DETECTION OF SWEET POTATO VIRUSES IN WESTERN KENYA, DEVELOPMENT OF A MULTIPLEX PCR TECHNIQUE FOR SIMULTANEOUS DETECTION OF MAJOR VIRUSES AND EVALUATION OF MEDICINAL PLANTS FOR ANTIFUNGAL AND ANTIBACTERIAL ACTIVITIES AGAINST THE CROP PATHOGENS

By

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A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry.

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DECLARATION

I hereby declare that this thesis is my original work and has not been submitted for award a degree or any other award in any university. This thesis has been submitted for examination with the approval of my supervisors.

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DEDICATION

To my husband, Peter Owiti Opiyo and children Jacqueline, Dixon and Linda.

Abstract

Sweet potato is an important food crop worldwide since it is drought tolerant and acts as a famine relief crop. However, its production is limited by viral, fungal and bacterial infections. Lack of rapid and sensitive techniques for detection of infections inhibits their control. Use of synthetic chemicals to manage microbial infections causes is discouraged since they ar non-biodegradable. The aim of this study was to identify viruses infecting sweet potato in western Kenya, develop a multiplex PCR protocol for detection of major viruses of the crop, and evaluate efficacy of medicinal plants for antimicrobial activity against the crop pathogens. Symptomatic sweet potato vines obtained from farmers' fields were testes for ten viruses using NCM-ELISA. The mPCR protocol for detection of SPFMV and SPCSV was optimized through variation of test parameters under standard PCR conditions. Extracts from *Warburgia ugandensis, Elaeodendron schweinfurthianum* and *Terminalia brownii,* which are traditionally used to manage microbial infections, were fractionated using chromatographic methods to obtain pure compounds. Structures of isolates were determined using spectroscopic and physical methods. Extracts and isolates were tested for antimicrobial activity against *Alternaria spp, Aspergillus niger, Fusarium oxysporum, F. solanum, Rhizopus stolonifer* (fungi), *Ralstonia solanacearum* and *Streptomyces ipomoeae* (bacteria). Five viruses namely SPFMV, SPCSV, SPMMV, SPCFV and CMV were detected. Eighty nine percent of samples had viral infection with over 80% showing multiple infections. Occurrence of CMV in Kenya was recorded for the first time. An mPCR protocol was successfully developed that detects SPFMV and SPCSV. Phytochemical studies afforded 26 compounds including 7α-acetylugandensolide that was isolated from *W. Ugandensis* for the first time. All the methanol, ethyl acetate and n-hexane extracts of the three plants were active against one or more of the test organisms. Methanol extract of *W. ugandensis* exhibited significantly higher activity (24.50 mm) than positive controls against *R. stolonifer*. Eighteen out of the twenty six compounds isolated exhibited antimicrobial activity against one or more of the test pathogens at concentrations ≤200 µg/ml. Polygodial, warbuganal, mukaadial, ugandensidial, ugandensolide, deacetoxyugandensolide and muzigadial, exhibited low MIC (MIC ≤ 50 µg/ml) against one or more pathogens that was comparable to those exhibited by standard drugs. This study provided a quantitative assessment of viruses infecting sweet potato in western Kenya and a simplified mPCR protocol for routine rapid detection of SPFMV and SPCSV. Scientific proof for the efficacy of extracts of *W. ugandensis, E. schweifurthianum* and *T. brownii* as antimicrobial agents against sweet potato pathogens and perhaps other crop pathogens was also provided.

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LIST OF ABBREVIATIONS AND ACRONYMS

A niger	*Aspergellus niger*
Altern spp	*Alternaria species*
AP	Alkaline Phosphate
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
bp	Base pair
C NMR	Carbon Nuclear Magnetic Resonance
cDNA	Complementary deoxyribonucleic acid
CH_2Cl_2	Dichloromethane
CIP	International Potato Centre
CMV	*Cucumber Mosaic Virus*
COSY	Correlation spectroscopy
C.V.	Coefficient of variation
d	Doublet
dd	Doublet of doublet
DEPT	Distortionless Enhancement of Polarization Transfer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2', 3'- Deoxyribonucleoside-5'- phosphate
EIMS	Electron Ionization Mass Spectroscopy
ELISA	Enzyme-Linked Immunosorbent Assay
ESI-MS	Electrospray Impact Mass Spectroscopy
EtOAc	Ethyl acetate
EtOAc	Ethyl acetate
F. oxy	*Fusarium oxysporum*
F. sol	*Fusafium solani*
FAO	Food Agriculture Organization
NMR	Nuclear Magnetic Resonance

^{1}H NMR	Proton Nuclear Magnetic Resonance
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
Hz	Hertz
IR	Infrared Spectroscopy
J	Coupling constant
L.S.D.	Least significant difference
m	Multiplet
m/z	Mass to charge ratio
M^{+}	Molecular ion
MeOH	Methanol
MeOH	Methanol
MHz	Mega Hertz
MIC	Minimum Inhibitory Concentration
MS	Mass Spectroscopy
NBT	Nitroblue tetrazolium
n-Hex	n-Hexane
NMR	Nnuclear Magnetic Resonance
NOESY	Nuclear Overhauser Effect Spectroscopy
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
q	Quartet
R_f	Rate of flow
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
s	Singlet
SPCaLV	*Sweet Potato Caulimo-Like Virus*
SPCFV	*Sweet Potato Chlorotic Fleck Virus*

SPCSV	*Sweet Potato Chlorotic Stunt Virus*
SPFMV	*Sweet Potato Feathery Mottle Virus*
SPLCV	*Sweet Potato Leaf Curl Virus*
SPMMV	*Sweet Potato Mild Mottle Virus*
SPMSV	*Sweet Potato Mild Speckling Virus*
SPVD	Sweet Potato Virus Disease
SPVG	*Sweet Potato Virus* G
SwPLV	*Sweet Potato Latent Virus*
t	Triplet
Taq	*Thermophilus acquaticus*
TBS	Tris buffered saline
T-TBS	Tris Buffered Saline supplemented with Tween-20
TLC	Thin Layer Chromatography
UV	Ultraviolet spectroscopy
δ	Chemical shift
ν_{max}	Maximum wavelength of absorption

CHAPTER ONE

1.0 INTRODUCTION

1.1 Sweet potato

Sweet potato (*Ipomoea batatas* L.) is one of mankind's highest yielding crops with higher food value and total production per unit area than other staple crops such as maize, rice, sorghum and millet (FAO, 2002). Worldwide, the importance of sweet potato is exceeded only by cereals (wheat, maize, rice and barley) and potato (*Solanum tuberosum*) (Carey *et al*., 1997; FAO, 2002). Although the crop is grown in more than 100 countries around the globe, about 90% is produced in Asia with just below 5% in Africa (Woolfe, 1992). Sweet potato serves as a staple diet in many parts of Africa (Carey *et al*., 1996). China is the world's highest producer with annual harvest of 100 million tones followed by Uganda, Nigeria, Indonesia and Vietnam (FAO, 2002).

Sweet potato is adaptable to a wide range of agro-ecological conditions and performs well in low - input agriculture (CIP, 1996). The production of the crop in Africa is concentrated in East Africa around the Great Lake regions (Gibson *et al*., 1997). The presence and the adaptation of sweet potato to the tropical areas where *per capita* incomes are generally low and its nutritional value make the crop an important component in food production and consumption. Sweet potato is an important food security and famine relief crop during seasons of crop failure (CIP, 1998). The production of the crop can be staggered, while harvesting can be done in piecemeal thereby ensuring continuous source of food for farm families.

In the last decade, the importance of sweet potato increased greatly in many African regions due to frequent droughts and prevalence of pests and diseases which adversely affect the production of staple food crops such as maize, cassava and banana (Thottappilly *et al.*, 1993; Otim-Nape *et al.*, 2000; Tushemereirwe *et al.*, 2004). Kenya is the seventh largest African sweet potato producer with average yield of 8.2 tones /ha against a potential of 50 tons/ha (FAO, 2002; 2003). With the increasing population and diminishing arable land per house hold, there is a need to increase sweet potato yields to meet the demand for the crop. The main sweet potato producing regions of Kenya are western, eastern, central and coastal areas (MOA, 1999).

Despite its high potential for food security, production of sweet potato is constrained by pests and diseases (Carey *et al*., 1997; Karyeija *et al.,* 1998; Gibson and Aritua, 2002; Aritua *et al.,* 2007). Several viruses (Is Hak *et al*., 2003; Mukasa *et al.,* 2003; Ateka *et al.,* 2004; Tairo *et al.,* 2004; Miano *et al*., 2006), fungi and bacteria (Lenne, 1991; Skuglund *et al.,*1990; Clark and Hoy, 1994; Onuegbu, 2002; Aritua *et al.,* 2007; Osiru *et al.,* 2007) infect the crop. *Sweet potato feathery mottle virus* (SPFMV) is the most widespread in many parts of the world (Moyer and Salazar, 1989; Nishiguchi *et al*., 1995; Sakai *et al.,* 1997). When infecting alone, SPFMV seem insignificant in its effects on sweet potato (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). A greater damage is realized when SPFMV infects sweet potato in the presence of *Sweet potato chlorotic stunt virus* (SPCSV), leading to sweet potato virus disease (SPVD), the most harmful disease of the crop (Geddes, 1990; Gibson *et al.,* 1998; Gibson and Aritua, 2002). Some of the fungi and bacteria associated with pathogenesis of sweet potato include *Macrophomina phaseoli, Diplodia gossypina, Monilochaetes infuscans, Alternaria spp, Aspergellus niger, Fusarium oxysporum, F. solanum, Rhizopus stolonifer* (Fungi), *Ralstonia solanacearum, Streptomyces scabies, S. ipomoea, Erwinia carotovora and E. chrysanthemi* (bacteria) (Clark and Moyer, 1988; Skuglund *et al.,* 1990; Lenne, 1991; Ristaino, 1993; Clark and Hoy, 1994; Clark *et al.,* 1998). Apart from reducing the yield, these infections cause rotting of sweet potato in the field, during storage and transportation, making the produce unpalatable (Person and Martin 1940; Clark and Moyer, 1988; Snowdown, 1991, Clark and Hoy, 1994; Clark *et al.,* 1998). Some of the symptoms exhibited by sweet potato infections are shown in Fig. 1 nd 2.

Lake Victoria Crescent has higher infection levels due to favourable climatic conditions for the pathogens infection and disease development (Lenne, 1991; Skuglund *et al.,* 1990; Aritua *et al.,* 2007; Osiru *et al.,* 2007). However, information on viruses infecting sweet potato in western Kenya is scanty.

Viral infections are mainly managed by prevention and controlling their spread (Aritua *et al.,* 1998; Gibson and Aritua, 2002; Ghosh and Aglave, 2007). Farmers use cultural practices such as crop rotation, removal and destruction of infected plants as soon as they are detected (Dent, 1995). Farmers also select relatively healthy vines as the parent of the next crop as a control measure (Aritua *et al.,* 1998; Gibson and Aritua, 2002). Such practices have not been effective due to absence of visible symptoms (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). Since in most, cases viral infections co-exist, rapid and effective methods of multiple virus detection

will enable selection of 'clean' planting materials. A number of enzyme-linked immunosorbent assays (ELISA) protocols have are uses for virus detection. However, the protocols are not applicable unless antibodies specific to the virus being tested is available. Polymerase chain reaction (PCR) technique is an alternative rapid method for virus detection. Several multiplex PCR protocols have been developed for virus detection in other crops such as citrus and pepper but there is none for detection of sweet potato viruses.

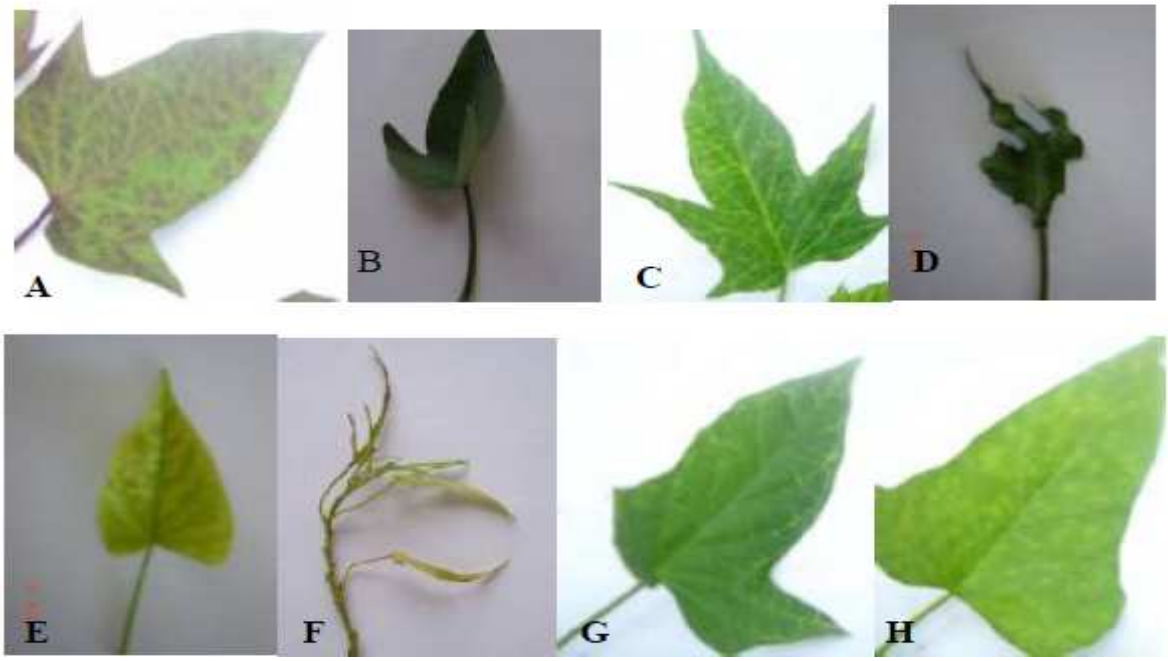


Fig. 1: Some virus symptoms observed on sweet potato plants (A) purpling of leaves in plants infected with SPCSV, (B) symptomless leaves of plants infected with SPFMV, (C) vein clearing in leaves of plants infected with SPMMV, (D) deformed leaves of plants infected with SPFMV and SPCSV, (E) interveinal chlorosis in leaves of plants infected with SPMMV, (F) chlorotic, small deformed leaves in plants infected with SPFMV and SPCSV, (G) severe symptoms in plants infected with SPFMV, SPCSV, SPMMV and SPCFV, (H) chlorotic spots on leaves of plants infected with SPFMV and SPMMV (Adapted from Nyaboga *et al.*, 2008).

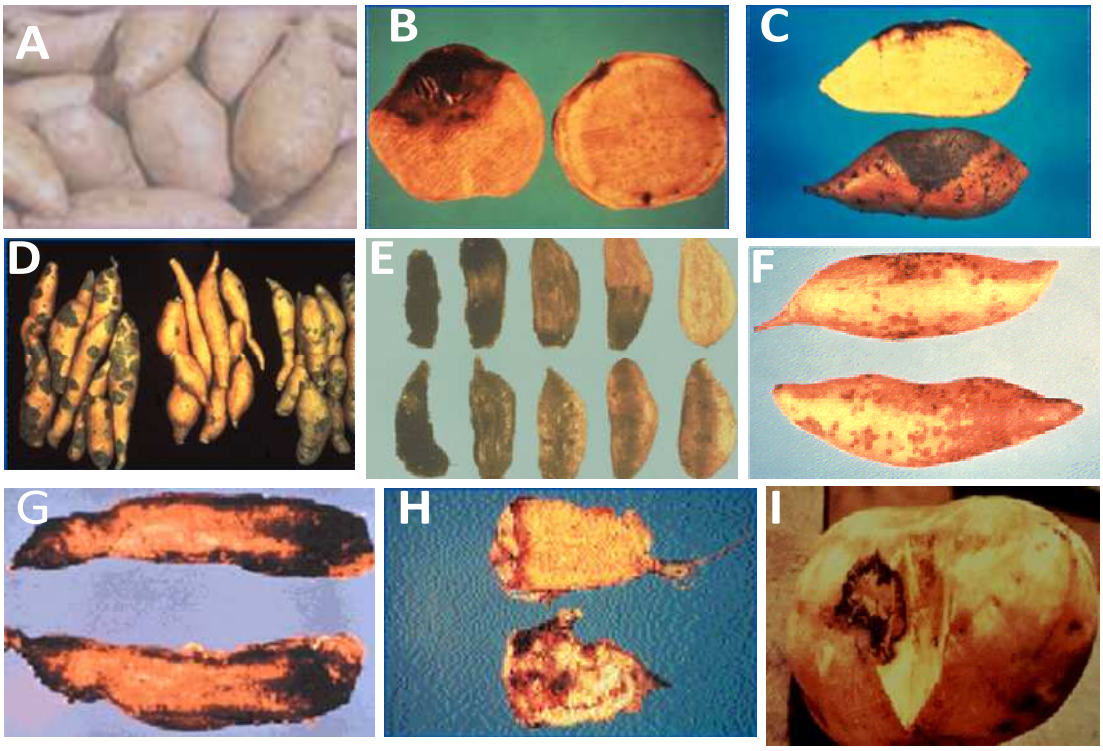


Fig. 2: Some of the symptoms exhibited by microbial infections in sweet potato; (**A**) Healthy roots, (**B**) Cross section of root with symptoms of Fusarium root rot (left) and Fusarium surface rot (right), (**C**) Internal and external views of roots infected with black rot (*Ceratocystis fimbriata*), (**D**) Roots with black rot lesions (left and right) and un-infected roots (center), (**E**) Internal and external views of roots with Java black, rot (*Diplodia gossypina*) at different stages of development (**F**) Sweet potatoes with symptoms of scurf (*Monilochaetes infuscans*), (**G**) Internal views of root with charcoal rot (*Macrophomina phaseoli*), (**H**) Internal and external views of roots infected with Rhizopus soft rot (*R. stolonifer*) and (**I**) Sweet potato with symptoms of bacterial soft rot (*Erwinia chrysanthami*) (Adapted from Sikaro, 1995).

The use of synthetic chemicals to manage infections in crops is discouraged since they are not easily biodegradable therefore accumulate in the environment and are gradually absorbed into the food chain (Khanna *et al.,* 1989). The use of biodegradable agrochemicals from natural origin is an attractive possibility. A biological method of control is preferred in most cases because it is selective with no side effect and it is usually cheap compared to the conventional synthetic chemicals. Resistance to biological control is rare and biological control agents are self-propagating and self-perpetuating (Okigbo and Ikediugwu, 2000; Okigbo, 2003, 2004, 2005). Extracts from medicinal plants are traditionally known to control diseases in plants and tuber crops (Sivropou *et al.,* 1997; Kim *et al.,* 1998; Perez *et al.,* 1999; Amadioha, 2003; Okigbo and Emoghene, 2004; Okigbo and Nmeka, 2005). Extracts of *Warburgia ugandensis, Terminalia brownii* and *Elaeodendron schweinfurthianum* are used in ethnomedicine to manage viral, fungal and bacterial infections (Kokwaro, 2009; Wilson and Woldo, 1979; Heine and Brenzinger, 1988). Phytochemical studies of *Warburgia, Terminalia and Elaendendron* species have lead to isolation of different compounds some of which have antimicrobial activities. Scientic proof for the medicinal claims is lacking It is necessary to evaluate the extracts of these plants for their possible use in managing infections of sweet potato.

1.2 Statement of the problem

Sweet potato is an important crop which acts as food security and famine relief crop. However, its production is constrained by viral, fungal and bacterial infections. Information on identity and distribution of viruses infecting in western Kenya is lacking. Effective methods of detecting multiple virus infection are lacking. Effective methods of managing the fungal and bacterial infections are lacking.

1.3 Objectives of the study

The aim of this study was to identify the viruses infecting ordinary sweet potato grown by rural folk in western Kenya, develop a protocol for simultaneous detection of major viral infections and investigate the efficacy of some tradidionally used medicinal plant extracts in the management of fungal and bacterial infections of the crop.

The specific objectives were to:-

i. Determine the identity and distribution of viruses which infect sweet potato in western Kenya.
ii. Develop a mPCR protocol for the simultaneous detection of SPFMV and SPCSV, the most economically important viruses of sweet potato.
iii. Isolate and characterize the chemical constituents of *W. ugandensis, E. schweifurthianum* and *T. brownii*.
iv. Evaluate the antimicrobial activity of *Warburgia ugandensis, Elaeodendron schweifurthianum* and *Terminalia brownii* extracts against fungi and bacteria which infect sweet potato.
v. Determine the antimicrobial principles from the plants.

1.4 Null hypotheses

i. No more viruses rather than the reported ones infect sweet potato in western Kenya with possible multiple interactions.
ii. Multiplex PCR-based technique may not enable simultaneous detection of several viruses.
iii. Medicinal plant extracts do not contain secondary metabolites which exhibit antimicrobial activity against fungi and bacteria that infect sweet potato.

1.5 Justification of the study

Sweet potato is important root crop in developing countries since it is inexpensive and rich source of carbohydrates, vitamins and essential minerals. It serves as an important source of quality nourishment to the urban and rural people who live below the poverty line. Increased cultivation of the crop is being encouraged in Africa, where vitamin A deficiency is a serious health problem (http://en.wikipedia.org/wiki/Sweet_potato 5th June, 2010). However, the production of sweet potato is limited by viral, fungal and bacterial infections (Carey *et al*., 1997). Losses induced by these infections range from 1% to 100% (Gibson *et al.,* 1998; FAO, 2002; Gutierrez *et al*., 2003). Information on the identity and distribution of sweet potato viruses in western Kenya, which is the main sweet potato producing region in the country, is scanty.

Since the control of viral diseases is largely preventive, reliable and rapid methods of detection and diagnosis are neccessary. Enzyme linked immunosorbent assay (ELISA) such as nitrocellulose membrane (NCM)-ELISA, double antibody sandwich (DAS)-ELISA and triple antibody sandwich (TAS)-ELISA have been used routinely for virus detection. However, the method is not applicable unless the antibody specific to the virus studied is available. Since the viruses co-infect sweet potato leading to more severe infections (Mukasa *et al.,* 2003; Ateka *et al.,* 2004; Tairo *et al.,* 2004), a technique that allows simultaneous detection is highly called for. There are reports of multiplex polymerase chain reaction (mPCR) systems for the detection of two or even more plant viruses infecting crop plants (Ghosh and Aglave, 2007). However such system for sweet potato has not been developed.

