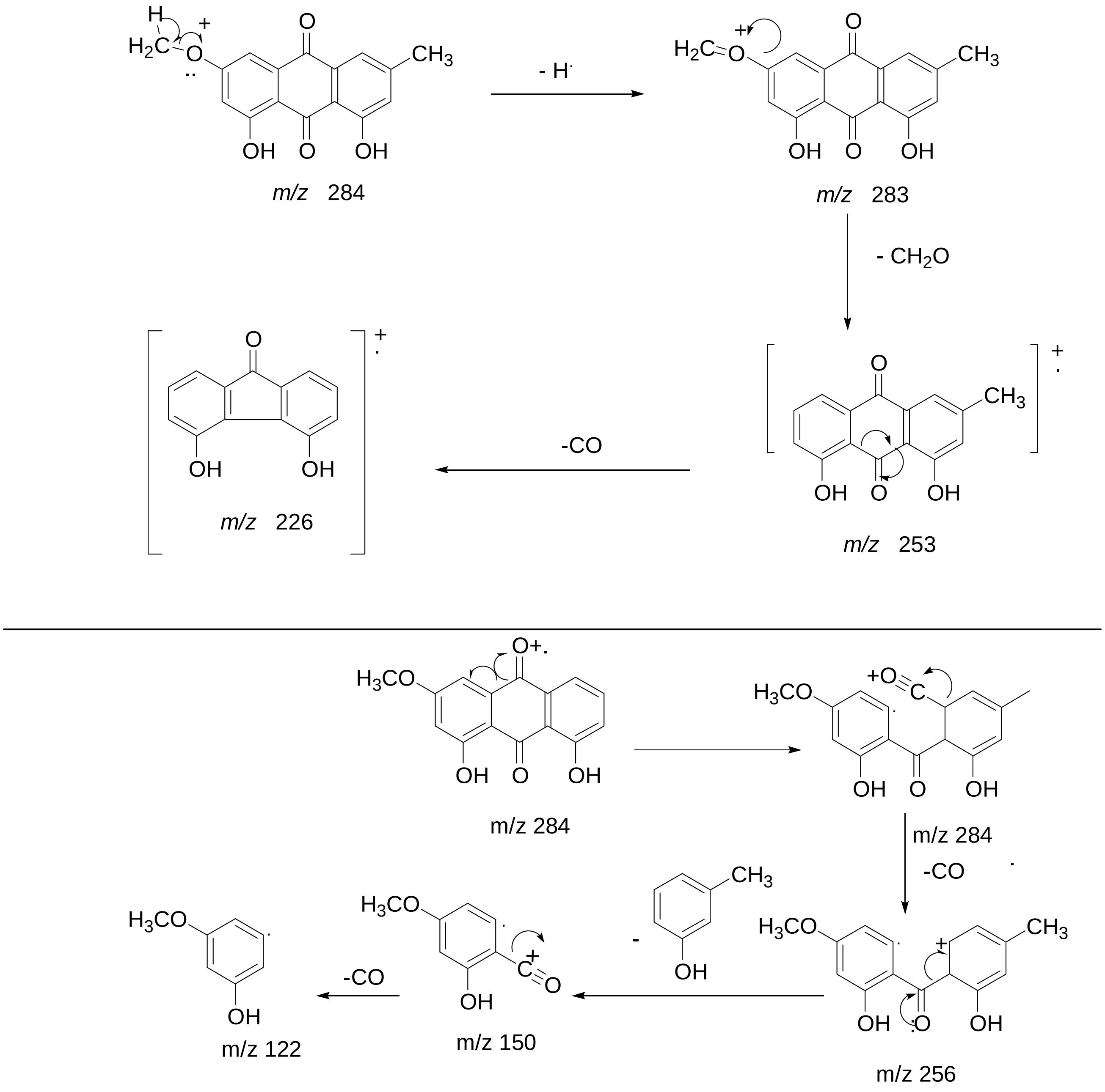


Table 13: ^{1}H-NMR (300MHz) in $CDCl_3$ of compound **55**.
(Melvyn and Morgan, 2001)

Proton	Chemical shift (Multiplicity, J Hz)	Integral	Physcion
1-OH	12.3 (s)	1H	12.33(s)
H-2	7.01 (s)	1H	7.15 (s)
3-CH_3	2.47 (s)	3H	2.38 (s)
H-4	7.69 (s)	1H	7.77 (s)
H-5	7.37 (d, 2.4)	1H	7.31 (d, 2.45Hz)
CH_3O-6	3.95 (s)	3H	3.96 (s)
H-7	6.69 (d, 2.4)	1H	6.70 (d, 2.45 Hz)
8-OH	12.12 (s)	1H	11.99 (s)

Table 14: ^{13}C NMR (75MHz) spectral data for compound **55** with that of authentic physcion (Stothers, 1972; Michiko and Koketsu, 1981).