Synthetic chemicals have been used to control infections in crops (Clark and Moyer, 1988). However, the use of such chemicals apart from their potential danger to both human health and environment (Cameron and Julian, 1984; Obagwu *et al.,* 1997; Osman and Al-Rehiayam, 2003; Masuduzzaman *et al.,* 2008; Siva *et al.,* 2008), are unaffordable for most farmers. Moreover, resistance by pathogens to such chemicals has rendered some of them ineffective (Cameron and Julian 1984; Zhonghua and Michailides, 2005). In order to fully exploit the potential of the sweet potato crop, there is a need to search for affordable, readily available, sustainable and environmentally friendly means of managing the problems posed by these pathogens.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution of sweet potato

Sweet potato (*Ipomoea batatas* Lam) originated from Central and South America, probably in the region between the Yucatan Peninsula of Mexico and the mouth of Orinoco River in Venezuela (Onwueme, 1978). Its dissemination to Polynesia is associated with voyages of early Peruvian or Polynesian explorers and traders. The crop was brought to Europe much later, in the 14th century by Christopher Columbus, a Portuguese explorer, on his return journey from South America. By the16th Century, the crop reached Africa, India and Eastern Asia through the Portuguese traders and explorers (Onwueme, 1978).

2.2 Importance of sweet potato

Staple crops such as maize, rice, sorghum and millet do not match the yield per unit area of sweet potato that also has higher food value (FAO, 2002). On a worldwide scale, the importance of sweet potato is exceeded only by cereals (wheat, maize, rice and barley) and potato (*Solanum tuberosum*) (Carey *et al*., 1997; FAO, 2002). Although the crop is grown in more than 100 countries around the globe, about 90% is produced in Asia with just below 5% in Africa (Woolfe, 1992). About 75% of African sweet potato production is concentrated in East Africa, especially around Lake Victoria, where it is a basic subsistence crop, grown by rural women near their homes to feed their families (Kapinga *et al.*, 1995; Gibson *et al.*, 1997; Gibson and Aritua, 2002). The crop is dependable since it is drought tolerant and acts as food security and famine relief crop during periods of crop failures (Karyeija *et al,* 1998). The presence and the adaptation of sweet potato to the tropical areas where *per capita* incomes are generally low and its nutritional value make the crop an important component in food production and consumption. The importance of sweet potato increased greatly in many African regions due to frequent droughts and prevalence of pests and diseases which adversely affect the production of staple food crops (Thottappilly *et al.*, 1993; Otim-Nape *et al.*, 2000; Tushemereirwe *et al.*, 2004).

Tuberous storage roots of sweet potato are generally eaten while steamed, baked, fried or boiled (Carey *et al.*, 1997). The crop is one of the most nutritionally complete foods, especially useful in vegetarian dishes (www.recipes4us.co.uk, 12th July, 2010). Besides simple starches, sweet potato is rich in carbohydrates, dietary fiber, beta carotene (a vitamin A equivalent nutrient), vitamin C, and vitamin B_6 (www.recipes4us.co.uk, 12th July, 2010). Important minerals found in the crop include calcium, phosphorous, iron, sodium and potassium (Woolfe, 1992). Young sweet potato leaves are also used as nutritious vegetables and are rich sources of vitamin A, B_2, iron and protein (Woolfe, 1992). Its vines are good dairy animal feed supplements because of the high protein content and are easily digestible (www.recipes4us.co.uk,11th, 12th July, 2010; www.cipotato.org/sasha/07, 11th August, 2010). Sweet potato is a very popular item of local and regional trade especially in East and Central Africa regions (CIP, 1996; 1998). It is also a potential source of raw material for industries which produce snacks, starch, flour, crisps dyes, ethanol and spirits (CIP, 1996; 1998). Due to the numerous uses of sweet potato, there is a need to improve its production in order to meet its demand.

2.3 Constraints to sweet potato production

Sweet potato withstands adverse climatic conditions better than most agricultural crops. However, a wide range of pathogens such as fungi, bacteria, nematodes and viruses infect the crop (Moyer and Salazar, 1989; Geddes, 1990; CIP, 1995; Carey *et al*, 1997). Apart from reducing photosynthetic area and transport of nutrients, these diseases also cause rotting of tubers both in fields and storage hence causing significant losses (Skoglund and Smit, 1994; Carey, 1996; Ristaino, 1993; Clark and Hoy, 1994; Clark and Moyer, 1988; Clark *et al.*, 1998). The persistence of high incidences of infections on sweet potato is attributed to use of infected planting materials. The vegetative propagation, usually done using cuttings from a previous crop to establish a new crop also increases the risk of build-up of the pathogens (Karyeija *et al.*, 1998). Search for effective method for managing the infections is necessary.

2.3.1 Virus infection in sweet potato

Among the biological factors, virus diseases rank second to weevil in causing yield reduction in sweet potato (Karyeija *et al.,* 1998; Gibson and Aritua, 2002). Several viruses have been reported infecting sweet potato including *Sweet potato feathery mottle virus* (SPFMV), *Sweet potato chlorotic stunt virus* (SPCSV), *Sweet potato mild mottle virus* (SPMMV), *Sweet potato chlorotic fleck virus* (SPCFV), Sweet *potato latent virus* (SPLV), Sweet potato caulimo-like virus (SPCa-LV), *Cucumber mosaic virus* (CMV), Sweet potato virus Y (SPVY), C-6, Sweet potato virus G (SPVG), *Sweet potato mild speckling virus* (SPMSV) and *Sweet potato leaf curl virus* (SPLCV) (Is Hak *et al*., 2003; Mukasa *et al.,* 2003; Ateka *et al.,* 2004; Tairo *et al.,* 2004; Miano *et al*., 2006). SPFMV, SPCSV, SPMMV, SPCFV and SPLCV have been detected in some parts of East Africa (Miano *et al*., 2006). However, there is little information on the distribution of sweet potato viruses in western Kenya, which is the main sweet potato producing region in the country.

Sweet potato feathery mottle virus (SPFMV) is the most prevalent and widespread in many parts of the world where sweet potato is cultivated (Moyer and Salazar, 1989; Nishiguchi *et al*., 1995; Sakai *et al.,* 1997). When infecting alone, SPFMV seem insignificant in its effects on sweet potato (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). A greater economic impact is realized when SPFMV infects sweet potato in the presence of *Sweet potato chlorotic stunt virus* (SPCSV), leading to sweet potato virus disease (SPVD), the most harmful disease of the crop in Africa and elsewhere (Geddes, 1990; Gibson *et al.,* 1998; Gibson and Aritua, 2002). In preveous studies (Ateka, 2004; Ateka *et al.,* 2004; Miano *et al.,* 2006; Nyaboga *et al.,* 2008), apparently symptomatic plants tested negative in NCM-ELISA indicating that there are viruses in sweet potato not yet identified. There is need for further tests to identify the unknown infections.

2.3.2 Fungal and bacterial infection in sweet potato

Several fungi are reported to be associated with reduction of yield and rotting of sweet potato including *Monilochaetes infuscans, Fusarium oxysporum, Ceratocysts fimbriata, Rhizopus stolonifer, Macrophomina phaseolina, F. solani* and *Botryodiplodia theobromae Alternaria spp. Penicillium* spp, *Certocystis fimbriata, Diaporthe batatalis, Aspergillus niger and A. flavus* have been implicated as fungi responsible for decay of Sweet potato roots (Clark and Hoy, 1994; Onuegbu, 2002). Fungi associated with post harvest fungal rots to include *Mortierella ramanniana, Rhizopus stolonifer, Mucor pusillus, Botrytis cinerea, Erysiphe polygoni* and *A. flavus* (Oyewale, 2006).

Bacteria that cause infection in sweet potato include *Agrobacterium tumefaciens, Erwinia carotovora, E. chrisanthemi, Streptomyces scabies, S. acidiscabies* and *Ralstonia salanacearum* (Clark and Moyer, 1988; Ristaino, 1993; Clark and Hoy, 1994; Barton *et al.*, 1997; Lemanga *et al.*, 1997; Clark *et al.*, 1998). These pathogens invade the host through wounds and cracks and sources of inocula include soil, infected mother plants or contaminated water and harvesting equipments (Sikaro, 1995). The infections create local discolouration and disruption of surrounding tissues of infected tubers (Snowdon, 1991), resulting in changes in appearance, deterioration of texture and flavor, hence reduction in the market value and misfortune to farmers (Person and Martin, 1940; Clark and Moyer, 1988; Clark *et al.*, 1998). Search for effective methods of cotroling these fungal and bacterial infections is necessary.

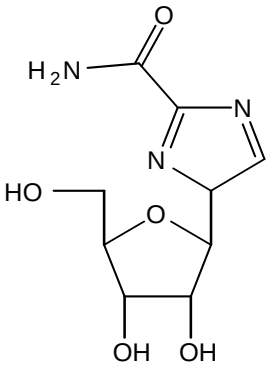
2.3.3 Management of sweet potato infections

Viral infections in plants are generally managed by prevention and controlling their spread (Aritua *et al.*, 1998; Gibson and Aritua, 2002; Ghosh and Aglave, 2007). Cultural practices such as crop rotation, removal and destruction of plants as soon as they become infected and eradication of sources of infection and inoculum are used to control the spread of sweet potato diseases (Dent, 1995). Farmers also select relatively healthy vines as the parent of the next crop as a control measure (Aritua *et al.*, 1998; Gibson and Aritua, 2002). These cultural methods of managing the crop infections have been ineffective since some infections do not exhibit visible symptoms, making it impossible to select safe seeds or to employ cultural

methods of management. Availability of fast and effective methods of virus detection may enable safe selection of clean planting materials and effective control of spread of the viruses.

While ELISA is used routinely for virus detection, the method is not applicable unless the antibody for the virus studied is available. Secondly, the ELISA technique is only sensitive during dry seasons of the year when the viruses concentrations in plant are high (Ghosh and Aglave, 2007). Polymerase chain reaction (PCR) is an alternative rapid virus detection method. Several singlex PCR (sPCR) – based methods have been reported for a number of sweet potato viruses (Nishiguchi *et al.,* 1995; Ki and Sun, 2002; Kokkinos and Clark, 2006). Since sweet potato infecting viruses in East Africa mostly occur as multiple infections (Gibson *et al*., 1998; Karyeija *et al*., 2000; Mukasa *et al*., 2003), availability of multiplex virus detection protocols could lower the time and cost of detection. There are reports of multiplex PCR (mPCR) systems for the detection of two or even more plant viruses infecting crop plants including rice, citrus, pepper, grapes potato and sugar beets (Manifra and Hadidi, 1994; Nie and Singh, 2000; Bertolini *et al.,* 2001; Meunier *et al.,* 2003; Periasamy *et al.,* 2006; Bhat and Siju, 2007; Ghosh and Aglave, 2007). However, there is no such system available for sweet potato.

Synthetic chemicals such as virazole [**1**], pyrazofurin [**2**], azaguanine [**3]**, carbaryl [**4]**, dichloronitroaniline [**5]** and blitox (copper oxychloride) [**6]** are used to manage plant infections (Lozoya *et al.,* 1984; Clark and Moyer, 1988). Such chemicals pose adverse effects on ecosystems and are possible carcinogenic risks (Cameron and Julian, 1984; Osman and Al-Rehiayam, 2003; Masuduzzaman *et al.,* 2008; Siva *et al.,* 2008). Some of these chemicals are not easily biodegradable therefore they accumulate in the environment and are gradually absorbed into the food chain (Khanna *et al.,* 1989). Furthermore, the synthetic chemicals are costly and are out of reach of most subsistence farmers (Obagwu *et al.,* 1997; Amienyo and Ataga, 2007). It is necessary to search for affordable environmentally friendly antimicrobial agents.



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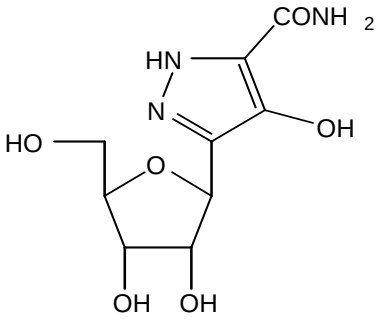

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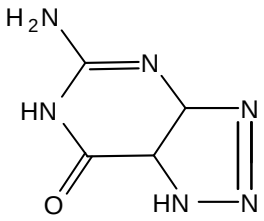

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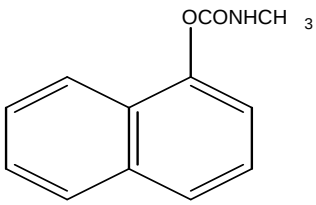

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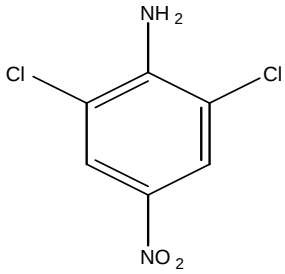


5

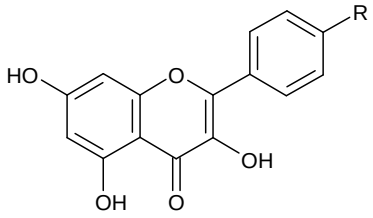
$3Cu(OH)_2.CuCl_2$

6

2.4 The use of plant extracts in disease management

The use of medicinal plants in managing diseases is widespread (Farnsworth, 1994). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Other than for the purposes of scientific inquiry, plants have served as models in drug development for three reasons. First, each plant is a unique chemical factory capable of synthesizing a large number of highly complex and unusual chemical substances. In United States of America, about 25 % of prescription drugs contain active principles that are still sourced from higher plants and there is increasing popularity in the use of plant-derived prescriptions (Farnsworth and Morris, 1976). The World Health Organization (WHO) estimates that about 80% of the population of the developing countries relies exclusively on plants to meet their healthcare needs (Farnswoth *et al.,* 1986). Secondly, the biologically active substances derived from plants have served as templates for synthesis of pharmaceutical formulations. Such compounds may have poor pharmaceutical and toxicological profiles that may be improved through derivatization. Thirdly, many highly active secondary metabolites from plants have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984).

In Nigeria, plant extracts have been used to control fungal diseases in cowpea (Amadioha, 2003), banana (Okigbo and Emoghene, 2004) and yam (Okigbo and Nmeka, 2005). Galangin [**7**] isolated from *Helichrysum aureonitens* showed antiviral activity against HSV-1 and Cox B1 (Meyer *et al.*, 1997), while quercetin [**8**] which is widely distributed in the plant kingdom exhibited activity against HIV (Kim *et al*., 1998; www.herbalextractsplus.com/ quercetin.cfm, 14th July, 2010; www.florahealth.com/flora/home /Canada/HealthInformation, 10th August, 2010). A triterpene lactone, lancilactone C [**9**] isolated from stems and roots of *Kadsura lancilimba* inhibited HIV replication with an EC_{50} value of 1.4 mg/ml (Chen *et al.*, 1999). Essential oil extracted from aerial part of *Salvia fructiosa* exhibited virucidal activity against Herpes Simplex Virus 1 and the active principles were found to be 1, 8-cineole [**10**], thujone [**11**] and camphor [**12**] (Sivropou *et al*., 1997; Deans and Waterman, 1993).



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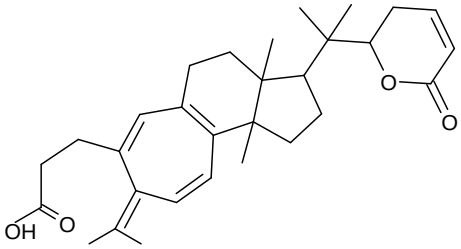
8 R = OH



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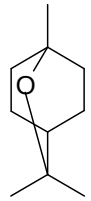

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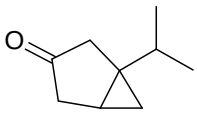

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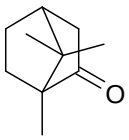


12

The essential oil of *Melaleuca alternifolia* was effective in decreasing local lesions of *tobacco mosaic virus* (TMV) in *Nicotiana glutinosa* (Bishop, 1995). Extracts of *Thuja occidentallis* and *Psidium guajava* inhibited the growth of *bean common masaic virus* (BCMV) (Prasad *et al.*, 2007) while the growth *tomato mosaic virus* in tomato (ToMV) was checked by extracts from decomposed tomato plants (Avgelis and Morios, 1989). Essential oils from *Ocimum sanctum* and *Peperomia pellucida* have inhibitory activity against *cowpea mosaic virus* (CPMV), *mung bean mosaic virus* (MBMV), *bean commonil mosaic virus* (BCMV) and *southern bean mosaic virus* (SBMV). *Ocimum sanctum* crude extracts concentration of 3,000 ppm yielded inhibition of 89.6, 90, 92.7, 88.2% against CPMV, MBMV, BCMV, and SBMV,

respectively. Other reports showed 62% inhibition against *tobacco mosaic virus* (TMV) by essential oil from fresh carrot leaves (Khanna. *et al.*, 1989) while *Tagetes minuta* oil was active against *carnation ring spot* (CaRSV) and *carnation vein mottle viruses* (CaVMV) (Khanna *et al.*, 1989).

Seed extracts of *Moringa oleifera* exhibited antimicrobial activity against soil pathogens namely *Pasturella multocida, Escherichia coli, Bacillus subtilis, Staphylocuccus aureus, Fusarium solani* and *Rhizopus solani* (Jabeen *et al*., 2008). Water extracts of *Alchornea cordifolia, Annona muricata, Allium sativum, Gacinia cola* and *Zingiber officinale* exhibited antifungal activity against *Aspergillus flavus, A. niger, Botryodiplodia theobromae, Fusarium solani, Fusarium oxysporum* and *Rhizopus stolonifer* which cause disease in sweet potato (Amienyo and Ataga, 2007). In another study (Mekbib *et al.*, 2007), crude extracts of medicinal plants including *Achyranthus aspera, Tribulus terrestris, Withania somnifera, Acacia seyal, Dolichos oliver, Cissus quadrangularis* and *Mirabilis jalapa* exhibited antimicrobial activity against plant and food-borne pathogens namely *Shigella sonnei, Staphylococcus epidermidis, S. faecalis, Salmonella typhimurium, Pseudomonas syringae, Ralstonia solanacearum, Xanthomonas campestris, Penicillium digitum, Geotrichum candidum and Phytophthora nicotianae.* Crude extracts of *Ocimum gratissimum* and *Aframomum melegueta* were active against fungal pathogen namely *Aspergillus niger, A. flavus, Fusarium oxsporium Rhizopus stolonifer, Botryodiplodia theobromae* and *Penicillium chrysogenum* which cause soft rot of yam tuber (Okigbo and Ogbonnaya, 2006). The need to investigate the antifungal and antibacterial activities of medicinal plant extracts against sweet potato pathogens is highly called for.

2.5 Plants and antimicrobial production

Plants have several ways of generating antimicrobial compounds to protect them against pathogens (Kuc, 1990). External plant surfaces are often protected by biopolymers e.g. waxes and fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, steroid glycoalkaloids and other compounds, which inhibit the development of fungi and bacteria (Kuc, 1985). Cell walls of some monocotyledons contain antimicrobial protein, referred to as thionins (Carr and Klessig, 1989).

Plant cells containing sequestered glycosides release them when ruptured by injury or infection. These glycosides may have antimicrobial activity against the invading pathogens or may be hydrolyzed by glycosidases to yield more active aglycones. In the case of phenolic compounds, these may be oxidized to highly reactive, antimicrobial quinone and free radicals (Kuc, 1985; Dean and Kuc, 1987). Thus, damage to a few cells may rapidly create an extremely hostile environment for a developing pathogen. This rapid, but restricted disruption of a few cells after infection can also result in the biosynthesis and accumulation of phytoalexins at sites of infections (Kuc, 1985; Carr and Klessig, 1989; Dean and Kuc, 1987). Phytoalexins are degraded by some pathogens and by the plant, thus they are transient constituents and their accumulation is a reflection of both biosynthesis and degradation (Kuc, 1985). Biopolymers including lignin, callose and suberin are often associated with the phytoalexin accumulation at the site of injury or infection. These provide both mechanical and chemical restriction of development of pathogens (Kuc, 1985; Carr and Klessig, 1989; Rao and Kuc, 1990). The macromolecules produced after infection or certain forms of physiological stress include enzymes which can hydrolyze the walls of some pathogens including chitinases and proteases (Carr and Klessig, 1989). Unlike the phytoalexins and structural biopolymers, the amounts of these enzymes increase systematically in infected plants even in response to localized infection. These enzymes are part of a group of stress or infection-related proteins commonly referred to as pathogenesis-related (PR) proteins. The function of many of these proteins is unknown. Some may be defense compounds while others may regulate the response to infection (Carr and Klessig, 1989; Boller, 1987; Rao and Kuc, 1990).

Another group of systematically produced biopolymer defense compounds comprises the peroxidases and phenoloxidases (Hammerschmidt *et al.*, 1982, Rao and Kuc, 1990). Both can oxidize phenols to generate protective barriers to infection, including lignin. Phenolic oxidation products can also cross-link to carbohydrates and proteins in the cell walls of the plants and fungi to restrict further microbial development (Stermer and Hammerschmidt, 1987). Peroxidases also generate hydrogen peroxide, which is strongly antimicrobial. Associated with peroxidative reactions after infection is the transient localized accumulation of hydroxyl radicals and super oxide anion, both of which are highly reactive and toxic to cells (Hammerschmidt *et al.*, 1982, Rao and Kuc, 1990).

Plants therefore have several mechanisms to counter antimicrobial attack. Some of the antimicrobial compounds in plants may be exploited for use against fungal and bacterial diseases. Plants have developed an arsenal of weapons to survive attacks by microbial invasions. These include physical barriers as well as chemical ones, i.e. the presence or accumulation of antimicrobial metabolites. These are either produced in plant (prohibitins) or are induced after infection, the so-called phytoalexins. Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors (Grayer *et al.*, 1994).

When an infection or damage to plant takes place, a number of processes are activated and some of the compounds produced become activated immediately whereas phytoalexins take two to three days to be produced. Sometimes it is difficult to determine whether the compounds are phytoalexins or prohibitins and moreover, the same compound may be antimicrobial in one species and a phytoalexinin another (Grayer *et al.*, 1994). Since the advent of antibiotics in 1950s, the use of plant derivative as antimicrobials has been virtually non-existence but the pace is rapidly on the increase as we begin to realize the need for new and effective treatments. Since the use antimicrobial principles from natural origin is environmentally safe and resistance is rear, further such geared towards identification of more of such principles is necessary.

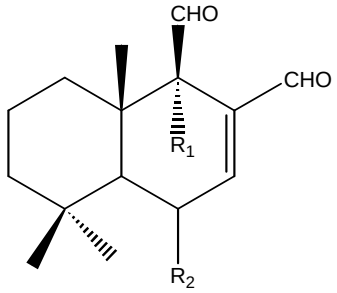
2.6 Medicinal plants used in this study

2.6.1 *Warburgia ugandensis*

The genus *Warburgia* (Conellaceae) consists of two species widely distributed in East Africa, namely *W. stuhlmanii* Engl. and *W. ugandensis*. *W. ugandensis* Sprague is one of the most highly utilized medicinal plants in tropical and subtropical Africa and is now highly endangered in the wild (Kioko *et al.*, 2005). It is rated as the second highest priority medicinal plant species in Kenya (Kariuki and Simiyu, 2005). Dried bark of the tree is commonly chewed and the juice swallowed as a remedy for stomach ache, constipation, toothache, venereal diseases, cough, fever, muscle pains, weak joints and general body pains (www Worldagroforetsrycentre. org/sea/products/AFDbases/af/asp/ Specie sInfo, 12th June, 2010). The leaf decoction baths are used as a cure for skin diseases while the bark, roots or leaves can be

boiled in water and drunk to treat malaria, although this causes violent vomiting (Kokwaro, 2009). In addition, the leaves of *W. ugandensis* are sometimes used locally as a spice for food.

Warburgia plants are characterized by the presence of drimane sesquiterpenes (Kioy *et al.*, 1990a; 1990b; Mohanlall and Odhiv, 2009; Xu *et al.*, 2009a). Some of the sesquiterpenes isolated from the plants include polygodial [**13**], warbuganal [**14**], mukaadial [**15**], ugandensidial [**16**], cinnamolide [**17**], 9α, 11α-dihydroxycinnamolide [**17a**], 9α, 11α-dihydroxy-6β-acetylcinnamolide [**17b**], 9α-hydroxycinnamolide [**17c**], 11α-hydroxycinnamolide [**17d**], cinnamolide-3β-ol [**18**], cinnamolide-3β-acetate [**19**], ugandensolide [**20**], deacetylugandensolide [**21**], muzigadial [**22**], 6β-hydroxymuzigadial [**23**], muzigadiolide [**24**] dendocarbins L [**25**] and M [**26**] (Kubo *et al.*, 1976; 1977; Kioy *et al.*, 1989; 1990a; 1990b; Mashimbye *et al.*, 1999; Manguro *et al.*, 2003a; 2003b; Wube *et al.*, 2005; Clarkson *et al.*, 2007; *Xu et al*., 2009a) . Drimane-type sesquiterpenes glycosides; mukadial 6-*O*-β-D-glucopyranoside [**27**] and mukadial 6-*O*-α-L-rhamnoside [**28**] were isolated from the methanolic leaf extract of *W. stuhlmannii* (Manguro *et al.*, 2003b).



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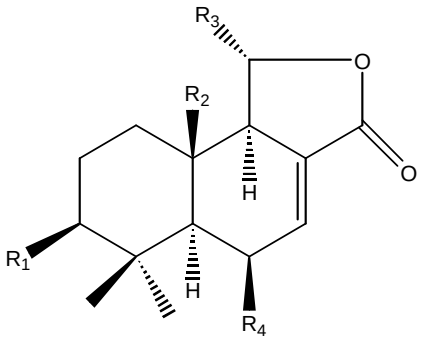
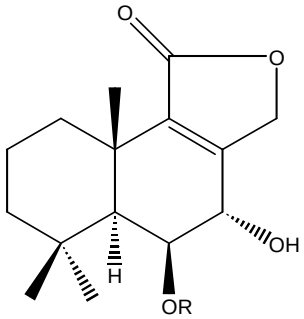
13	H	H
14	OH	H
15	OH	OH
16	OH	OAc



	R_1	R_2	R_3	R_4
17	H	H	H	H
17a	H	OH	OH	H
17b	H	OH	OH	OAc
17c	H	OH	H	H
17d	H	H	OH	H
18	OH	H	H	H
19	OAc	H	H	H



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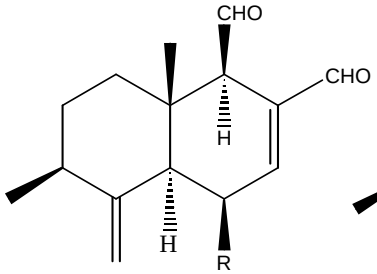
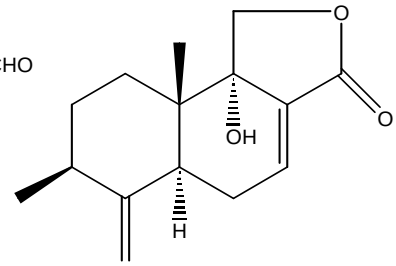
21 R- H



22 R = H
23 R = OH



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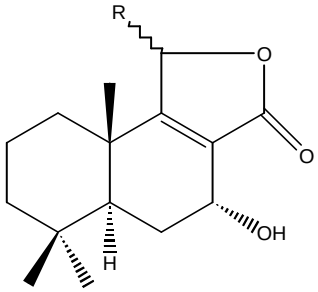
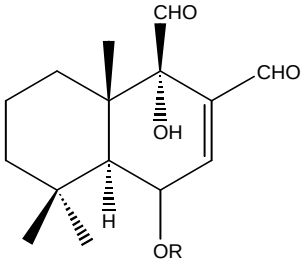
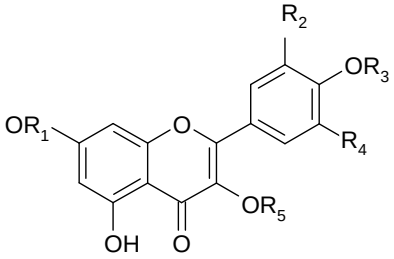


25 R = Beta OH
26 R= Alpha OH



27 R = Glu
28 R = Rha

Flavonoids and flavonol glycosides including Kaempferol [**29**], kaempferol 3-glucoside [**30**], kaempferol 3-rhamnoside [**31**], kaempferol 3-arabinose [**32**], kaempferol 7-glucoside [**33**], kaempferol 3-rhamnoside 7, 4′-digalactoside [**34**], kaempferol 3, 7, 4′-triglucoside [**35**], kaempferol 3-rhamnoside 4′-galactoside [**36**], quercetin [**37**], quercetin 3-glucoside [**38**], quercetin 3-rhamnoside 7, 4′-digalactoside [**39**], quercetin 3-rhamnoside [**40**], myricetin [**41**], myricetin 3-galactoside [**42**] and myricetin 3-rhamnoside [**43**] (Manguro *et al.*, 2003a; 2003b).



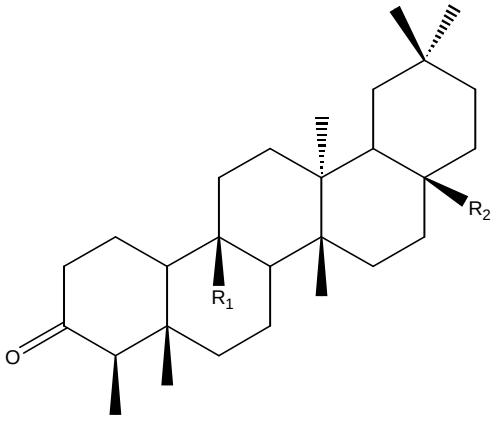
	R_1	R_2	R_3	R_4	R_5
29	H	H	H	H	H
30	H	H	H	H	Glu
31	H	H	H	H	Rha
32	H	H	H	H	Ara
33	Glu	H	H	H	H
34	Gal	H	Gal	H	Rha
35	Glu	H	Glu	H	Glu
36	H	H	Rha	H	Rha
37	H	OH	H	H	H
38	H	OH	H	H	Glu
39	Gal	OH	Gal	H	Rha
40	H	OH	H	H	Rha
41	H	OH	H	OH	H
42	H	OH	H	OH	Gal
43	H	OH	H	OH	Rha

Extracts from *Warburgia* species have been reported to exhibit numerous biological activities including antibacterial, antifungal, antiviral activity and trypanocidal effects (Kubo and Nakanishi, 1979; Kioy *et al*., 1990a; Wube *et al.,* 2005; Clarkson *et al.,* 2007; Mbwambo *et al.,* 2009). Crude extract from stem bark of *W. ugandensis* showed activity against *Mycobacterium tuberculosis* H37Rv and M. Bovis BCG Pasteur (Madikane *et al.,* 2007). A study (Rugutt *et al.,* 2006) showed that crude extracts from leaves and stem bark of *W. ugandensis* to be active against soil pathogens namely *Fusarium oxysporum, Alternaria passiflorae,* and *Aspergillus niger*. Olila *et al.* (2001; 2002) demonstrated that this plant has both anitiviral, antibacterial and antifungal activities. Some of the antimicrobial principles from *Warburgia* species include polygodial [**13**], warbuganal [**14**] and muzigadial [**22**] (Kioy *et al*., 1990a; Wube *et al.,* 2005; Rugutt *et al.,* 2006). However, there is little or no work on the evaluation of efficacy of extracts *Warburgia* species in the management of plant pathogens especially plant fungal and bacterial infections.

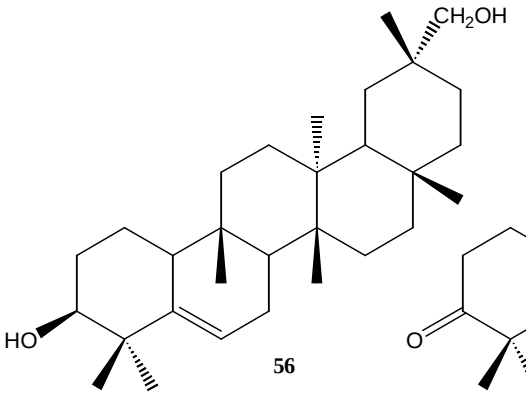
2.6.2 *Elaeodendron schweinfurthianum*

Elaeodendron species are used widely for a number of medicinal applications. For example, Kokwaro (2009) reported that dry powdered roots of *E. schweinfurthianum* are used in the management of wounds and primary symptoms of syphilis. Chewing of the plant has been said to cure diarrhoea. On the other hand, ingestion of *E. schweinfurthianum* leaves, fruits or bark is said to cause sudden death. The genus is characterized by the presence of terpenoids, (Anjaneyulu and Rao, 1980b; Weeratunga *et al*., 1982; Weeratunga and Kumar, 1985; Kubo *et al.*, 1990), steroids (Shimada *et al*., 1982; Yasuko *et al*., 1985) and flavonoids (Weeratunga *et al*., 1985).

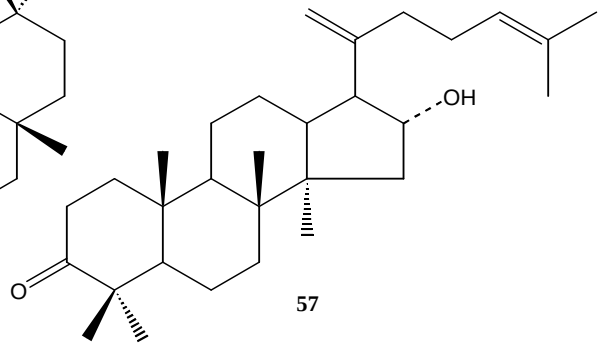
Friedooleanane derivatives; friedelin [**44**], canophyllal [**45**] canophyllol, [**46**], 28-acetoxycanophyllol [**47**], methylfriedoolean-3-one-28-oate [**48**], friedelan-3-one-25-al [**49**], 25-hydroxyfriedelin [**50**], 25-acetoxyfriedeline [**51**], elaeodendrol [**52**] and elaeodendadiol [**53**] were isolated from root bark of *E. glaucum* (Anjaneyulu and Rao, 1980a). Weeratunga and Kumar (1985) isolated 25, 28-dihydroxyfriedoolean-3-one [**54**], 25-hydroxy-3-oxofriedoolean-28-al [**55**] and friedoolean-5-ene-3β, 29-diol [**56**] from the root bark of *E. balae*. In other studies (Kubo *et al.*, 1990; Anjeneyulu and Rao, 1980b), elabunin [**57**], lupeol [**58**] and its derivatives; betulin [**49**], 23-hydroxybetulin [**60**] and betulinic acid [**61**] were isolated from *E. buchananii*. A sesquiterpene of the eudesmane type, with five hydroxyl functions, mutangin [**62**] was isolated from *E. buchananii* (Tsanou *et al*., 1993).



	R_1	R_2
44	Me	Me
45	Me	CHO
46	Me	CH_2OH
47	Me	CH_2OAc
48	Me	OAc
49	CHO	Me
50	CH_2OH	Me
51	CH_2OAc	Me
52	Me	OH
53	CH_2OH	Me
54	CH_2OH	CH_2OH
55	CH_2OH	CHO



56



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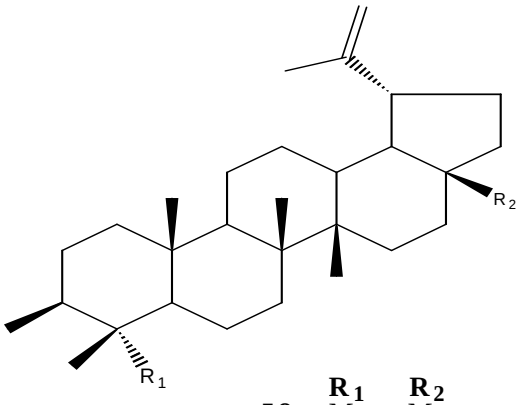
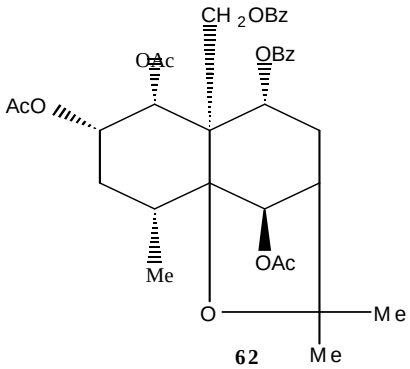


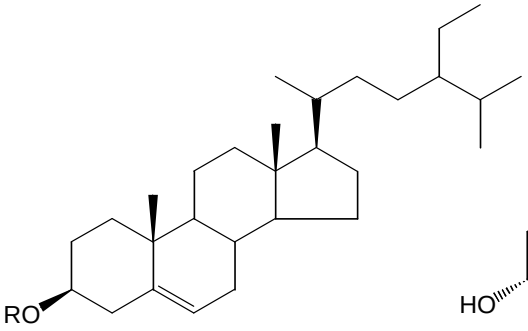
	R_1	R_2
58	Me	Me
59	Me	OH
60	OH	OH
61	Me	CO_2Me



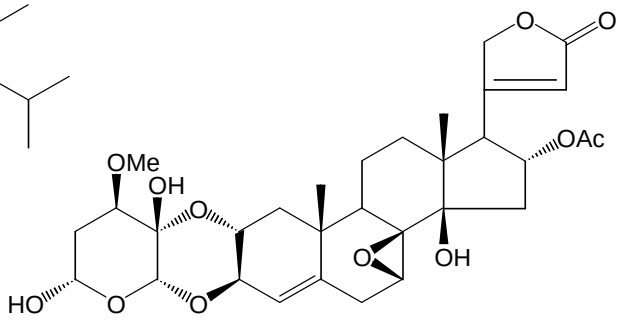
62

Phytochemical studies of *Elaeodendron* species resulted into the isolation of β-sitosterol [**63**], sitosterol-3β-glucoside [**64**], Buchaninoside [**65**] (Yasuko *et al.,* 1995), elaeodendroside A, D, E, F, G, I, J, H, O, B and C [**66** - **76**] and elaeodendrogenin [**77**] (Shimada *et al*., 1982).

Elaeocyanidin [**78**] ourateacatechin [**79**] and ouratea-proancyanidin A [**80**] were isolated from ethyl acetate extract of the root bark of *E. balae* (Weeratunga *et al*., 1985).

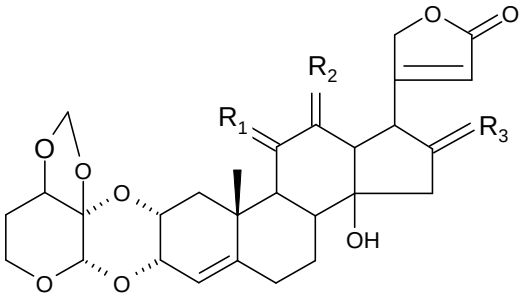


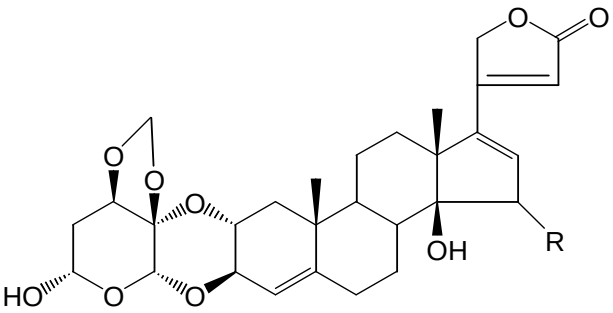
63 R = H
64 R = Glu



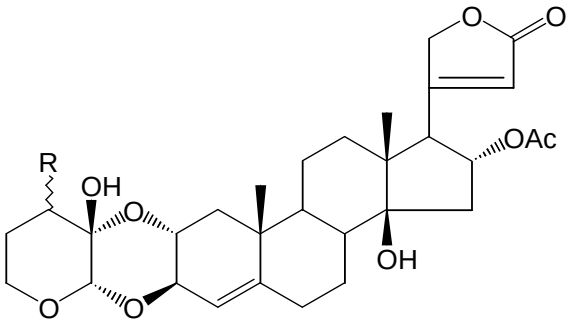
65

	R_1	R_2	R_3
156	O	α OH, H	H_2
157	H_2	H_2	H_2
158	H_2	H_2	β OAc, H
159	β OH, H	α OH, H	H_2
160	α OH, H	α OH, H	H_2
161	H_2	α OH, H	H_2
162	α OH, H	O	H_2

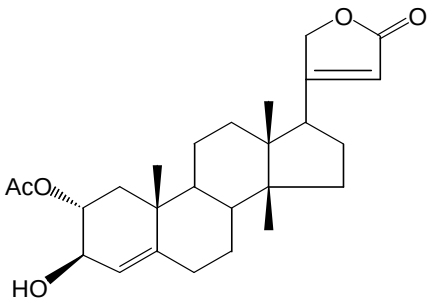




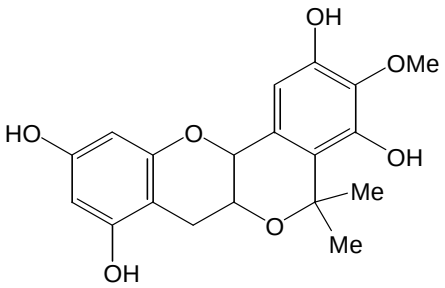
73 R = H
74 R = OH



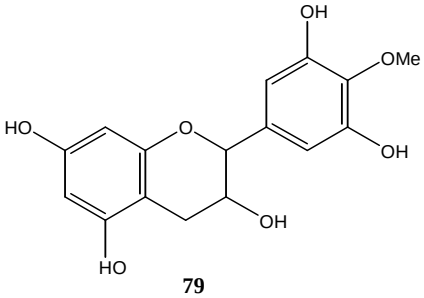
75 R = Alpha OMe
76 R = Beta OMe


O
O
AcO
HO

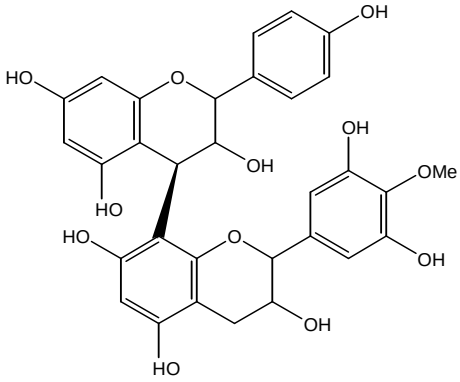
77


OH
OMe
HO
O
OH
Me
O
Me
OH

78


OH
OMe
HO
O
OH
OH
HO

79


OH
HO
O
OH
OH
OMe
HO
HO
O
OH
OH
HO

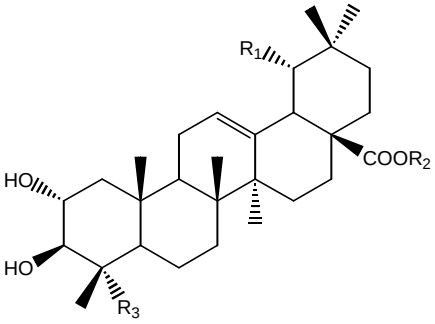
80

Previous biological activity studies of *Elaeodendron* species showed that their extracts are cytotoxic. In a study by Shimada *et al*. (1982), ethanol extract of seeds of *E glaucum* showed inhibitory *in-vitro* activity against cells derived from human nasopharynx carcinoma. The cytotoxic compounds from *E glaucum* are elaeodendroside A, D, E, I, and O, compounds **66**, **67**, **68**, **71**, **74** and lupeol [**58**] (Shimada *et al.*, 1982; 1985; Kupchan *et al*., 1977). Phytochemistry, antifungal and antibacterial activities of *E. scweifurthianum* have not been reported.

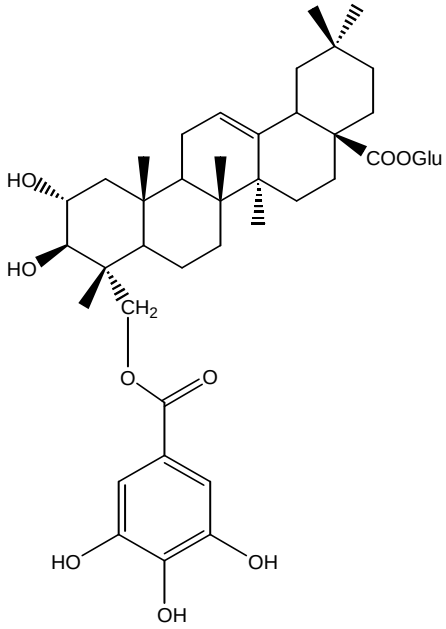
2.6.3 *Terminalia brownii*

Terminalia brownii Fries (Combretaceae) is found in many parts of Africa and it has different uses. The leaves are used by traditional healers in Tanzania to control diarrhoea and stomach ache, gastric ulcers, colic, and heartburn (Mbuya *et al.,* 1994; Fyhrquist *et al.,* 2002). In the Democratic Republic of Congo barks from the stems, branches, and trunks are used to manage urogenital infections, urethral pain, endometritis, cystitis, leucorrhoea, syphilis, and gonorrhoea (Dhetchuvi and Lejoly, 1990). Traditional healers in Kenya use leaves of the plant to treat malaria (Heine and Heine, 1988). The decoction of the stem bark, trunk and branches is taken orally to treat dysmenorrhoea, nervosity, hysteria, epilepsy, beriberi, dyspepsia, stomachache, gastric ulcers, and colitis (Lindsay and Hepper, 1978; Fyhrquist *et al.,* 2002). Stem barks are chewed to treat cough and as emetic, infusion of barks and leaves are mixed with meat to treat hepatitis (Timberlake, 1987). Traditional healers in Ethiopia use the stem and barks to treat jaundice, hepatitis, liver cirrhosis, and yellow fever (Kokwaro, 2009; Wilson and Woldo, 1979; Heine and Brenzinger, 1988).