Carbon	Compound **55**	DEPT	Physcion
C-1	162.5	C	162.7
C-2	113.6	CH	106.8
C-3	148.4	C	148.4
C-4	124.4	CH	124.2
C-4a	108.1	C	1081
C-5	135.2	CH	135.5
C-5a	165.2	C	165.2
C-6	106.7	C	106.8
C-7	166.5	CH	166.6
C-8	110.2	C	110.3
C-8a	133.2	C	133.3
C-9	190.8	C	191.0
C-9a	121.2	C	121.3
C-10	181.9	C	181.8
3-CH_3	22.1	CH_3	22.0
6-OCH_3	56.05	OCH_3	56.0

4.5.5 Antimicrobial results of isolated/purified compounds

Bioassay was carried out on the four isolated compounds (Table **15**). However, antifungal tests were not carried because of the small amount of the compounds.

Table 15: Antibacterial results of selected isolated/purified compounds

	Inhibition zones (mm ± S.D, n= 3)			
Compounds	***P. aeruginosa***	***B. subtillis***	***S. aureus***	***E. coli***
Stigmasterol [**52]**	10.4±1.0	9.3±0.7	10.8±1.2	8.5±0.5
β-Sitosterol [**53]**	10.8±1.2	8.7±1.3	8.0±1.0	9.5±1.5
Chrysophanol [**54]**	14.4±0.6	12.8±0.2	14.3±0.7	9.4±0.6
Physcion [**55]**	9.5±0.5	8.2±0.8	13.5±0.5	10.1±0.9
+ve (control) (Gentamicin)	16.7±1.3	14.3±1.7	20.2±0.8	20.2±1.8
Negative control	6.0±0.0	6.0±0.0	6.0±0.0	6.0±0.0

The four compounds exhibited mild to low antibacterial activity between Gram-positive and Gram-negative bacteria, the fact that the four compounds showed moderate activity against the four strains of bacteria is an indication that they are not potent.

From the resulting data, compound **54** with inhibition zones of 12.8 and 14.3 mm showed good activity against the Gram- positive bacteria; *B. subtilis* and *S. aureus*. It also showed good activity against the Gram-negative bacteria with the inhibition zone of 14.4 mm. On the other the hand, compound **55** exhibited a mild activity against the Gram-negative (*E. coli*) by exhibiting an inhibition zone of 10.1 mm.

When the measured zones were compared with that due to the standard drug (Gentamicin) it was seen that compound **54** and **55** were more active of the four tested compounds and this is evident by the results in table 15 above.

The Gram-negative bacteria are among the notorious pathogens found in the hospitals that have acquired resistance to several antibiotics in the past. Therefore the search for the bioactive metabolites that could enable the synthesis of drugs should be encouraged. The usage of the *Senna didymobotyra* in traditional medicine by different communities could be justified by the antibacterial activity observed so far.

4.6 Conclusion and recommendation

4.6.1 Conclusion

- This study has shown that *S. didymobotrya* root bark contain anthraquinone and triterpenoids, in addition to other compounds. Four compounds were isolated of which two triterpenes stigmasterol [**52**] and β-sitosterol [**53**], two anthraquinone chrysophanol [**54**] physcion **[55].**

- The hexane extract of *S. didymobotrya* displayed the highest toxicity against brine shrimp and was the most bioactive extract. The hexane and DCM crude extracts of *Senna didymobotrya* exhibited moderate activity towards Gram-positive bacteria and mild activity against Gram-negative.

- At concentrations of 750 and 1000 ppm, hexane and DCM extracts of *Senna didymobotrya*, respectively showed comparable adulticidal activity against bean weevil that compliments the positive control, actellic super. This support the traditional use of this plant material as grain protectant against destructive pests.

- The result presented in this study demonstrates a possible scientific rationale for the incorporation of the root bark of *Senna didymobotrya* into traditional medicine and methods of grain protection, and are likely to have been selected after empirical demonstration of their efficacy over long period of time.

4.6.2 Recommendations

1) The three plants that were studied are on high demand and this is due to their traditional uses. On this grounds, there is need to conserve these plants from extinction so that the future generations may use them. These can be achieved by sensitizing and encouraging farmers on the importance of preserving the existing plants and even planting more for potential application by farmers in different eco-regions.

2) The significant role played by trace compounds in the studied plants in overall biological activities of extracts need further study.

3) Standardized laboratory tests need to be undertaken to determine the seed viability, toxicological properties and residual effects of the plant materials against bean weevil.

4) The crude extracts showed good adulticidal activity and this may be due to synergistic effects of different compounds, hence there is need to extend the bioassay work on the this plants to include blending of the extracts. This might improve the activity of the biopesticide.

5) There is the need for more thorough investigation into such practices to facilitate their improvement and adoption for the control of stored product insect pest especially in the rural communities.

6) The cost of extraction and purification of pure compounds is high for resource poor farmers, thus the use of crude extracts developed into powdered dusts or appropriate fumigant may be recommended. However, conclusive recommendation of their use can only be made after exhaustive analysis of the effect of this crude on the quality of grain safety.

7) The isolated compounds from *Senna didymobotrya* showed broad biological activity ranging from antibacterial, antifungal and adulticidal hence, more work should be carried on both pharmacological and biological activities.

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