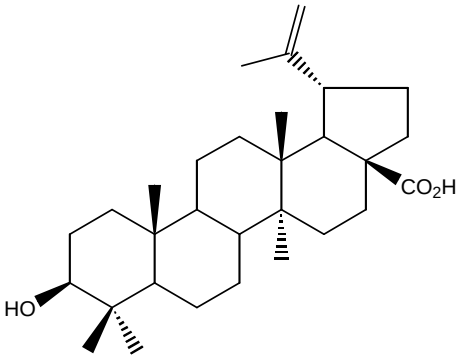
Phytochemical studies of some *Terminalia* species have reported the presence of terpenoids and flavonoids (Reddy *et al.,* 1994; Fayez and Mahmoud, 1998; Garcez *et al.,* 2006; Cao *et al.,* 2010). Terpenoids previously isolated from *Terminalia* species include arjunic acid [**81**], arjunetin [**82**], arjungenin [**83**], arjunglucoside I [**84**], arjunolic acid [**85**], arjunlucoside II [**86**], 23-galloylarjunglucoside II [**87**], betulinic acid [**88**], ursonic acid [**89**] (Kong *et al.,* 1954; Row *et al.,* 1970; Honda *et al.,* 1976a; 1976b; Tsuyuki *et al.,* 1979; Anjaneyulu and Rama, 1982; Reddy *et al.,* 1994; Pettit *et al.,* 1996; Conrad *et al.,* 1998; Singh *et al.,* 2004; 2008; Garcez *et al.,* 2006; Haque *et al.,* 2008). Steroids such as sitosterol [**63**] and stigmasterol [**90**] were reported from the species (Garcez *et al.,* 2006; Haque *et al.,* 2008).



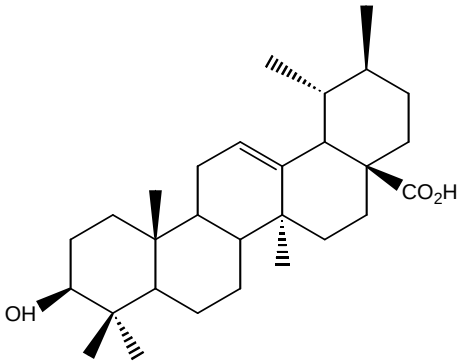
	R_1	R_2	R_3
81	OH	H	CH_3
82	OH	Glu	CH_3
83	OH	H	CH_2OH
84	OH	Glu	CH_2OH
85	H	H	CH_2OH
86	H	Glu	CH_2OH



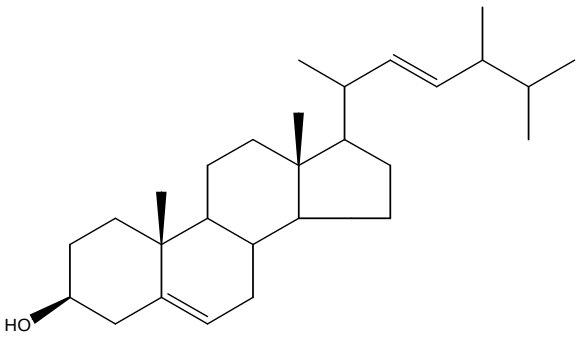
87



88



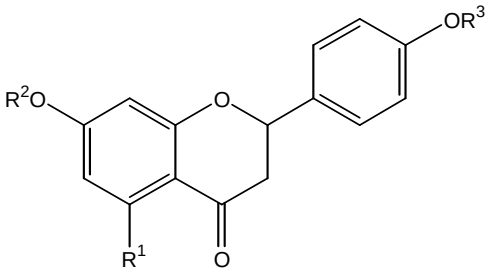
89



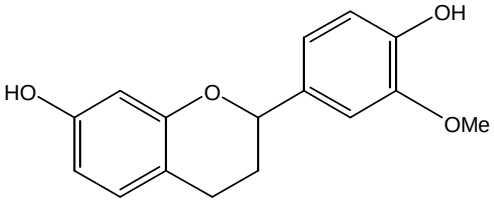
90

Flavonoids isolated from Terminalia species include naringenin-4′, 7-dimethyl ether [**91**], isosakuranetin [**92**], naringenin [**93**], liquiritigenin-4′, 7-dimethyl ether [**94**], liquiritigenin-4′-methyl ether [**95**], liquiritigenin-7-methyl ether [**96**], sukuranetin [**97**], 7, 4′-dimethyl 3′-methylflavone [**98**] and two chalcones [**99** and **100**] (Sharma *et al.,* 1982; Garcez *et al.,* 2006).

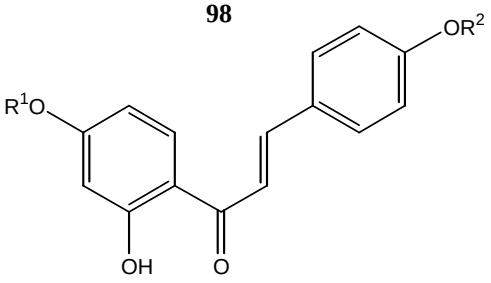
Biological activities of *Terminalia* species include antifungal, antibacterial (Dwivedi and Udupa, 1989; Conrad *et al.*, 1998; Moshi and Mbwambo, 2005; Mbwambo *et al.*, 2007; Singh *et al.*, 2008), antioxidant, antitumor (Garcez *et al.,* 2006), feeding deterrent and growth inhibitor (Singh *et al.,* 2004). Singh *et al* (2008) reported the antimicrobial principles to be arjunic acd [**81**], arjungetin [**82**] and arjungenin [**83**]. Phytochemistry, antifungal and antibacterial activities of *T. brownii* have not been reported.



	R^1	**R^2**	**R^3**
91	OH	CH_3	CH_3
92	OH	H	CH_3
93	OH	H	H
94	H	CH_3	CH_3
95	H	H	CH_3
96	H	CH_3	H
97	OH	CH_3	H



98



	R^1	**R^2**
99	H	H
100	CH_3	H

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Establishment of the incidence and identity of sweet potato viruses in western Kenya

3.1.1 Survey of sweet potato viruses

In western Kenya, sweet potato is widely grown in Bungoma, Busia, Kakamega, Teso and Vihiga districts in Western Province which lie between latitude 0° 30' N to 0° 58' S and longitude 33° 58' E to 35° 38' E; Homa Bay, Kisii, Kisumu, Rachuonyo and Siaya districts in Nyanza Province located between latitude 0° to 1°' N and longitude 33° 55' E to 35° 38' E (Appendix B). A survey was conducted in sweet potato fields in the ten districts in April 2009 as previously described (Tairo *et al.*, 2004). Sweet potato fields with a 3 to 5-month-old crop were randomly sampled along rural roads or paths at approximately 2-5 km intervals. A total of 327 vines from symptomatic ordinary sweet potato plants were collected and transferred to Jomo Kenyatta University of Agriculture and Technology (JKUAT), Department of Horticulture where they were planted in 15-20 cm diameter pots in sterile soil (solar sterilization) enriched with diammonium phosphate fertilzer in an insect-proof screenhouse and watered regularly. Plants were sprayed regularly with Malathion against aphids and whiteflies to avoid virus spread among the plants. The samples were tested for virus infections after one month.

3.1.2 Serological analysis of sweet potato samples

Symptomatic sweet potato samples collected during the survey were subjected to serological testing for SPFMV, SPCSV, SPMMV, SPCFV, SPMSV, SPCa-LV, SPLV, C-6, SPVG and CMV using nitrocellulose membrane enzyme-linked immunosorbent assay (NCM-ELISA) using standard NCM-ELISA kit obtained from the International Potato Center, CIP, Lima, Peru (Gibb and Padovan, 1993). The Kit contained polyclonal antibodies specific to SPFMV, SPCSV, SPMMV, SPCFV, SPMSV, SPCa-LV, SPLV, C-6, SPVG and CMV as well as negative and positive controls.

Three leaf discs (1–cm diameter) from a composite sample of three leaves taken from different points (bottom, middle and top) of sweet potato plant (one month old) were ground in 1 ml of extraction buffer (Appendix C) in plastic bags. The ground sample was allowed to stand for 30 – 45 minutes at room temperature for the sap to phase out. Using a clean pipette each time, 15 µl of clear supernatant of each sample was blotted at the center of a square made on the nitrocellulose membrane. The membrane was allowed to dry at the room temperature for about 15 – 30 minutes. Once dry, the membrane was immersed in 30 ml blocking solution (Appendix C) in a Petri dish for 1h. The blocking solution was discarded and the membrane immersed in a Petri dish with the primary antibody diluted (1:1000, v/v) in antibody solution. The membrane was then incubated at room temperature overnight, with a constant agitation on an orbital shaker (50 rpm). The primary antibody solution was discarded and unbound antibodies removed from the membranes by washing with constant agitation in T-TBS (Appendix C) four times for three minutes each time at 100 rpm. The membranes were then immersed in 30 ml of goat anti–rabbit alkaline phosphatase (GAR – AP) solution (1:1000 v/v) in a Petri dish for 1h. The substrate solution, nitro blue tetrazolium chloride / 5-bromo-4-chloro–3– indolyl phosphate (NBT/BCIP), was added and the reaction allowed to proceed for 30 min at room temperature. Positive and negative reactions were determined by visual assessment with different grades of purple colour indicating positive reactions. The substrate solution was discarded after 30 min of incubation and membranes washed twice with distilled water to stop any further colour development.

3.1.3 Total ribonucleic nucleic acid extraction from *Cucumber mosaic virus* (CMV) infected samples

Fresh young leaf tissue (100 mg) was ground to fine powder in liquid nitrogen using a mortar and pestle. Total RNA was extracted from leaves of sweet potato samples determined to be infected with CMV from the serological analysis and from a healthy control. The RNA was extracted using RNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions (Qiagen, 2006). The integrity of the extracted RNA was evaluated by electrophoresis in 1% agarose gel stained with ethidium bromide (Sigma). Total RNA was amplified using CMV-specific primers, 5'-GCC GTA AGC TGG ATG GAC AA- 3' and 5'-TAT GAT AAG AAG CTT GTT TCG CG-3' forward and reverse primers, respectively (Wylie *et al.*, 1993). The primers were synthesized by Sigma, Life Sciences Corporation (Germany).

3.1.4 Reverse transcription and polymerase chain reaction (RT-PCR)

Reverse transcription (RT) was performed in 18.8 µl reaction mixture using Omniscript® Reverse Transcription Kit according to the manufacturer's instructions (Qiagen, 2004). Three (3) µl of total RNA (1 µg/µl), 0.2 µl of reverse primer, 0.3 µl of random primer and 9.5 µl of RNase-free H_2O were incubated at 70 ºC for 10 min then cooled in ice. For the first strand cDNA synthesis, 2.0 µl of 10x strand buffer, 0.3 µl of 10 mM dNTP mix, 0.5 µl of Omniscript® Reverse Transcriptase (Qiagen Inc., Valencia, CA), 0.5 µl of RNase inhibitor and 2.5 µl of RNase - free H_2O were added to the reaction tube and the mixture further incubated at 42 ºC for 1 h. PCR was performed using the Taq PCR Master Mix Kit according to the manufacturer's protocol (Qiagen, 2002). PCR reaction mixture, 20.0 µl, consisted of 10.0 µl of PCR Master Mix (containing 25nM $MgCl_2$, dNTP mix 10 mM each, Taq polymerase and 10x QIAGEN PCR buffer), 4.0 µl of Q – Solution (Qiagen), and 0.3 µl each of forward and reverse primer, 1.0 µl of cDNA (1 µg/µl) and 4.4 µl of RNase free H_2O was prepared. PCR was carried out using the following cycling conditions: one cycle at 95 ºC, 5 min for initial denaturation followed by 35 cycles 96 ºC, 5 sec for denaturation, 61 ºC, 5 sec for primer annealing and 72 ºC, 30 sec for extension and a final extension at 72 ºC for 1 min. PCR products were analyzed by electrophoresis using 1% agarose gel stained with ethidium bromide and DNA bands were visualized under UV light (254 nm).

3.2 Development of a multiplex PCR technique for detection of SPFMV and SPCSV

3.2.1 RNA extraction from SPFMV and SPCSV infected samples

Total RNA was extracted from leaves of sweet potato samples infected with both SPFMV and SPCSV and a healthy control using RNeasy® Plant Mini Kit (Qiagen Inc., Valencia, CA) according to the manufacturer's instruction (Qiagen, 2006). Two sets of primers specific to SPFMV and SPCSV (Table 1) were developed and obtained from a commercial source (Sigma, Life Sciences Corporation, Germany). The SPFMV primers were designed from highly conserved regions of the SPFMV strains prevalent in East Africa namely the East African strain (SPFMV-EA), ordinary strain (SPFMV-O), and the common strain (SPFMV-C) (Ateka, 2004; Mukasa *et al*., 2003).

Table 1: Virus-specific primer pairs used to amplify SPFMV and SPCSV in mPCR

Virus	Primer name	Primer /Sequence	Expected product size (bp)
SPFMV	SPFMV-F	5'-GGACGAGACACTAGCAA-3'	703
	SPFMV-R	5'-TTCTTCTTGCGTGGAGACGT-3'	
SPCSV	SPCSV-F	5'-ACGTTGGTTGGCGTTGA-3'	235
	SPCSV-R	5'-ATCTGCGGGAACTGACACG-3'	

3.2.2 Optimization of multiplex conditions

The reactions were optimized by varying the amount of complementary DNA (cDNA) template of each target. Optimization was carried out by methodical variation of test parameters under standard PCR conditions. The cDNA template amounts tested ranged from 1.0 to 3.0 µg per PCR reaction mix of 20 µl and 40 µl for singlex PCR (sPCR) and mPCR, respectively. The annealing temperature ranged from 60 to 63 °C whereas the number of cycles ranged from 30 to 35.

3.2.3 Singlex PCR and RT-PCR assay

The designed specific primer sets of SPFMV and SPCSV were tested against templates of RNA extracted from leaves of sweet potato plants showing SPVD symptoms separately in Singlex PCR (sPCR). Reverse transcription (RT) was performed using Omniscript® Reverse Transcriptase according to the manufacturer's protocol (Qiagen, 2006). Reverse primers, SPFMV-R and SPCSV-R (Table 1) specific to SPFMV and SPCSV, respectively were used. DNA amplification was performed using the Qiagen's Taq PCR Master Mix Kit (Qiagen) following the manufacturer's instructions(Qiagen, 2002). PCR was carried out using the following parameters: initial denaturation at 95 ºC, 5 min followed by 35 cycles 96 ºC, 5 sec for denaturation, 63 ºC, 5 sec for primer annealing and 72 ºC, 30 sec for extension and a final extension at 72 ºC for 1 min.

3.2.4 Multiplex PCR and RT-PCR assay

The mPCR, similar to the sPCR, consisted of a two step reaction procedure which included reverse transcription followed by PCR amplification. Total RNA was subjected to cDNA synthesis in a 19.0 µl as follows: Three µg of total RNA, 0.2 µl each of SPFMV-R and SPCSV-R primers (Table 1), 0.3 µl of random primer and 9.5 µl of RNase free H_2O were incubated at 70 ºC for 10 min. To the reaction mixture, 2.0 µl of 10x strand buffer, 0.3 µl of 5 mM dNTP mix, 0.5 µl of Omniscript® Reverse Transcriptase (Qiagen), 0.5 µl of RNase inhibitor and 2.5 µl of RNase free H_2O were added. The resulting master mix was incubated at 42 ºC for 1 hr followed by 70 ºC for 10 min. The mPCR reaction mixture consisted of 1.0 to 3.0 µg of the synthesized cDNA (containing cDNA for SPFMV and SPCSV), 20 µl PCR Master Mix (Qiagen), 0.3 µl of each of the two set of primers, 8.0 µl of Q-Solution (Qiagen), and the final volume made up with RNase free H_2O. The mPCR was performed using the following parameters; initial denaturation at 95 ºC for 5 min followed by 30 - 35 cycles 96 ºC, 5 sec for denaturation; 60 – 63 ºC, 5 sec for primer annealing and 72 ºC, 30 sec for extension and a final extension at 72 ºC for 10 min to determine the annealing temperature for the two primers.

3.2.5 Evaluation of the developed mRT-PCR protocol

Thirteen symptomatic and one healthy sweet potato samples collected from farmers' fields were tested for SPFMV and SPCSV infection using the developed protocol. Two µg of cDNA template was used in the PCR under the following parameters: one cycle at 95 ºC for 5 min followed by 35 cycles 96 ºC, 5 sec for denaturation; 63 ºC, 5 sec for primer annealing and 72 ºC, 30 sec for extension and a final extension at 72 ºC for 10 min.

3.3 Phytochemical studies of *W. ugandensis, E. schweinfurthianum* and *T. brownii* plants

3.3.1 General experimental procedure

Melting points were determined on a Gallenkamp (Loughborough, UK) melting point apparatus and are uncorrected. Distilled solvents were used for extraction and chromatographic separations. Analytical thin layer chromatograms (TLC) were run on 0.2mm thick layer of silica gel, Merck. Column chromatography was performed on silica gel 70-230 mesh (Sigma). The UV spectra were run on Pye Unicam SP8-150 UV/visible spectrophotometer (Cambridge, UK. IR data were recorded on a Perkin-Elmer FTIR 600 series spectrophotometer (Waltham, MA, USA). The NMR data were measured in $CDCl_3$ and $CDCl_3$-DMSO-d_6 on a Bruker NMR ultrashield TM (Darmstadt, Germany) operating at 360 and 90 MHz ^{1}H and ^{13}C for, respectively. The MS data were obtained on a Varian MAT 8200A instrument (Bremen, Germany).

3.3.2 Collection of plant materials

The stem bark of *W. ugandensis* was obtained from species grown near St. Mary's Hospital along Nakuru-Gilgil highway (latitude 0° 24' 42.49" S and longitude 36° 15' 10.59" E) in August 2008. *E. schweinfurthianum* stem bark was collected from Shimba Hills (latitude 4° 15' 53.84" S and longitude 39° 22' 19.61" E) in September 2008. Stem bark of *T. brownii* was collected near Kindu Bay Mission Hospital along Kendu Bay - Oyugis road (latitude 0° 22' 22.00" S and longitude 34° 39' 09.05" E) in November 2008. The collection process of the various plant specimens was supervised by Mr. Mutiso of the Department of Botany, University of Nairobi. Identification of the plants was done at the Kenya National Museum and at the Department of Botany, University of Nairobi herbaria after comparison with authentic samples. *Warburgia ugandensis* (2008/08/01/SAO/CHEMMK), *E. schweinfurthianum* (2008/09/04/SAO/CHEMMK) and *T. brownii* (2008/11/15/SAO/ CHEMMK).

3.3.3 Preparation and solvent extraction of plant materials

Plant materials were cut into small pieces and air-dried for one month under a shade. The material specimens were ground into fine powder in readiness for extraction. The sample preparation and extraction procedure were carried out as described by Harbone (1994). Cold sequential extraction was done with distilled organic solvents of increasing polarity, (*n*-hexane, ethyl acetate and methanol). Powdered plant (2 kg) material was soaked in n-hexane (3 x 3 L) solvents and left to stand for seven days, with occasional shaking. The macerate was filtered and the filtrate concentrated under vacuum using rotary evaporator. The same extraction procedure was repeated with ethyl acetate and finally with methanol.

The stem bark of *W. ugandensis* afforded 20 g, 105 g and 215 g of n-hexane, ethyl acetate and methanol extracts, respectively. Extracts from *E. schweinfurthianum* were obtained in 15 g, 100 g and 210 g of n-hexane, ethyl acetate and methanol extracts, respectively. *T. brownii* stem bark sequential solvent extraction produced 18 g, 75 g and 150 g of n-hexane, ethyl acetate and methanol extracts, respectively. Extracts were stored at 4 ^{0}C for phytochemical and antimicrobial activity studies against sweet potato fungi and bacteria.

3.3.4 Isolation of compounds from *W. ugandensis*

For chromatographic separation, portions of n-hexane (15 g) and ethyl acetate (65 g) extracts were combined because the two extracts showed similar profiles on thin TLC. The combined extract (80 g) was dissolved in minimum amount of ethyl acetate and adsorbed into silica gel before evaporation to dryness. The resulting dark brown oily material was loaded on top of silica gel packed glass column (5 x 60 cm, 200 g) and eluted with increasing gradient of EtOAc in n-hexane, EtOAc, CH_2Cl_2-MeOH mixture with increasing concentration of MeOH and elution was finally concluded with neat MeOH. The whole process was done under medium pressure.

A total of 400 fractions (each 20 ml) were sampled and their homogeneity monitored by TLC using solvent systems: n-hexane-ethyl acetate (9:1, 4:1, 3:2, 2:1 and 1:1) and CH_2Cl_2-MeOH (95:5 and 9:1). The eluants were combined to afford the following fraction pools.

I) Fraction 15-60 (n-hexane - EtOAc 95:5 eluate, 15 g) was an oily substance with aromatic smell. It was kept for future GC - MS analysis.

II) Eluate (n-hexane- EtOAc 9:1, and 4:1, fractions 64 -100, 10 g) showed four sports R_f values 0.53 (major component), 0.42, 0.35 and 0.22, which turned greenish purple with anisaldehyde – conc. H_2SO_4 mixture after spraying and heating at 100 °C. The fractions were combined and from this pool crystallized out white needles which were filtered and further purified by re-crystallization (n-hexane- EtOAc, 95:5) to give polygodial [**13**], R_f = 0.53 (n-hexane- EtOAc, 4:1), 120 mg. The mother liquor was evaporated under reduced pressure and upon flash chromatography using (n-hexane – EtOAc 9:1) followed by the same solvent system in the ratios 19:3 and 4:1 to give further **13**, 25 mg, cinnamolide [**17**] (n-hexane- EtOAc, 4:1, R_f = 0.42, 73 mg), warbuganal [**14**] (n-hexane- EtOAc 4;1, R_f 0.35, 65 mg) and 9-deoxymuzigadial [**101**] (n-hexane- EtOAc 4:1, R_f = 0.22, 45 mg).

III) Pool III (n-hexane- EtOAc 4:1, 3:2 and 1:1, fractions 102-220, 15 g) contained one major spot R_f value 0.33 (n-hexane- EtOAc 3:2) which masked other spots. This pool also crystallized out to give amorphous powder which was further purified by re-crystallization in n-hexane- EtOAc 19:3 to afford white plate-like material, muzigadial [**22**], 2000 mg. The mother liquor was evaporated under reduced pressure and further purification by repeated flash chromatography over silica gel column (3 x 60 cm, 100 g) using (n-hexane - EtOAc (9:1, 4:1 and 3:2) and collecting 10 ml each gave a further polygodial [**13**], 15 mg, warburganal [**14**, 20 mg] and muzigadial [**22**, 68 mg]. The other secondary metabolites isolated from this eluate included bemadienolide [**102**, n-hexane – EtOAc 3:2, R_f = 0.22, 55 mg] and drimenin [**103**, n-hexane – EtOAc 3:2, R_f = 0.18, 64 mg].

IV) Eluate IV (n-hexane - EtOAc, 1:2 and EtOAC, fractions 225 -300, 11 mg) afforded five spots of R_f values 0.36, 0.32, 0.29, 0.26 and 0.18 using eluent: n-hexane – EtOAc, 1:1. The spots upon spraying with anisaldehyde – conc. H_2SO_4 mixture and heating turned greenish purple. A change of developing reagent to iodine vapor revealed an additional spot of R_f value 0.34. This pool was resolved into individual compounds by fractionation over silica gel column 3.5 x 60 cm, 150 g) using n-hexane- EtOAc (3:2) to give further **14**, **22** and **102** in 16, 29 and 10 mg, respectively. Also isolated from this fraction were 3β-acetoxycinnamolide [**19**, R_f = 0.26,

65 mg], 7α-acetoxyugandensolide [**104**, R_f = 0.34, 85 mg] and ugandensidial [**16**, R_f = 0.18, 54 mg].

V) Eluate V (EtAOc, fractions 310 - 360, 9 g) showed three spots R_f values 0.61 (major), 0.59 and 0.46 using CH_2Cl_2-MeOH, 97:3 as the developing solvent. Application of this pool to repeated silica gel column (2.5 x 60 cm, 120 g) eluting with CH_2Cl_2-MeOH (99:1) and collecting 10 ml each afforded mukaadial [**15**, R_f = 0.61, 100 mg], 6α-hydroxymuzigadial [**23**, R_f = 0.59, 55 mg] and ugandensolide [**20**, R_f = 0.46, 72 mg].

VI) Eluate VI (CH_2Cl_2-MeOH, 95:5 and 9:1, fractions 365 - 400, 8 g) showed one component with R_f = 0.41 after development with CH_2Cl_2-MeOH (95:5) and spraying with anisaldehyde – conc. H2SO4 mixture. The compound was obtained in pure form by re-crystallization in n-hexane-CH_2Cl_2-MeOH (97:2.5:0.5) to give white crystals, deacetoxyugandensolide [**21]**, 45 mg.

Polygodial [13]: white needle crystals (n-hexane- ethyl acetate mixture), $C_{15}H_{22}O_2$, R_f value of 0.53 (n-hexane: ethyl acetate 4:1), mp 58 -59 °C (lit. [Jansen *et al.,* 1988] 57 °C); $[\alpha]_D$ -131° (MeOH, c, 0.35); UV λ_{max} (CH_3CN) 226 nm ; IR ν_{max} (KBr) cm^{-1}: 1730, 1680, 1645; ^1H and ^{13}C NMR δ (ppm): See Table 4; EIMS *m/z* (rel. int.): 234 $[M]^+$ (1), 206 (55), 191 (25), 121 (65), 109 (60), 41 (100).

Warbuganal [14]: White crystals (n-hexane- ethyl acetate mixture), $C_{15}H_{22}O_3$, R_f 0.35 (n-hexane: ethyl acetate 4:1), mp 134 -135 °C (lit. [Kioy *et al.,* 1989] 134 -136 °C); $[\alpha]_D$ -216° (MeOH, c, 1.0); UV λ_{max} (CH_3CN) 223 nm ; IR ν_{max} (KBr) cm^{-1}: 3451, 2947, 2921, 2868, 2803, 1714, 1681, 1644; ^1H and ^{13}C NMR: δ (ppm): See Table 5; ESI-MS *m/z:* 273 $[M+Na]^+$.

Mukaadial [15]: White needdle crystals (CH_2Cl_2-MeOH), $C_{15}H_{22}O_4$, R_f 0.61 (CH_2Cl_2-MeOH 97:3), mp 228-229 °C (lit. [Kioy *et al.,* 1989] 230 °C); $[\alpha]_D$ -35° (MeOH, c, 0.5); UV λ_{max} (CH_3CN) 218 nm ; IR ν_{max} (KBr) cm^{-1}: 3350, 2990, 2923, 2798, 2701, 1720, 1696, 1466; ^1H and ^{13}C NMR: δ (ppm): See Table 6; EIMS *m/z* (rel. int.): 266 $[M]^+$ (5), 248 (100), 236 (15), 230 (46), 220 (23), 202 (21), 175 (27), 160 (6), 138 (8), 104 (10), 94 (4).

Ugandensidial [16]: White needles (n-hexane- ethyl acetate), $C_{17}H_{24}O_5$, R_f 0.18 (n-haxane: ethyl acetate 1:1), mp 136 - 137 °C (lit. [Kioy *et al.,* 1990a] 138 -140 °C); $[\alpha]_D$ -360° (MeOH, c, 2.5); UV λ_{max} (CH_3CN) 220 nm ; IR ν_{max} (KBr) cm^{-1}: 3428, 3007, 2945, 2848, 1743, 1781, 1692, 1462; ^1H

and ^{13}C NMR: δ (ppm): See Table 7; EIMS *m/z* (rel. int.): 308 $[M]^+$ (5), 280 (34), 237 (12), 220 (50), 148 (56), 109 (60), 60 (80), 43 (100).

Muzigadial [22]: White plate-like material (n-hexane: ethyl acetate mixture), $C_{15}H_{20}O_3$, R_f 0.33 (n-hexane: ethyl acetate 3:2), mp 123 -125 °C (lit. [Kubo *et al.*, 1977] 122 - 124 °C); $[\alpha]_D$ -230° (MeOH, c, 2.0); UV λ_{max} (CH_3CN) 228 nm ; IR ν_{max} (KBr) cm^{-1}: 3455, 2966, 2921, 2870, 1731, 1671; ^1H and ^{13}C NMR: δ (ppm): See Table 8; EIMS *m/z* (rel. int.): 248 $[M]^+$ (10), 237 (100), 219 (19), 109 (43)69 (50), 41 (52).

6α-Hydroxymuzigadial [23]: White crystals (CH_2Cl_2-MeOH mixture), $C_{15}H_{20}O_4$ R_f 0.59 (CH_2Cl_2-MeOH 97:3), mp 150-152 °C; $[\alpha]_D$ -97° (MeOH, c, 0.35); UV λ_{max} (CH_3CN) 210 nm ; IR ν_{max} (KBr) cm^{-1}:3360, 2958, 2860, 1727, 1678, 1639, 1454, 1387, 1318, 1171, 1135, 1055, 1029, 986, 897; ^1H and ^{13}C NMR: δ (ppm): See Table 9; EIMS *m/z* (rel. int.): 265 $[M+1]^+$ (3), 248 (12), 247 (100), 235 (5), 229 (8), 219 (7), 201 (13), 173 (15), 109 (4), 95 (1).

9-Deoxymuzigadial [101]: Pale yellow oil, $C_{15}H_{20}O_2$, R_f 0.22 (n-hexane: ethyl acetate 4:1); $[\alpha]_D$ -78° (MeOH, c, 0.35); UV λ_{max} (CH_3CN) 216 nm ; IR ν_{max} (KBr) cm^{-1}: 2960, 2922, 2870, 1714, 1680, 1655, 1408, 1340, 1205, 1140, 1022, 994, 840; ^1H and ^{13}C NMR: δ (ppm): See Table 10; EIMS *m/z* (rel. int.): 232 $[M]^+$ (100), 217 (39), 214 (90), 203 (59), 199 (57), 189 (48), 173 (70), 159 .

Ugandensolide [20]: White needle crystals (CH_2Cl_2-MeOH), $C_{17}H_{24}O_4$, R_f 0.57 (CH_2Cl_2-MeOH 97:3), mp 215-218 °C (lit. [Brooks and Drafan, 1969a] 218 °C); $[\alpha]_D$ +23° (MeOH, c, 1.0); UV λ_{max} (CH_3CN) 220 nm ; IR ν_{max} (KBr) cm^{-1}: 3451, 29.31, 2887, 1733, 1672, 1463, 1370, 1342, 1251, 1203, 1142, 1063, 1027; ^1H and ^{13}C NMR: δ (ppm): See Table 11; EIMS *m/z* (rel. int.): 308 $[M]^+$ (10), 266 (96), 248 (96), 233 (28), 215 (20), 177 (28), 163 (35), 69 (28), 55 (26), 43 (100).

7α-Acetoxyugandensolide [104]: White gummy material, $C_{19}H_{26}O_6$, R_f 0.34 (n-hexane-ethyl acetate 1:1), mp 228-230 ^oC; $[\alpha]_D$ +24° (MeOH, c, 0.01); UV λ_{max} (CH_3CN) 218 and 228 nm ; IR ν_{max} (KBr) cm^{-1}: 2966, 2915, 1758, 1728, 1681, 1458, 1373, 1245, 1196, 1095, 1043, 1009, 964, 742; ^1H and ^{13}C NMR: δ (ppm): See Table 12; EIMS *m/z* (rel. int.): 350 $[M]^+$ (2), 307 (23), 290 (65), 274 (15), 248 (18), 247 (100), 232 (35), 206 (22), 42 (84).

Deacetoxyugandensolide [21]: White needle crystals (n-hexane-EtOAc-MeOH 97:2.5:0.5), $C_{15}H_{22}O_4$, R_f value 0.41 (n-hexane: ethyl acetate 7:3), mp 260-264 °C (lit. [Kioy *et al.*, 1990a] 260 - 265 °C); $[\alpha]_D$ +70° (MeOH, c, 1.0); UV λ_{max} (CH_3CN) 220 nm; IR ν_{max} (KBr) cm^{-1}: 3350, 2930,

1727, 1672, 1468, 1344, 1281, 1063, 1015, 966, 787, 687, 554, 462; ^{1}H and ^{13}C NMR: δ (ppm): See Table 13; EIMS *m/z* (rel. int.): 266 $[M]^{+}$ (100), 248 (35), 215 (45), 151 (55), 136 (75), 69 (88), 41 (100), 39 (25).

Cinnamolide [17]: White crystals (n-hexane-ethyl acetate mixture), $C_{15}H_{22}O_{2,}$ $R_{f,}$ 0.42 (n-hexane-ethyl acetate 4:1), mp 126-128 °C (lit. [Kubo *et al.*, 1990a] 128 - 129 °C); $[\alpha]_D$ -29° (MeOH, c, 0.1); UV λ_{max} (CH_3CN) 224 nm ; IR ν_{max} (KBr) cm^{-1}: 2918, 2862, 1754, 1689, 1458, 1388, 1328, 1219, 1037, 1010, 962, 742, 675, 628; ^{1}H and ^{13}C NMR: δ (ppm): See Table 14; EIMS *m/z* (rel. int.): 234 $[M]^{+}$ (7), 219 (7), 124 (89), 111(30), 109 (100), 69 (28).

3β-Acetoxycinnamolide [19]: White crystals (n-hexane-ethyl acetate), $C_{17}H_{24}O_{4,}$ R_f value 0.26 (n-hexane-ethyl acetate 4:1), mp 152-155 °C (lit. [Kioy *et al.*, 1990a] 153 - 155 °C); $[\alpha]_D$ +7° (MeOH, c, 0.1); UV λ_{max} (CH_3CN) 221 nm; IR ν_{max} (KBr) cm^{-1}: 2970, 2955, 1754, 1728, 1682, 1482, 1461, 1444, 1422, 1396, 1376, 1244, 1201, 1140, 1095, 1041, 1040, 967, 742; ^{1}H and ^{13}C NMR: δ (ppm): See Table 15; EIMS *m/z* (rel. int.): 292 $[M]^{+}$ (100), 232 (45), 231 (5), 122 (6), 121 (26), 106 (7), 95 (3)

Bemadienolide [102]: White crystals (n-hexane- ethyl acetate mixture), $C_{15}H_{20}O_{2,}$ R_f value 0.22 (n-hexane: ethyl acetate 3:2), mp 124-126 °C (lit. [Canonica *et al.*, 1969b] 124 - 124 °C); $[\alpha]_D$ -181° (MeOH, c, 0.35); UV λ_{max} (CH_3CN) 210 nm ; IR ν_{max} (KBr) cm^{-1}: 2978, 1760, 1640, 1461, 1398, 1246; ^{1}H and ^{13}C NMR: δ (ppm): See Table 16; EIMS *m/z* (rel. int.): 232 $[M]^{+}$ (27), 217 (10), 203 (18), 176 (15), 173 (18), 161 (26), 149 (39), 147 (100), 131 (20), 119 (32), 91 (28), 41 (28).

Drimenin [103]: White crystals (n-hexane-ethyl acetate), $C_{15}H_{22}O_{2,}$ R_f 0.18 (n-hexane: ethyl acetate 3:2), mp 131-133 °C (lit. [Wenkert *et al.*, 1964] 132 - 133 °C); $[\alpha]_D$ -42° (MeOH, c, 0.76); UV λ_{max} (CH_3CN) 226 nm ; IR ν_{max} (KBr) cm^{-1}: 2923, 2848, 1761, 1706, 1487, 1385, 1365, 1274, 1172, 1134, 1003, 985, 933, 861, 809, 715, 560; ^{1}H and ^{13}C NMR, ^{13}C NMR: δ (ppm): See Table 17; EIMS *m/z* (rel. int.): 235 $[M]^{+}$ (100), 191 (10), 189 (10), 179 (7), 139 (6), 124 (28), 111 (25), 95 (12), 91 (5).

3.3.5 Isolation of compounds from *E. shweinfurthianum*

Isolation of compounds from n-hexane extract: The crude extract (10 g) was mixed with silica gel in a minimum amount of n-hexane in a round bottom flask. The solvent was removed in a *vacuo* to obtain a free flowing brown substance. The extract was fractionated over silica gel column (2.5 x 60 cm, 100g) using n-hexane with gradient of ethyl acetate up to 100%. A total of 100 fractions each of 20 ml were collected and their composition monitored by TLC using solvent systems: n-hexane ethyl acetate, 9:1 and 4:1. Fractions showing similar TLC profiles were combined resulting into three pools (I-III). Pool I (fractions 1-23, 1 g) did not show any spot on TLC and was discarded. Pool II (fractions 31-68, 3 g) crystallized out to give a white compound upon which, on further purification using n-hexane-ethyl acetate (9:1) gave α-amyrin acetate [**105]** 56 mg. The mother liquor of this pool upon TLC analysis using n-hexane-EtOAc (9:1) showed one spot of R_f values 0.45, and was subjected to further column chromatography with n-hexane- EtOAc (9:1) to afford stigmasterol [**90**] (78 mg). Pool III (fractions 73-100, 3 g) also crystallized out and after re-crystallization (n-hexane-EtOAc, 9:1) afforded further stigmasterol [**90**] 45 mg.

Isolation of compounds from ethyl acetate extract: Ethyl acetate extracts of *E. shweinfurthianum* (75 g) was fractionated over in silica gel column (3.0 x 60 cm, 200 g) using n-hexane-ethyl acetate (10% increment of ethyl acetate), ethyl acetate neat and finally with CH_2Cl_2-MeOH (with 10% and 20% increment of MeOH) to yield 251 fractions (20 ml each). The composition of the fractions was monitored by TLC using n-hexane-ethyl acetate (4:1, 3:2 and1:1) and CH_2Cl_2-MeOH (9:1 and 4:1) as solvent systems. Fractions showing similar TLC profiles were combined resulting in five pools (I-V).

Pool I (fractions 1-44, 8.2 g) which gave one spot on TLC R_f value 0.23 (n hexane-ethyl acetate 9:1) on subjection to further fractionation eluting with n hexane-ethyl acetate (95:5, 9:1) gave α-amyrin acetate [**105**] 30 mg, . Pool II (fractions 45-113, 15 g) which was eluted with n-hexane: ethyl acetate mixture (9:1) showed three spots of R_f values 0.23, 0.33 and 0.42 on TLC with the same solvent system. Further column chromatography with n-hexane: ethyl acetate mixture (95:5, 9:1, 4:1) further afforded α-amyrin acetate [**105**] 72 mg, 3-oxofriedooleanane [**44**] 65 mg and β-sitosterol [**63**] 54 mg. Pool III (fractions 114-163, 17g) which was eluted with n-

hexane: ethyl acetate mixture (4:1) gave three major spots on TLC using the same solvent system. On subjection to further column chromatography eluting with n-hexane: ethyl acetate mixture (9:1, 4:1, 3:2) pool III yielded stigmasterol [**90**] 78 mg, 3-oxofriedoolean-28-al [**45**] 80 mg and 3α-hydroxyfriedooleanane [**106**] 83 mg. Pool IV (fractions 164-211, 12.5 g) showed two major spots on TLC with n-hexane: ethyl acetate mixture (7:3) and on further fractionation eluting with n-hexane: ethyl acetate mixture (4:1, 7:3) afforded α-amyrin [**107**] 77 mg and 3-oxofriedoolean-29-ol [**108**] 93 mg. Pool V (fractions 212-151, 8.9 g) was further subjected to column chromatography using of n-hexane: ethyl acetate mixture (7:3, 3:2) to yield lanosterol [**109**] 74 mg.

β-Sitosterol [63]: white crystals (n-hexane-ethyl acetate), $C_{29}H_{50}O$, R_f value 0.42 (n-hexane: ethyl acetate 9:1), mp 134-135 °C(lit. [Abu-Mustafa *et al.,* 1960] 136 - 137 °C); $[\alpha]_D$ +49° (MeOH, c, 0.35); UV λ_{max} (CH_3CN) 202 nm; IR ν_{max} (KBr) cm^{-1}: 3404, 2924, 1635, 1455; ^1H and ^{13}C NMR: δ (ppm): See Table 18; ESI-MS *m/z* 437 $[M+Na]^+$.

Stigmasterol [90]: white crystals (n-hexane-ethyl acetate), $C_{29}H_{50}O$, R_f 0.16 (n-hexane: ethyl acetate 9:1), mp 169-170 °C (lit. [Shammel *et al.,* 1996] 169.5 °C); $[\alpha]_D$ +51° (MeOH, c, 2.0); UV λ_{max} (CH_3CN) 257 nm; IR ν_{max} (KBr) cm^{-1}: 3356, 2941, 2869, 1663, 1647, 1457, 1381, 1369; ^1H and ^{13}C NMR: δ (ppm): See Table 18; EIMS *m/z* (rel. int.): 412 $[M]^+$ (24), 369 (4), 314 (1), 300 (4), 271 (36), 255 (8), 246 (10), 231 (4), 171 (4), 159 (8), 147 (14), 133 (13), 119 (21), 107 (31), 81 (67), 55 (100), 45 (85).

Lanosterol [109]: White crystals (5% MeOH in CH_2Cl_2), $C_{30}H_{50}O$, R_f value 0.46 (n-hexane: ethyl acetate 4:1), mp 144-145 °C (lit. [Clayton and Bloch, 1955] 132 - 133 °C); $[\alpha]_D$ +62° (MeOH, c, 1.12); UV λ_{max} (CH_3CN) 202 nm; IR ν_{max} (KBr) cm^{-1}: 3462, 2928, 1644, 1445; ^1H and ^{13}C NMR: δ (ppm): See Table 18; ESI-MS *m/z* 449 $[M+Na]^+$.

3-Oxofriedooleanane [44]: White crystals (n-hexane-ethyl acetate mixture), $C_{30}H_{50}O$, R_f value 0.33 (n-hexane-ethyl acetate 9:1), mp 260 -261 °C (lit. [Sod *et al.,* 2009] 258.1 – 261.3 °C); $[\alpha]_D$ -28° (MeOH, c, 1.12); IR ν_{max} (KBr) cm^{-1}: 1706, 2927-2869, 1450, 1339; ^1H and ^{13}C NMR: δ (ppm): See Table 19 & 20; EIMS *m/z* (rel. int.): 426 $[M]^+$ (100), 411 (20), 344 48), 341 (10), 302 (36), 273 (50), 246 (34), 218 (36), 205 (42), 179) (34), 137 (28), 125 (60), 123)62), 109 (72), 95 (82), 69 (88), 55 (59), 41 (32).

3α-Hydroxyfriedooleanane [106]: White crystals (CH_2Cl_2-MeOH 9:1), $C_{30}H_{52}O$, R_f value 0.28 (n-hexane ethyl acetate 4:1) mp 290-292 °C (lit. [Budzikiewics *et al.,* 1963] 303 – 304.3 °C); $[\alpha]_D$ +18° (MeOH, c, 1.12); IR ν_{max} (KBr) cm^{-1}: 3476, 2933, 2869, 1449, 1338; ^1H and ^{13}C NMR: δ (ppm): See Table 18 & 20; EIMS *m/z* (rel. int.): 428 $[M]^+$ (30), 413 (22), 395 (30), 248 (12), 220 (30), 206 (22), 177 (21), 165 (67), 137 (26), 123 (48), 108 (74), 95 (100)69 (90), 55 (44), 41 (22).

3-Oxofriedoolean-29-ol [108]: White crystals (n-hexane- ethyl acetate mixture), $C_{30}H_{50}O_2$, R_f value 0.38 (n-hexane: ethyl acetate 4:1), mp 250-252 °C (lit. [Abbas *et al*., 2007] 252 - 253 °C); $[\alpha]_D$ +87° (MeOH, c, 1.0); ^1H and ^{13}C NMR: δ (ppm): See Table 19 & 20; ESI-MS *m/z*: 465 $[M+Na]^+$.

3-Oxofriedoolean-28-al [45]: White needles (5% MeOH in CH_2Cl_2), $C_{30}H_{48}O_2$, R_f value, 0.22 (n-hexane- ethyl acetate 4:1), mp 263-265 °C (Mahato and Kundu, 1994] 262 - 263 °C);; $[\alpha]_D$ +65° (MeOH, c, 1.0); ^1H and ^{13}C NMR: δ (ppm): See Table 19 & 20; ESI-MS *m/z*: 463 $[M+Na]^+$.

α-Amyrin [107]: White crystals (5% MeOH in CH_2Cl_2), $C_{30}H_{50}O$, R_f value of 0.32 (n-hexane: ethyl acetate 4:1), mp 180-181 °C (lit. [Goldstein and Jenkins, 2006] 181 – 181.5 °C); $[\alpha]_D$ +83° (MeOH, c, 1.12); ^1H and ^{13}C NMR: δ (ppm): See Table 21; ESI-MS *m/z*: 449 $[M+Na]^+$.

α-Amyrin acetate [105]: White crystals (n-hexane-ethyl acetate mixture), $C_{32}H_{52}O_2$, R_f 0.23 (n-hexane: ethyl acetate 9:1) mp 214-215 °C (lit. [Goldstein and Jenkins, 2006] 225 – 226 °C); $[\alpha]_D$ +77° (MeOH, c, 1.22); ^1H and ^{13}C NMR: δ (ppm): See Table 21; ESI-MS *m/z*: 491 $[M+Na]^+$.

3.3.6 Isolation of compounds from *T. brownii*

The composition of n-hexane extract of *T. brownii* was monitored by TLC and was found to contain no major spot/ compound and was discarded. Ethyl acetate extract (75 mg) was subjected to column chromatography over silica gel eluting with n-hexane-ethyl acetate (10% increment of ethyl acetate), ethyl acetate neat and finally with CH_2Cl_2-MeOH (with 10% and 20% increment of MeOH) to give 132 fractions (each 20 ml). The composition of the fractions was monitored by TLC eluent: n-hexane-ethyl acetate (4:1, 3:2 and1:1) and CH_2Cl_2-MeOH (9:1 and 4:1). Fractions showing similar TLC profiles were combined, resulting to five pools (I-V). Pool I (fractions 3-16, 6 g) showed an intense purple colouration upon spraying with

anisaldehyde – conc. H_2SO_4 mixture and heating. The pool contained mainly fatty acids and was discarded. Pool II (fractions 18-32, 8.5 g) which showed two spots on TLC with n-hexane- ethyl acetate (9:1) was subjected to further column chromatography eluting with n-hexane: ethyl acetate (95:5, 9:1, 85:15, 4:1). Recrystalization was done in CH_2Cl_2 – EtAOC mixture to afford β-sitosterol [**63**], 85 mg and stigmasterol [**90**] 65 mg. Pool III (fractions 37-66, 10.5 g) also showed two spots on TLC with n-hexane- ethyl acetate (4:1) was further fractionated by column chromatography eluting with n-hexane: ethyl acetate (4:1, 75:25, 7:3, 65:35, 3:2) to give monogynol A [**110**], 95 mg and betulinic acid [**88**], 98 mg on recrystalization with CH_2Cl_2 – EtAOC mixture . Pool IV (fractions 72-101, 9.6 g) afforded arjungenin [**83**], 65 mg after repeated column chromatography eluting with EtOAc followed by EtOAc: MeOH mixture (95:5, 9:1).

Monogynol A [110]: White crystals (CH_2Cl_2 - MeOH), $C_{30}H_{52}O_2$, R_f value of 5.8 (n-hexane: ethyl acetate 9:1), mp 237-238 °C (lit. [Ulubleen *et al.*, 1994] 232 °C); $[\alpha]_D$ +39° (MeOH, c, 1.28); UV λ_{max} (CH_3CN) 210 nm; ^{1}H NMR ($CDCl_3$, 360 MHz) δ ppm: 3.20 (1H, dd, *J* = 7.9, 3.6 Hz, H-3), 1.78 (1H, m, H-19), 1.74 (1H, m, H-1), 1.61 (1H, m, H-2), 1.49 (1H, m, H-18), 1.36 (1H, m, H-7), 1.23 (1H, m, H-9), 1.18, 1.09, 1.05, 0.97, 0.95, 0.85, 0.84, 0.76 (24H, s, 8 x Me); ^{13}C NMR: δ (ppm): See Table 22; ESI-MS m/z 467 $[M+Na]^+$.

Butelinic acid [88]: White crystals from dichloromethane – methanol mixture, $C_{30}H_{48}O_3$, R_f value 0.18 (n-hexane: ethyl acetate 7:3), mp 280 - 282 °C (lit. [Sod *et al.*, 2009] 279 - 281 °C); $[\alpha]_D$ +10° (MeOH, c, 1.0); UV λ_{max} (CH_3CN) 220 nm; IR ν_{max} (KBr) cm^{-1}: 3455 (OH), 3072 (=CH_2), 2942, 2869, 1687, 1642, 1458, 1387; ^{1}H NMR ($CDCl_3$, 360 MHz) δ ppm: 4.73 (1H, *br*. s, H-29a), 4.60 (1H, *br*. s, H-29b), 3.20 20 (1H, dd, *J* = 8.2, 6.0 Hz, H-3), 2.99 20 (1H, dt, *J* = 10.8, 5.4 Hz, H-19), 2.24 20 (1H, m, H-16), 1.97 20 (1Hm, H-22), 1.69, 0.97, 0.97, 0.96, 0.83, 0.75 (18H, s, 6 x Me); ^{13}C NMR: δ (ppm): See Table 22; EIMS m/z (rel. int.): 456 $[M]^+$ (65), 438 (18), 248 (70), 207 (65), 189 (100).

Arjungenin [83]: White amorphous solid from CH_2Cl_2-MeOH mixture, $C_{30}H_{48}O_6$, R_f value of 0.32 (n-hexane: ethyl acetate 1:1), mp 282-283 °C. lit. [Honda *et al.*, 1976a] 293-294 °C); $[\alpha]_D$ +29° (MeOH, c, 2.0); UV λ_{max} (CH_3CN) 214 nm; IR ν_{max} (KBr) cm^{-1}: 3404, 2935, 2720, 1705, 1640, 1462, 1362, 1067, 1024; ^{1}H NMR ($CDCl_3$, 360 MHz) δ ppm: 5.22 (1H, *br*. s, H-12), 4.48 (1H, d, *J* = 8.1 Hz, H-3), 4.01 (1H, m, H-2), 3.75 (1H, d, *J* = 10.1, Hz, H-23a), 3.36 (1H, d, *J* = 10.0 Hz,

H-23b), 3.33 (1H, m, H-19), 1.27, 1.08, 0.89, 0.87, 0.83, 0.64 (18H, s, 6 x Me); ^{13}C NMR: δ (ppm): See Table 22**.** ESI-MS m/z 527 $[M+Na]^+$.

3.4 Evaluation of antifungal and antibacterial activity of plant extracts

3.4.1 Isolation of spoilage fungi and bacteria from decayed sweet potato

Deteriorating sweet potato tubers were obtained from the open market (Maseno). Pieces 3 x 3 x 2 mm cut from advancing edge of a rot were surface sterilized in 70% alcohol for 1 min, dried on sterile tissue paper and plated out on Nutrient Agar (NA) and Potato Dextrose Agar (PDA). The plates were incubated at room temperature for up to 5 days after which fungal and bacterial growth associated with rot affected tissue identified with the aid of the appropriate taxonomic keys (Ainsworth *et al.,* 1973). The isolates were maintained on PDA slants at 4°C till needed.

3.4.2 Antifungal and antibacterial assay of crude extracts

Antifungal and antibacterial activity of the methanol, ethyl acetate and n-hexane extracts of *W. ugandensis, E. schweifurthianum and T. brownii* was evaluated by the agar diffusion method (Barry *et al.,* 1979). Test fungi used were *Alternaria spp, A. niger, F. oxysporum, F. solanum* and *R. stolonifer* while test bacteria used were *R. solanacearum* (gram negative) and *S. ipomoeae* (gram positive). The test was performed in sterile petri-dishes (90 mm diameter) containing 20 ml PDA and NA for fungi and bacteria, respectively. PDA and NA media were prepared by reconstituting 39 and 28 g in 1 litre of distilled water and heated to dissolve completely. The media were sterilized by autoclaving at 120 °C for 20 min. Inoculation was done by spreading 0.5 ml of spore suspension (1 x 10^5 colony forming unit/ ml) of the test pathogen on the surface of the solidified agar (Kariba *et al.,* 2001). Paper disc (Whatmann No. 1, 5 mm diameter) were impregnated with 100 µl of the plant extracts (5 mg/ml) using a sterile micropipette and left for 30 min to dry at room temperature in the hood. The dried discs were placed on the surface of the solidified inoculated agar and incubated at 28 ºC for 48 h for fungi and 37 ºC for 24 h for bacteria. Blitox, dithane M-5 and streptomycin sulphate (10µg/ml) were used as positive controls while DMSO without plant extract was used as a negative control. All tests were done in triplicates. The presence of zones of inhibition around the disc was interpreted

as an indication of antimicrobial activity. Inhibition zones were measured as described by Reiner (1982).

3.4.3 Antifungal and antibacterial assay of pure isolates and minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of pure isolates were determined using the method described by Anastasiah and Ndalut (2005).) at varying concentrations of 200, 100, 50, 25, 12.5, 6.25, 3.13 and 1.56 µg/ml. Sterile paper discs were impregnated with 100 µl of the reconstituted samples. The dried discs were transferred aseptically into PDA and NA plates previously inoculated with test fungi and bacteria, repectively as described in Section 3.4.2. MIC (in µg/ml) was regarded as the lowest concentration that produced a visible zone of inhibition.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Viruses infecting sweet potato in western Kenya

Symptomatic sweet potato samples collected from farmers' fields in Western Kenya were tested for SPFMV, SPCSV, SPMMV, SPCFV, SPMSV, SPCa-LV, SPLV, C-6, SPVG and CMV using NCM-ELISA. Out of the 327 samples tested, 89% were infected whereas (11%) gave negative results (Table 2). Samples from Western Province showed 94% infection whereas those from Nyanza Province showed 84% infection. Samples from Bungoma, Busia, Kakamega, Kisii and Rachuonyo showed 100% infection. Siaya District of Nyanza Province showed the least frequency of infection (60%).

Five viruses namely SPFMV, SPCSV, SPMMV, SPCFV and CMV were detected from the serological analysis (Table 3). SPFMV, which was the most widespread, was detected in samples from all the 10 districts surveyed with frequency of detection ranging from 60% to 100%. A total of 293 (89%) of the samples tested reacted with SPFMV-specific antibody. SPCSV was the second most widespread and was detected in 179 (55%) of the samples. SPMMV, SPCFV and CMV were also detected in the samples at 17%, 12% and 5% respectively. SPMMV was detected in 9 out of 10 districts whereas SPCFV was detected in 8 out of 10 districts. CMV, which was the least widespread, was detected in 4 out of 10 of the districts surveyed namely Bungoma (14%), Teso (11%), Kisii (10%), and Rachuonyo (15%).

Previously, SPFMV, SPCSV, SPMMV, SPCFV and SPMSV were reported to infect sweet potato in Kenya (Ateka *et al.,* 2004; Miano *et al.*, 2006; Nyaboga *et al.,* 2008). The widespread occurrence of SPFMV in the region is in agreement with previous reports that the virus occurs virtually everywhere sweet potato is grown including countries in tropical and sub-tropical areas as well as temperate regions (Moyer and Salazar, 1989; Salazar and Fuentes, 2001). The widespread occurrence of SPFMV (89%) as compared to the other four viruses detected might be related to the way farmers select their planting materials. Since sweet potato plants that are singly infected with SPFMV exhibit mild or no clearly visible symptoms (Gibson

et al., 1997), farmers find it difficult to distinguish and exclude SPFMV- infected cuttings from the planting materials they select for the next crop, thereby maintaining this virus.

Table 2: Proportion of samples per district that reacted positive for one or more viruses tested from NCM-ELISA

Province	District	Number of samples tested	Plants that reacted positive to one or more viruses (%)
Western	Bungoma	36	100
	Busia	33	100
	Kakamega	27	100
	Teso	27	85
	Vihiga	30	78
	Mean	**30**	**93**
Nyanza	Homa Bay	30	90
	Kisii	27	100
	Kisumu	30	70
	Rachuonyo	57	100
	Siaya	30	60
	Mean	**36**	**84**
Overall mean		**33**	**89**

Table 3: Proportion (%) of sweet potato samples that reacted positive for different viruses detected from NCM-ELISA

Province	District	No. of samples tested	SPFMV	SPCSV	SPMMV	SPCFV	CMV
Western	Bungoma	36	100	92	17	25	14
	Busia	33	100	91	9	18	0
	Kakamega	27	89	44	10	10	0
	Teso	27	89	56	33	0	11
	Vihiga	30	90	50	10	10	0
	Mean	**30**	**94**	**67**	**16**	**13**	**5**
Nyanza	H/bay	30	90	50	10	5	0
	Kisii	27	100	44	11	6	10
	Kisumu	30	70	50	20	20	0
	Rachuonyo	57	100	42	32	21	15
	Siaya	30	60	30	0	0	0
	Mean	**36**	**84**	**43**	**15**	**10**	**5**
Overall mean		**33**	**89**	**55**	**15**	**10**	**5**

SPFMV = Sweet potato feathery mottle virus; SPCSV = Sweet potato chlorotic stunt virus; SPMMV = Sweet potato mild mottle virus; SPCFV = Sweet potato chlorotic fleck virus; CMV = Cucumber mosaic virus.

The high frequency of detection of the dual infection of SPFMV and SPCSV in this study concurred with findings from previous studies (Mukasa *et al*., 2003; Ateka *et al.,* 2004; Tairo *et al*., 2004; Miano *et al.,* 2006; Nyaboga *et al*., 2008). Co-infection of SPFMV and SPCSV causes sweet potato virus disease (SPVD) which is economically the most important disease of sweet potato (Gibson *et al*., 1998; Mukasa *et al*., 2003). The viral mixtures of infections lead to synergistic effect which results in severer damage to sweet potato than it would be achieved if an individual virus was infecting the crop alone (Gutierrez *et al*., 2003). All CMV infected plants carried SPFMV and this observation was in agreement with previous reports (Cohen *et al.,* 1988), suggesting a synergistic co-existence between the two viruses.

Over 80% of the samples tested showed presence of mixed infections. The most common infection combination was that of SPFMV and SPCSV and was detected in 52% of the samples (Fig. 3). Other dual infection combinations detected were SPFMV + SPMMV (13%), SPFMV + SPCFV (14%), SPFMV + CMV (4%), SPCSV + SPMMV (9%), SPCSV + SPCFV (10%) and SPCFV + SPMMV (5%). The most widespread triple infection combination detected was involving SPFMV + SPCSV + SPMMV which was detected in 9% of the samples. Other triple infection combinations detected include SPFMV + SPCSV + SPCFV (2%) and SPFMV + SPCSV + CMV (1%). A complex virus infection involving 4 viruses (SPFMV + SPCSV + SPMMV + SPCFV) was detected in 1% of the samples.

Out of the 327 symptomatic samples tested, 89% were infected by at least one of the viruses tested (Table 2). The high incidence of viral infection could be attributed to the piece-meal harvesting and continuous cropping which are practiced in the region, thereby providing a continuous reservoir of the infecting pathogens. Whereas samples were selected on the basis of presence of virus-like symptoms, 11% did not react with any of the ten virus-specific antibodies used in this study. This observation suggests the presence of variants of known viruses that are not recognized by the antibodies used or new viruses. More assays targeting viruses other than the ones tested in this study is necessary.

Total RNA extracted from samples that reacted positively with antibodies specific to CMV were subjected to RT-PCR using primers specific to CMV to confirm that the positive colour reaction observed in the NCM-ELISA did not result from contamination or an artifact. Analysis of PCR products by electrophoresis using 1% agarose gel showed successful DNA

amplification from twelve CMV-infected sweet potato samples. PCR products of expected size were observed at 670 bp from the infected samples whereas no product was observed from the healthy control. This is the first time that CMV is reported in sweet potato in Kenya.

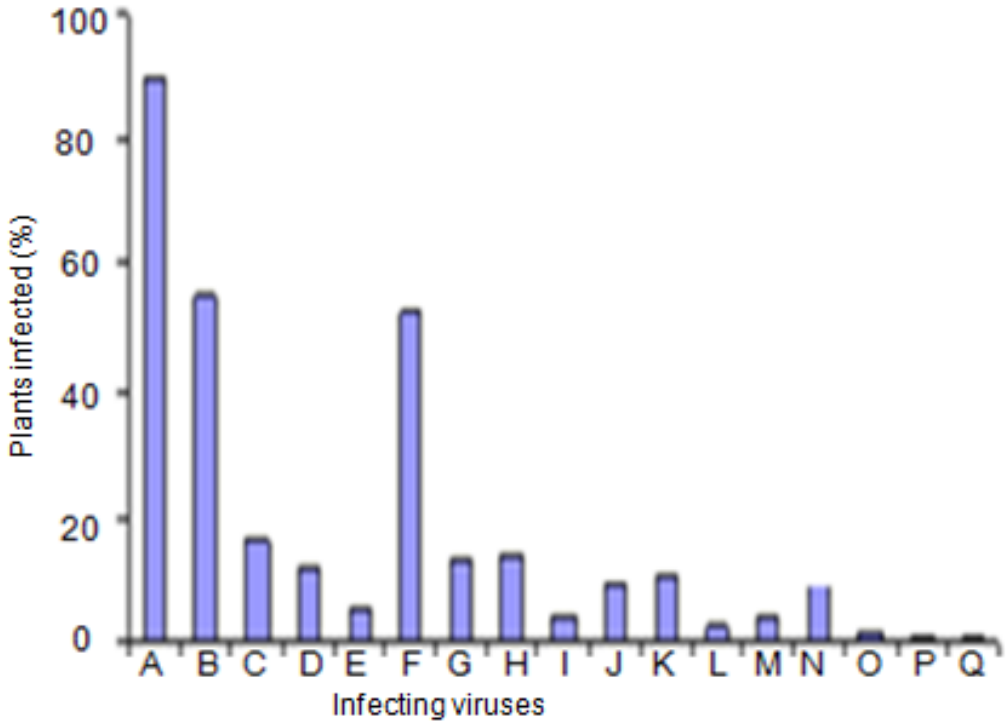


Fig. 3: Proportion (%) of single and mixed virus infections detected by NCM-ELISA in symptomatic sweet potato plants in western Kenya. SPFMV (**A**), SPCSV (**B**), SPMMV (**C**), SPCFV (**D**), CMV (**E**), SPFMV + SPCSV (**F**), SPFMV + SPMMV (**G**), SPFMV + SPCFV (**H**), SPFMV + CMV (**I**), SPCSV + SPMMV (**J**), SPCSV + SPCFV (**K**), SPCSV + CMV (**L**), SPCFV + SPMMV (**M**), SPFMV + SPCSV + SPMMV (**N**), SPFMV + SPMMV + SPCFV (**O**), SPFMV + SPCSV + CMV (**P**), SPFMV + SPCSV + SPMMV + SPCFV (Q).

4.2 Multiplex PCR protocol for detection of SPFMV and SPCSV

Two PCR primers set specific to SPFMV and SPCSV were selected for optimization of the mPCR. Different amplification assays with varying amounts of (1.0 – 3.0 μg), varying annealing temperatures (60 – 63 ºC) and varying number of cycles (30 – 35) were also performed to optimize the multiplex reaction conditions. The best amplification was obtained with 2.0 μg of the cDNA template with 35 cycles compared to 1.0 μg and 3.0 μg (results not shown). There was no significant difference between 60 ºC and 63 ºC for the annealing temperature; although better results were obtained by 63 ºC than 60 ºC. PCR products of expected sizes for SPFMV and SPCSV (703 and 235 bp, respectively) were obtained from sweet potato leaf samples infected with SPFMV and SPCSV by both sPCR and mPCR (Fig. 4) while no amplification products were obtained from healthy plants.

Thirteen sweet potato samples showing sweet potato virus disease (SPVD) symptoms tested in PCR assay were analyzed using NCM – ELISA to confirm the PCR results. Antibodies specific to SPFMV and SPCSV obtained from International Potato Centre (CIP, Lima, Peru) were used. All the samples tested, including the positive control reacted positively (spots developed a purple coloration after the final colour development reaction) with both SPFMV and SPCSV antibodies. Spots from healthy sweet potato samples (negative control) did not show any apparent colour change on nitrocellulose membrane.

In East Africa, the presence of high incidences associated with virus diseases in sweet potato is attributed to the use of infected planting materials (the most common virus source). The vegetative propagation usually done using cuttings from a previous crop to establish a new crop (Karyeija *et al.,* 1998) also increase the risk of build up of viruses. To reduce the chances of virus transmission through use of infected cuttings, use of virus tested planting materials is necessary. Results from this study show the successful use of a simplified mPCR as a rapid assay for the simultaneous detection of SPFMV and SPCSV leading to improved turnaround time and reduced cost of virus detection. Since the primers used for SPFMV amplification were broad based, this increases the chances of detection and are therefore appropriate for routine assays.

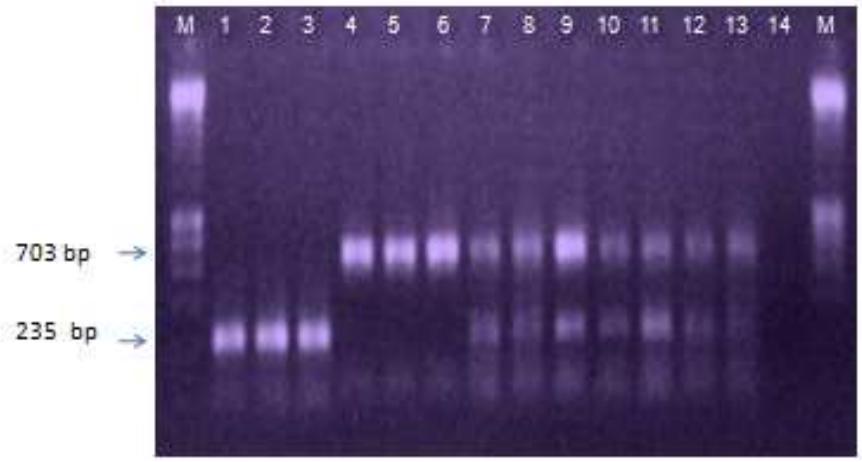


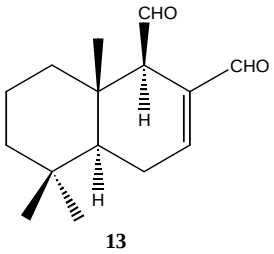
Fig. 4: Electrophoresis profile of DNA amplified products from total RNA obtained from 13 symptomatic and 1 healthy sweet potato samples by singlex and multiplex PCR. Lane M: DNA size marker (200 bp); Lanes 1 – 3: singlex PCR using primers specific to SPCSV; Lanes 4 – 6: singlex PCR using primers specific to SPFMV; Lane 7 – 13: multiplex PCR with mixed viral cDNA's using two primer pairs specific to SPFMV and SPCSM; Lane 14: healthy control.

4.3 Results from phytochemical studies

Crude extracts from *W. ugandensis, E. schweifurthianum* and *T. brownii* were subjected to repeated column chromatography on silica gel (see materials and methods section) to afforded 26 compounds consisting of one new and 25 known compounds. The structures of the isolates were determined using physical and spectroscopic methods.

4.3.1 Identification of compounds from *W. ugandensis*

Fractionation of extracts from *W. ugandensis* led to the isolation of 14 compounds identified as polygodial [**13**], warburganal [**14**], mukaadial [**15**], ugandensidial [**16**], muzigadial [**22**], 6α-hydroxymuzigadial [**23**], 9-deoxymuzigadial [**101**], ugandensolide [**20**], 7α-acetoxyugandensolide [**104**], deacetoxyugandensolide [**21]**, cinnamolide [**17**], 3β-acetoxycinnamolide [**19**], bemadienolide [**102**] and drimenin [**103**]. Compounds **103 and 104** were isolated from the plant for the first time.



13

Polygodial [**13**] was isolated as white crystals with R_f value of 0.53 (n-hexane: ethyl acetate 4:1), mp 58 – 59 ^oC and $[\alpha]_D$ -131° (MeOH, c, 0.35). The electron ionization mass spectrum (EIMS) 70 eV of **13** (Appendix 1) gave a molecular ion peak at *m/z* 234, corresponding to a molecular formula $C_{15}H_{22}O_2$, a fact which was supported by the ^{13}C NMR and DEPT spectrum (Table 4) which showed 15 distinct carbon signals consisting of three methyl, four ethylene, five methine and three quaternary carbons atoms. The ^1H and ^{13}C NMR data were similar to those reported for polygodial (Kubo *et al.,* 1983; Kioy *et al.,* 1990a; Barrero *et al.,* 1995).

Peaks at δ 201.90 and 193.21 in the ^{13}C NMR spectrum corresponding to an isolated and α,β-unsaturated aldehyde groups, respectively, suggesting **13** to be an 8, 9-dialdehyde sesqiterpene derivative. Similarly peaks at δ 154.41 and 138.06 inferred the presence of a double bond between C-7 and C-8 (Kubo *et al.,* 1976; Fukuyama *et al.,* 1982; Kioy *et al.,* 1990b: Barrero *et al.,* 1995). The carbon signals at δ 33.01, 21.86 and 15.18 in the ^{13}C NMR spectrum were assigned to the methyl carbons at C-13, C-14 and C-15, respectively. The presence of the two aldehyde groups were confirmed by ^1H NMR spectrum (Appendix 1) which showed two singlets at δ 9.53 and 9.47 integrating for one proton each while the peak at δ 7.17 (dd, J = 6.0,

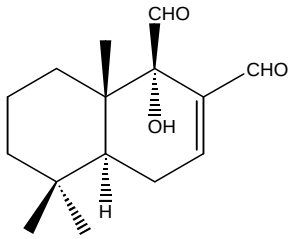
3.0 Hz) was assigned to the olefinic proton at C-7. In the EIMS spectrum, diagnostic peaks at *m/z* 206 $[M\text{-}CHO]^+$, 191, 121, 109 and 45 were in agreement with those previously reported for polygodial (Kubo *et al.*, 1976; Kioy *et al.*, 1990a). On the basis of the spectral data aided by comparison with already published literature (Kubo *et al.*, 1976; Kioy *et al.*, 1990b; Mashimbye *et al.*, 1999; Barrero *et al.*, 1995), compound **13** established to be polygodial.

Table 4: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 13 in $CDCl_3$

C	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{13}C (δ_C)*
1	39.44	CH_2	2.29 m;1.2 m	39.52
2	17.92	CH_2	1.50 m	17.98
3	41.63	CH_2	1.17 m	41.68
4	33.01	C	-	33.09
5	49.03	CH	1.78 dd (12,2, 2.5)	48.92
6	25.13	CH_2	2.54 dddd (20.6, 5.1, 2.5, 2.3) 2.24 ddd (20.6, 12.0, 2.4)	25.18
7	154.41	CH	7.17 dt (6.0, 3.0)	154.21
8	138.06	C	-	139.25
9	60.18	CH	3.25 ddd (4.6, 2.4, 2.2)	60.25
10	36.75	C	-	36.82
11	201.90	CH	9.86 d (4.5)	201.85
12	193.21	CH	9.41	193.16
13	33.01	CH_3	0.94 s	33.09
14	21.86	CH_3	0.97 s	21.93
15	15.18	CH_3	0.92 s	15.25

*^{13}C NMR literature data of polygodial [**13]** (Barrero *et al.*, 1995)

Warburganal [14] R_f value of 0.35 (n-hexane: ethyl acetate 4:1) was isolated as white crystals and had melting point of 134 -135 °C $[\alpha]_D$ -216° (MeOH, c, 1.0). The ESI-MS spectrum of **14** showed quasimolecular ion peaks at *m/z* 273 $[M+Na]^+$, suggesting the molar mass to be 250 ($C_{15}H_{22}O_3$). Its NMR data (Table 5) were similar to those of polygodial [**13**] except for the presence of peaks at δ 4.09 and 77.65 in the ^{1}H and ^{13}C NMR spectra, respectively, which suggested the presence of OH group at C-9 (Kubo *et al.*, 1976; Fukuyama *et al.*, 1982; Ayer and Talamas, 1988; Kioy *et al.*, 1989; 1990a). This was further supported by the ^{13}C NMR spectrum (Appendix 2) which displayed a total of 15 carbon atoms whose multiplicity assignments using DEPT experiments (Appendix 2)



14

established the presence of three methyl, four methylene, four methine and four quaternary carbons. The peaks at δ 202.27 and 192.70 in the ^{13}C NMR confirmed the presence of isolated and α,β-unsaturated aldehyde groups. On the other hand, signals at δ 157.63 and 140.26 confirmed the presence of a tri-substituted double bond between C-7 and C-8 while the peaks at δ 33.03, 22.09 and 15.18 were assigned to the three methyl groups at C-13, C-14 and C-15, respectively (Fukuyama *et al.,* 1982). The fragment ion peaks in the EIMS (70 eV) spectrum at *m/z* 232 $[M\text{-}H_2O]^+$, 221 $[M\text{-}CHO]^+$, 204, and 189 further supported the presence of a dialdehyde drimane sesquiterpene (Kubo *et al.,* 1976; Ayer and Talamas, 1988; Kioy *et al.,* 1989; 1990a; Mashimbye *et al.,* 1999).

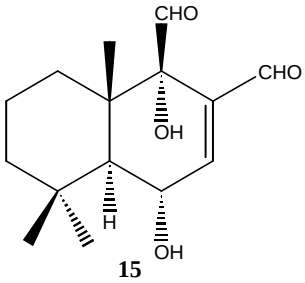
Further examination of the ^{1}H NMR spectrum revealed an olefinic peak at δ 7.28 (dd, J = 4.8, 2.8 Hz) which confirmed the presence of the tri-substituted carbon-carbon double bond between C-7 and C-8. The signals at δ 9.73 and 9.41 were assigned to the aldehyde carbon atoms at C-11 and C-12, respectively while the three singlets at δ 1.09, 0.99 and 0.95, integrating for three protons each, confirmed the three methyl groups at C-13, C-14 and C-15, respectively. On the basis of the spectral data (Table 5) as well as comparison with the reported data in the literature (Kubo *et al.,* 1976; Fukuyama *et al.,* 1982; Ayer and Talamas, 1988; Kioy *et al.,* 1989; 1990a; Mashimbye *et al.,* 1999), compound **14** was concluded to be warburganal.

Table 5: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 14 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{13}C (δ_C)*
1	41.26	CH_2	1.80 m, 1.26 m	41.3
2	17.72	CH_2	1.69 m	17.7
3	31.12	CH_2	1.54 m	31.2
4	33.04	C	-	33.0
5	41.68	CH	1.89 dd, 11.8, 5.1	41.7
6	25.92	CH_2	2.58 dt, 21.6, 5.1 2.35 ddd, 21.6, 11.8, 3.0	25.9
7	157.63	CH	7.28 dd, 4.9, 2.8	157.2
8	140.26	C	-	140.5
9	77.65	C	4.09 s (OH)	77.1
10	41.43	C	-	41.4
11	202.27	CH	9.73 s	202.0
12	192.70	CH	9.41 s	192.5
13	33.01	CH_3	1.09 s	33.0
14	22.09	CH_3	0.99 s	22.0
15	15.18	CH_3	0.95 s	17.0

*^{13}C NMR data of warbuganal from literature (Ayer and Talamas, 1988)

Mukaadial [15], R_f value 0.61 (CH_2Cl_2-MeOH, 97:3), mp 228-229 °C, $[\alpha]_D$ -35° (MeOH, c, 0.5), isolated as white crystals, showed a molecular formula $C_{15}H_{22}O_4$ as determined by ^{1}H, ^{13}C NMR, DEPT) data (Table 6) and a molecular ion peak, $[M]^+$ at *m/z* 266 in the EIMS spectrum. The IR spectrum showed significant peaks at 3347, 2798, 2701, 1720 and 1697 cm^{-1} suggesting OH, aldehyde functional groups and carbon-carbon double bond (Kubo *et al.*, 1983; Kioy *et al.*, 1989).



15

^{13}C NMR and DEPT spectra of **15** (Appendix 3) showed 15 distinct resonances attributed to three methyl, three methylene, two methine, three quaternary, two olefinic and two carbonyl carbons. The spectrum resembled closely that of polygodial [**13**], with the major difference being the presence of two peaks at δ 77.40 and 66.98 for hydroxylated quaternary and methine carbons at C-9 and C-6, respectively (Kubo *et al.*, 1983; Kioy *et al.*, 1989; 1990a; 1990b). Peaks at δ 203.49 and 193.47 were assigned to the aldehyde carbons at C-11 and C-12, respectively while peaks at δ 158.99 and 138.76 were attributed to the olefinic carbons at C-7 and C-8, respectively. The peaks at δ 36.24, 22.48 and 17.89 confirmed the presence of the three

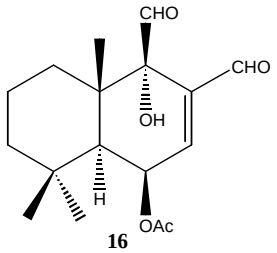
methyl groups at C-13, C-14 and C-15, a fact which was further supported by the presence of three singlets at δ 1.15, 1.08 and 1.03 integrating for three protons each in the ^{1}H NMR spectrum (Appendix 3). Other significant peaks in the ^{1}H NMR spectrum were at δ 9.64(s) and 9.40(s) which confirmed the presence of the two –CHO functional groups; δ 7.03 (s) indicating a vinylic proton (R_2C=CHR); δ 5.15 (s) and 5.04 (s) assigned to the hydroxyl groups and δ 4.41(ddd, *J* = 10.8, 9.0 and 2.4 Hz) assigned to the proton at C-6 (Kubo *et al.*, 1983; Kioy *et al.*, 1989; 1990a; Ying *et al.*, 1995).The large coupling costant observerd between H-5 and H-6 showed H-6 to be equitorial position thus suggesting the stereochemistry at C-6 to be a shown in **15** (Kioy *et al.*, 1989). Based on the spectroscopic data as well as comparison with the reported data in literature, compound **15** was identified as mukaadial.

Table 6: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of 15 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	13C (δ_C)*
1	42.82	CH_2	1.76 ddd (13.2, 13.2, 3.4) 1.42 m	42.4
2	32.84	CH_2	1.75 m	32.6
3	17.77	CH_2	1.37 m	17.3
4	36.24	C	-	36.2
5	47.43	CH	1.89 d (10.8)	47.9
6	66.98	CH	4.41 ddd (10.6, 9.0, 2.4) 5.04 d (4.5)	67.2
7	158.99	CH	7.03 s	158.7
8	138.76	C	-	139.3
9	77.40	C	5.15 s	77.6
10	43.09	C	-	43.1
11	203.49	CH	9.64 s	202.5
12	193.47	CH	9.40 s	192.3
13	17.89	CH_3	1.03 s	17.1
14	22.48	CH_3	1.08 s	21.9
15	36.24	CH_3	1.15 s	35.8

*^{13}C NMR (90 MHz, pyridine-d_5) data of mukaadial from literature (Kioy *et al.*, 1989)

Ugandensidial [16] was isolated as white needles from n-hexane: ethyl acetate, R_f value 0.18 (n-haxane: ethyl acetate 1:1), mp 136-137 °C, $[\alpha]_D$ -360° (MeOH, c, 2.5). The NMR data of compound **16** (Table



16

7) were similar to those of mukaadial [**15**] except for the presence of two extra peaks at δ 169.95 and 21.46 in the ^{13}C NMR spectrum supported by a singlet at δ 2.15 in the ^{1}H NMR spectrum (Appendix 4), integrating for three protons, which suggested that **16** is the acetylated mukaadial (Brooks and Draffan, 1969a; Al-Said *et al.,* 1990; Kioy *et al.,* 1990a).The ^{13}C NMR spectrum of **16** displayed a total of 17 distinct carbon signals corresponding to four methyl, three methylene, five methine and five quaternary carbon atoms. Peaks at δ 201.02 and 192.94 suggested the presence of an isolated and a conjugated carbonyl carbon, respectively, while those at δ 148.54 and 140.89 confirmed the presence of a trisubstituted carbon-carbon double bond at C-7, thus confirming the basic skeleton to be a drimane type sesquiterpene (Brooks and Draffan, 1969a; Al-Said *et al.,* 1990; Kioy *et al.,* 1990). Signals at δ 76.60 and 66.97 confirmed the presence of the two hydroxylated carbons atoms at C-9 and C-6.

Table 7: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 16 in $CDCl_3$

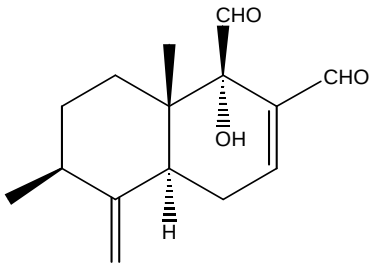
Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{1}H (δ_H,)*
1	44.00	CH_2	1.79 m	nr
2	32.57	CH_2	1.60 m	nr
3	17.59	CH_2	1.55 m	nr
4	33.91	C	-	-
5	44.87	CH	2.05 d (4.7)	2.04
6	66.97	CH	5.90 t (4.7)	5.89
7	148.54	CH	7.01 d (4.7)	7.00
8	140.89	C	-	-
9	76.60	C	4.10 t (1.4)	4.10
10	41.54	C	-	-
11	201.02	CH	9.77 s	9.76
12	192.94	CH	9.49 s	9.48
13	17.67	CH_3	1.17 s	1.17
14	24.74	CH_3	1.34 s	1.34
15	31.80	CH_3	1.03 s	1.03
$CH_3\underline{C}O$	169.95	C	-	-
$\underline{C}H_3CO$	21.46	CH_3	2.15 s	2.14

*^{1}H NMR (250 MHz, $CDCl_3$) data of ugandensidial from literature (Kioy *et al.,* 1990a)

nr = not recorded

Examination of the ^{1}H NMR spectrum futher showed the presence of three tertiary methyl groups (δ 1.03 s, 1.34 s 1ndd 1.17 s) and an olefinic proton (δ 7.01 s), thus further supporting the aforesaid facts (Brooks and Draffan, 1969b; Kioy *et al.*, 1989; Fukuyama *et al.*, 1985). The peaks at δ 9.77 and 9.49 as usual were assigned to the aldehyde protons at C-11 and C-12, respectively, while a triplet at δ 5.90 (t, J = 4.7 Hz) was assigned to the proton at C-6 (Kioy *et al.*, 1990a). The stereochemistry at C-6 was assigned as shown due to the small coupling constant between H-5 and H-6. Based on the NMR data (Table 7), the EIMS diagnostic peaks at m/z 280, 279 [M-CHO], 248 [M-HOAc], 237, 109 and 69 as well as comparison with data from the literature, compound **16** was identified as ugandensidial.

Muzigadial [22], mp 123-125 °C and R_f value of 0.33 (n-hexane: ethyl acetate 3:2), was isolated as white plate-like material from n-hexane - ethyl acetate mixture with $[\alpha]_D$ -230° (MeOH, c, 2.0). Infrared (IR) spectrum of **22** showed significant absorption peaks at 3455 cm^{-1} suggesting the presence of OH group; 1722 cm^{-1} indicating the presence of >C=O carbon and 1685 cm^{-1} indicating the presence of C=C (Kubo *et al.*, 1977; Kioy *et al.*, 1990a; Mashimbye *et al.*, 1999; Rabe *et al.*, 2000).



22

The EIMS spectrum of **22** which gave a molecular ion peak at *m/z* 248 $[M]^{+}$ corresponding to the molecular formula $C_{15}H_{20}O_3$ and was further supported by ^{13}C NMR and DEPT spectrum (Appendix 5) which afforded 15 distinct signals consisting of four quaternary, five methine, four methylene and two methyl carbon atoms suggested a drimane type sesquiterpene. The four olefinic peaks at δ155.64, 151.61, 139.95 and 106.07 in the ^{13}C NMR spectrum (Table 8) confirmed the presence of exocyclic and internal carbon-carbon double bonds at C-4 and C-7 (Al-Said *et al.*, 1990; Ying *et al.*, 1995). The presence of the two aldehyde groups at C-11 and C-12 was confirmed by the two carbonyl peaks at δ 201.21 and 192.66 while the signal at δ 77.61 confirmed the presence of the oxygenated quaternary carbon C-9 (Kioy *et al.*, 1989; Kioy *et al.*, 1990a; Ying *et al.*, 1995).

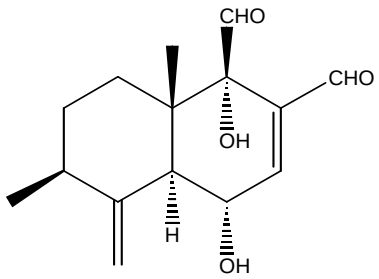
Above evidence was further supported by the ^{1}H NMR spectrum of **22** which was in agreement with data previously reported for compound **22** (Kioy *et al.,* 1990; Ying *et al.,* 1995). A singlet at δ 0.88 and a doublet at δ 1.08 (J = 6.6 Hz), integrating for three protons each, were assigned to the methyl groups at C-15 and C-14, respectively. The peaks at δ 9.64 and 9.43 in the ^{1}H NMR spectrum further confirmed the presence of two aldehyde protons (CHO) while the olefinic signals at δ 4.93 and 4.75 confirmed the presence of a terminal carbon-carbon double bond between C-4 and C-13 (Kioy *et al.,* 1990a; Ying *et al.,* 1995). The peak at δ 7.24 (t, J = 3.5 Hz) was assigned to the proton at C-7. On the basis of the above evidences as well as comparison with literature data (Kioy *et al.,* 1990a; Ying *et al.,* 1995; Mashimbye *et al.,* 1999; Rabe *et al.,* 2000) compound **22** was identified as muzigadial.

Table 8: ^{13}C (90 MHz, $CDCl_3$) and ^{1}H (360 MHz) NMR data of 22 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, J in Hz)	13C (δ_C)*
1	31.70	CH_2	2.01 m;1.68 m	31.7
2	30.83	CH_2	1.71 m	30.9
3	38.18	CH	1.10 m	38.2
4	151.61	C	-	151.6
5	40.21	CH	2.65 m	40.2
6	27.59	CH_2	2.63 m; 2.55 m	27.6
7	155.64	CH	7.24 t, 3.5	155.8
8	139.95	C	-	139.9
9	77.61	C	4.05 s (OH)	77.6
10	42.33	C	-	42.4
11	201.21	CH	9.64 s	201.3
12	192.66	CH	9.43 s	192.3
13	106.07	CH_2	4.93 *br* s; 4.75 *br* s	106.1
14	18.38	CH_3	1.08 d, 6.6	18.4
15	15.05	CH_3	0.88 s	15.1

*^{13}C NMR data of muzigadial from literature (Ying *et al.,* 1995)

6α-Hydroxymuzigadial [23]



23

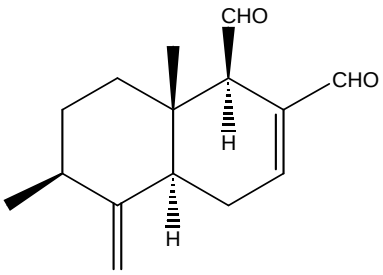
Compound **23** was isolated as white crystals from n-hexane- ethyl acetate mixture and had an R_f value 0.59 (CH_2Cl_2-MeOH 97:3), mp 150-152 °C and $[\alpha]_D$ -97° (MeOH, c, 0.35). The IR spectrum of **23** exhibited significant absorption peaks at 3360, 1727 and 1678 cm^{-1} suggesting the presence of a hydroxyl, carbonyl carbon and C=C (Wube *et al.,* 2005). The ^{13}C NMR data (Table 9) were similar to those reported for muzigadial [**22**] (Kubo *et al.,* 1977; Kioy *et al.,* 1990a; 1990b; Rabe *et al.,* 2000) except for presence of an oxymethine peak at δ 65.94 which suggested an additional OH group at C-6 (Kioy *et al.,* 1990a; 1990b; Brooks and Draffan, 1969a). The ^{13}C NMR spectrum (Appendix 6) showed a total of 15 carbons corresponding to two methyl, three methylene, six methine and four non-protonated carbons. The presence of dialdehyde functional groups was confirmed by ^{1}H-^{13}C detected heteromolecular multiple quantum coherence (HMQC) experiments where the carbon peaks at δ 200.53 (C-11) and 192.61 (C-12) correlated with protons at δ 9.66 and 9.50, respectively. The four olefinic peaks in the ^{13}C NMR spectrum at δ 148.78, 153.92, 139.07 and 106.79 confirmed the presence of two carbon-carbon double bonds in the molecule. The methylene peak at δ_C 106.79 coupled with two vinylic protons at δ_H 5.13 and 5.02. Similarly, the methine peak at δ_C 153.92 coupled to a proton at δ 7.09 in the HMQC spectrum, thus allowing the placement of the carbon-carbon double bonds at C-4 and C-7 (Brooks and Draffan, 1969a; Kioy *et al.,* 1989; 1990a; Ying *et al.,* 1995). The stereochemistry of C-6 OH group was established as axial because of the large coupling constant observed between the 6-H and 5-H (Kubo *et al.,* 1977; 1983; Kioy *et al.,* 1990a; Rabe *et al.,* 2000 Wube *et al.,* 2005). The proton signals at δ 1.12 and 0.95, integrating for three protons each, and correlated to the carbon peaks at δ 18.15 and 15.78, respectively, confirmed the methyl carbons at C-14 and C-15.

The above data was further supported by the EIMS data which afforded a molecular ion peak at *m/z* 265 $[M+1]^+$ corresponding to the molecular formula $C_{15}H_{20}O_4$. Other diagnostic peaks in the CI-MS spectrum were those at *m/z* 247 $[M-H_2O]^+$, 235 [M-CHO), 229 and 173. On the basis of the spectral data as well as comparison with literature data, compound **23** was identified as 6α-hydroxymuzigadial.

Table 9: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of 23 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)
1	31.62	CH_2	2.02 m, 1.69 m
2	31.57	CH_2	1.73 m, 1.03 m
3	38.62	CH	2.02 m
4	148.87	C	-
5	50.18	CH	2.63 d (9.6)
6	65.94	CH	4.67 ddd (9.6, 8.1, 2.4)
7	153.92	CH	7.09 d (9.5)
8	139.75	C	-
9	77.30	C	4.12 s
10	44.02	C	-
11	200.53	CH	9.65 s
12	192.61	CH	9.50 s
13	106.72	CH_2	5.13 s,5.02 s
14	18.15	CH_3	1.12 d, 6.6
15	15.78	CH_3	0.95 s

9-Deoxymuzigadial [101]



101

Compound **101** was isolated as Pale yellow oil, R_f value 0.22 (n-hexane: ethyl acetate 4:1), $[\alpha]_D$ -78° (MeOH, c, 0.35). The ESI-MS spectrum of **190** which showed a molecular ion peak at *m/z* 255 $[M+Na]^+$ corresponding to a molecular mass of 232 amu The ^{13}C NMR and DEPT spectra (Appendix 7) which displayed 15 distinct carbon signals corresponding to two methyl, four methylene, six methine and three quaternary carbon atoms suggested **101** to be a sesquiterpene. The ^{1}H and ^{13}C NMR data of **101** (Table 10) exhibited signals that were similar to those previously reported for muzigadial [**22**] (Kubo *et al.*, 1977; El-Feraly *et al.,* 1978; Al-Said *et al.,* 1990), except for the absence of the oxygenated carbon signal at C-9. Instead, a methine signal at δ 58.3 attributed to the same carbon was observed suggesting **101** to be deoxymuzigadial (Kioy *et al.*, 1989; 1990a; Al-Said *et al.,* 1990). The ^{13}C NMR spectrum signals at δ 201.21 and 192.66 confirmed the presence of an isolated and a conjugated carbonyl carbons at C-11 and C-12, respectively, while the peaks at δ 155.64, 151.61, 139.95 and 106. 07 confirmed the presence of internal and exocyclic double bonds (Al-Said *et al.*, 1990;

Ying *et al.,* 1995). Other diagnostic peaks in the ^{13}C NMR spectrum were observed at δ 15.05 and 18.58 corresponding to the methyl carbons at C-15 and C-14, respectively.

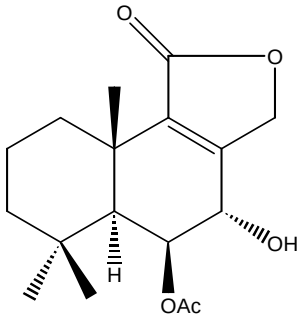
The ^{1}H NMR spectrum displayed three olefinic peaks δ 7.23 dd (*J* = 4.0, 2.3 Hz), 4.93 dd (*J* = 1.5, 1.5 Hz) and 4.75(s) further confirmed the presence of the double bonds (Al-Said *et al.,* 1990). The two singlets at δ 9.64 and 9.44 were assigned to the aldehyde protons at C-11 and C-12, respectively. Based on the spectral data as well as comparison with literature data, compound **101** was concluded to be 9-deoxymuzigadial (Brooks and Draffan, 1969b; Kioy *et al.,* 1990a).

Table 10: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 101 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{13}C (δ_c)*
1	31.70	CH_2	1.92 m, 1.72 m	31.4
2	30.83	CH_2	1.68 m, 1.15 m	31.6
3	38.18	CH	2.03 m	38.5
4	151.61	C	-	151.3
5	42.33	CH	2.51 m	45.8
6	27.59	CH_2	2.48 m	27.0
7	155.64	CH	7.23 dd (4.0, 2.3)	152.9
8	139.95	C	-	137.9
9	58.26	CH	2.65 m	58.3
10	40.21	C	-	38.2
11	201.21	CH	9.64 s	201.1
12	192.66	CH	9.44 s	193.3
13	106.07	CH_2	4.93 dd (1.5, 1.5), 4.75 s	106.0
14	18.38	CH_3	1.07 d (6.3)	18.4
15	15.05	CH_3	0.78 s	13.5

*^{13}C NMR (90 MHz, $CDCl_3$) data of 9-deoxymuzigadial (Al-Said *et al.,* 1990)

Ugandensolide [20]



20

Compound **20** was isolated as white needle crystals from CH_2Cl_2-MeOH (9:1), mp 215-218 °C, R_f value 0.46 (CH_2Cl_2-MeOH 97:3), $[\alpha]_D$ +23° (MeOH, c, 1.0). The EIMS spectrum of **20** showed an ion peak at *m/z* 308 corresponding to a molecular formula $C_{17}H_{24}O_4$. Other diagnostic peaks in the EIMS spectrum were observed at m/z 290 $[M-H_2O]^+$, 248 $[M-HOAc]^+$, 230, 202 and 175.

The ^1H and ^{13}C NMR data (Table 11) were similar to those previously reported for ugandensolide (Brooks and Draffan, 1969a; Canonica *et al.,* 1969a; Kioy *et al.,* 1990a; 1990b; Mohanlall and Odhiv, 2009). In the ^{13}C NMR spectrum (Appendix 8) 17 distinct peaks corresponding to four methyl, four methylene, three methine and six non-protonated carbon atoms were observed, suggesting an acetylated drimane sesquiterpene. The presence of an acetate group was supported by the signal at δ 2.03 s in the ^1H NMR spectrum integrating for three protons, and a signal at δ 170.96 in the ^{13}C NMR spectrum. The ^{13}C NMR spectrum als exhibited a carbonyl peak at δ 171.85 suggesting the presence of a lactone system (Canonica *et al.,* 1969a; Kioy *et al.,* 1990a; 1990b). Olefinic peaks at δ 154.09 and 137.95 confirmed the presence of a carbon-carbon double bond, which was confirmed to be tetra-substituted by the absence of olefinic peak in the ^1H NMR spectrum (Brooks and Draffan, 1969a, Kioy *et al.,* 1989; 1990a).

Table 11: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 20 in $CDCl_3$

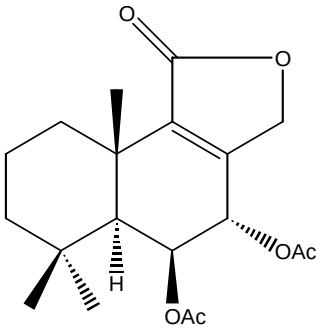
Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{1}H (δ_H,)*
1	42.99	CH_2	2.53 d (12.8)	2.55
2	18.26	CH_2	1.68 m	nr
3	36.29	CH_2	1.18 m	nr
4	35.27	C	-	-
5	49.23	CH	1.63 (1.5)	1.61
6	73.78	CH	5.34 s	5.36
7	66.04	CH	4.17 dd (5.5, 1.0)	4.21
			3.57 d (5.5)	-
8	154.09	C	-	-
9	137.95	C	-	-
10	33.32	C	-	-
11	171.85	C	-	-
12	69.70	CH_2	4.85 d (17.2)	4.91
			4.61 dd (17.2, 1.0)	4.65
13	20.71	CH_3	1.43 s	1.47
14	33.07	CH_3	1.02 s	1.05
15	21.36	CH_3	1.00 s	1.02
$CH_3\underline{C}O$	170.96	C	-	-
$\underline{C}H_3CO$	23.04	CH_3	2.03 s	2.09

*^{1}H NMR (250 MHz, $CDCl_3$) data of ugandensolide (Kioy *et al.*, 1990a)

nr = not reported.

The OH proton at δ 3.57 was coupled with a proton at δ 4.17 dd (*J* = 5.5, 1.0 Hz) confirming the OH to be secondary. The peaks at δ 73.78, 66.04 and 69.70 in the ^{13}C NMR spectrum confirmed the presence of two oxymethine and lactone methylene carbons at C-6, C-7 and C-12, respectively. The three singlets at δ 1.00, 1.02 and 1.43, integrating for three protons each, were assigned to the tertiary methyl carbons at C-13, C-14 and C-15 (Brooks and Draffan, 1969b; Kioy *et al.*, 1990a). A broad singlet at δ 5.34 was assigned to the proton on the carbon bearing the acetate (-C<u>H</u>-OAc) while two doublet at δ 4.85 and 4.61, *J* = 17.2 Hz each, were assigned to the lactone methylene protons, -O-C<u>H</u>$_2$- (Brooks and Draffan, 1969a). The rest of the NMR peaks were assigned as shown in Table 11. Based on the spectral data as well as comparison with literature data, compound **20** was identified as ugandensolide

7α-Acetylugandensolide [104]



104

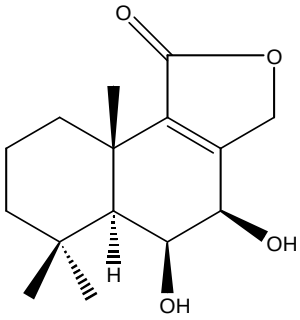
Compound **104** R_f 0.34 (n-hexane - ethyl acetate 1:1), mp 228-230 ^oC, $[\alpha]_D$ +24° (MeOH, c, 0.01); was isolated as white gummy material. High resolution mass spectrum (HRMS, 70 eV) spectrum (Appendix 9) displayed a molecular ion peak at *m/z* 350 $[M]^+$ corresponding to $C_{19}H_{26}O_6$ formula. The IR spectrum showed the presence of an ester carbonyl (1732 cm^{-1}), lactone (1680 cm^{-1}) and olefinic bond (1632 cm^{-1}). The ^{13}C NMR spectrum exhibited 19 distinct carbon signals corresponding to five methyl, four methylene, three methine and seven quaternary carbon atoms. The ^{13}C NMR data (Table 12) were similar to those previously reported for ugandensolide [**20**], except for the presence of two additional peaks at δ 169.78 and 20.76 which suggested the compound to be acetylated ugandensolide (Kioy *et al.*, 1989; 1990a; 1990b), confirmed by the ^1H NMR spectrum (Appendix 9) exhibited a singlet at δ 2.08 integrating for three protons. The absence of the dialdehyde peaks in ^{13}C NMR spectrum and the presence of peaks at δ 170.90 and 70.22 signified lactone carbonyl and methylene carbons, respectively (Canonica *et al.*, 1969a; Kioy *et al.*, 1990a). Peaks at δ 69.64 and 66.60 which correlated to protons δ 5.54 (tr, *J* = 1.7 Hz Hz) and 5.15 (dd, *J* = 1.3 Hz) in the HMBC spectrum were assigned to the oxymethine carbons at C-6 and C-7 respectively further confirmed the diacetyl groups. The stereochemistry at C-6 and C-7 followed from the small vicinal coupling constants of protons at H-5, H-6 and H-7 (Brooks and Draffan, 1969a; Kioy *et al.*, 1990a).

Table 12: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data compound 104 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)
1	43.06	CH_2	2.56 d tr d (13.1, 3.5, 1.6) 1.19 tr dd (13.2, 3.8, 0.8)
2	18.26	CH_2	1.80 m; 1.62 m
3	36.27	CH_2	1.53 m; 1.29 tr dd (13.6, 4.1, 0.8)
4	35.41	C	-
5	50.54	CH	1.54 d (1.7)
6	69.64	CH	5.54 tr (1.7)
7	66.60	CH	5.15 dd (1.3)
8	150.62	C	-
9	140.29	C	-
10	33.34	C	-
11	170.90	C	-
12	70.22	CH_2	4.71 dd (17.8, 1.1) 4.6 1 d (17.7)
13	22.85	CH_3	1.05 s
14	33.14	CH_3	1.03 s
15	20.56	CH_3	1.51 s
6-$CH_3\underline{C}O$	171.06	C	-
6-$\underline{C}H_3CO$	21.27	CH_3	2.13 s
7-$CH_3\underline{C}O$	169.78	C	-
7-$\underline{C}H_3CO$	20.76	CH_3	2.08 s

The position of carbon – carbon double bond was confirmed to be between C-8 and C-9 by the presence of quarenary carbon peaks at δ 150.62 and 140.29 (Canonica *et al.*, 1969a; Kioy *et al.*, 1990a; 1990b). Other diagnostic peaks in ^{13}C NMR observed at δ 22.85, 33.14 and 20.56 were assigned to methyl carbons (C-13, C-14 and C-15). In the HMQC spectrum, peaks at δ 4.71 (dd, *J* = 17.8, 1.1 Hz) and 4.61 (d, *J* = 17.7 Hz) which correlated to the carbon peak at δ 70.22 further confirmed oxymethylene protons (-OCH_2). On the basis of the above spectral evidence together with further diagnostic peaks in the EIMS spectrum *at m/z* 307 $[M\text{-}CH_3CO]^+$, 290 $[M\text{-}HOAc]^+$, 232 $[M\text{-}OAc\text{-}OAc]^+$ and 247 $[M\text{-}OAc\text{-}CH_3]^+$, compound **104** was identified as 7α-acetylugandensolide. Synthetic **104** was previously reported by Brooks and Draffan (1969b) but it was isolated from *W. ugandensis* in this study.

Deacetoxyugandensolide [21]



21

Compound **21,** white needles from n-hexane-ethyl acetate-MeOH (97:2.5:0.5), had mp 260-264 °C, R_f value 0.4 (CH_2Cl_2-MeOH, 95:5) and $[\alpha]_D$ +70° (MeOH, c, 1.0). IR spectrum showed significant peaks at 3350 (OH) and 1727 (>C=O) cm^{-1}. The EIMS of the compound gave an ion peak at *m/z* 266 corresponding to a molecular formula $C_{15}H_{22}O_4$ and was supported by the ^{13}C NMR spectrum (Appendix 10) which gave 15 distinct carbon signals attributed to three methyl, four methylene, three methine and five non-protonated carbons. The ^{1}H and ^{13}C NMR data of **21 (Table 13)** were in agreement with those previously reported for deacetoxylugandensolide (Brooks and Draffan, 1969a; Kioy *et al.,* 1989; 1990a; Ying *et al.,* 1995). The ^{13}C NMR peaks at δ 171.46 was assigned to the lactone carbonyl carbon at C-11 while the olefinic signals at δ 155.59 and 134.25 confirmed the presence a carbon - carbon double bond between C-8 and C-9, a fact further supported by the absence of a vinylic proton peak in the ^{1}H NMR spectrum, thus confirming the double bond to be tetrasubstituted (Brooks and Draffan, 1969b; Kubo *et al.,* 1983; Kioy *et al.,* 1989; 1990a; 1990b). Similarly, the ^{13}C NMR peak at δ 68.19 was assigned to the lactone oxymethylene carbon (-OCH_2R) while those at δ 70.12 and 67.64 were assigned to the oxymethine carbons (-$OCHR_2$) at (C-6 and C-7). Peaks at δ 32.90, 22.83 and 19.94 were assigned to the tertiary methyl carbons atoms positioned at C-13, C-14 and C-15.

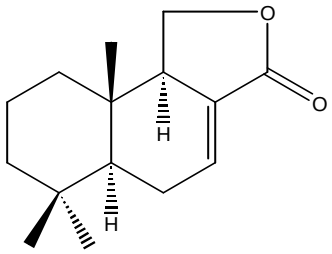
The ^{13}C NMR data were supported by the ^{1}H NMR spectrum which gave peaks at δ 5.17 (s) and 4.30 (s) assigned to oxymethine protons (H-6 and H-7) while signals at δ 4.88 d (*J* = 17.2 Hz) and 4.65 d (*J* = 17.2 Hz) were assigned to the lactone oxymethylene protons. The presence of the three tertiary methyl groups was confirmed by the singlets at δ 1.49, 1.24 and 1.03 (Brooks and Draffan, 1969a; Kioy *et al.,* 1989, 1990a; 1990b). Based on the spectral data as well as comparison with literature data, compound **21** was identified as deacetoxyugandensolide.

Table 13: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 21 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{13}C (δ_c)*
1	42.43	CH_2	2.42 m	42.4
2	17.85	CH_2		17.8
3	35.80	CH		35.9
4	34.75	C	-	34.7
5	49.00	CH	2.83 m	49.2
6	70.12	CH	5.71 s, 4.12 s (OH)	71.0
7	67.64	CH	4.30 s, 3.94 s (OH)	68.8
8	155.59	C	-	156.9
9	134.25	C	-	135.7
10	33.24	C	-	33.6
11	171.46	C	-	171.6
12	68.19	CH_2	4.88 d (17.2), 4.65 d (17.2)	69.3
13	19.94	CH_3	1.49 s	20.0
14	32.90	CH_3	1.24 s	32.7
15	22.83	CH_3	1.03 s	27.7

*^{13}**C** NMR (90 MHz, $CDCl_3$) data of deacetoxylugandensolide (Kioy *et al.*, 1990a)

Cinnamolide [17]



17

Compound **17** was isolated as white crystals from n-hexane-ethyl acetate mixture and had mp 126-128 °C, R_f value 0.42 (n-hexane-ethyl acetate 4:1) and $[\alpha]_D$ -29° (MeOH, c, 0.1). Its IR spectrum showed diagnostic peaks at 1754 and 1689 cm^{-1} sugesting the presence of carbonyl and alkene functional groups. The EIMS spectrum (Appendix 11) afforded a molecular ion peak at *m/z* 234 suggesting a formula $C_{15}H_{22}O_2$ and was supported by ^{13}C NMR spectrum and DEPT experiments which exhibited 15 carbon signals attributed to three methyl, five methylene, three methine and four non-protonated carbon atoms. The NMR data of **17** (Table 14) were similar to those reported for cinnamolide (Canonica *et al.,* 1967a; Kubo *et al.,* 1976; Kioy *et al.,* 1989; 1990a; 1990b; Ying *et al.,* 1995). Infact, the ^{13}C NMR peak at δ 169.suggested the presence of lactone carbonyl carbon while peaks at δ 135.90 and 126.98 inferred the presence of a tri-substituted carbon-carbon double bond at C-7 (Canonica *et al.,* 1967a; Kubo *et al.,* 1976). The carbon-carbon double bond was confirmed to be tri-substituted by the presence of an olefinic peak at δ

6.88 dd (J = 4.5, 3.5 Hz) in ^{1}H NMR spectrum assigned to H-7. The ^{13}C NMR peaks at δ 32.52, 21.00 and 13.07 confirmed the presence of three tertiary methyl carbons at C-13, C-14 and C-15, respectively (Kubo *et al.,* 1976; Canonica *et al.,* 1967a).

In the ^{1}H NMR spectrum, diagnostic peaks at δ 4.38 d (J = 10.0 Hz) and 4.04 d (J = 10.0 Hz) were assigned to the lactone oxymethylene protons at C-11 while those at δ 0.81 s, 0.92 s and 0.95 s integrating for three protons each, signified the methyl protons at C-13, C-14 and C-15 (Kioy *et al.,* 1989; 1990a; Ying *et al.,* 1995). Based on the spectral data as well as comparison with literature data, compound **17** was identified as cinnamolide.

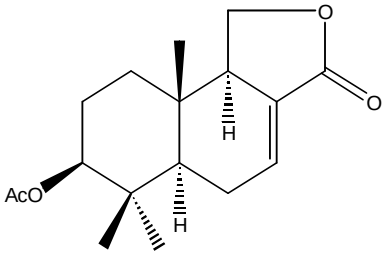
Table 14: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 17 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, J in Hz)	^{1}H (δ_H,)*
1	39.13	CH_2	1.60 m, 1.22 m	nr
2	18.19	CH_2	1.50 m	nr
3	41.86	CH_2	1.30 m	nr
4	33.92	C	-	-
5	50.49	CH	1.40 dd (11.7, 5.3)	1.38
6	24.66	CH_2	2.39 m, 2.12 m	2.10
7	135.90	CH	6.88 dd (4.5, 3.5)	6.87
8	126.98	C	-	-
9	49.37	CH	2.83 dd (9.2, 3.5)	2.80
10	32.82	C	-	-
11	66.88	CH_2	4.38 d (10.0), 4.04 d (10.0)	4.38, 4.04
12	169.81	C	-	-
13	32.52	CH_3	0.92 s	0.92
14	21.00	CH_3	0.95 s	0.94
15	13.07	CH_3	0.81 s	0.81

*^{1}H NMR (250 MHz, $CDCl_3$) data of cinnamolide from literature (Kioy *et al.,* 1990)

Nr = not reported

3β-Acetoxycinnamolide [19]



19

Compound **19** was isolated as white crystals from n-hexane-ethyl acetate and had mp 152-155 °C, R_f value 0.26 (n-hexane-ethyl acetate 4:1), and $[\alpha]_D$ +7° (MeOH, c, 0.1). The EIMS spectrum of **19** gave a molecular ion peak at *m/z* 292 corresponding to molecular formula $C_{17}H_{24}O_4$. This was supported by ^{13}C NMR and DEPT spectra (Appendix 12) which showed 17 carbon peaks corresponding to four methyl, four methylene, four methine and five quaternary carbon atoms. The ^{1}H and ^{13}C NMR data of **19** (Table 15) were similar to those reported for 3β-acetoxylcinnamolide (Canonica *et al.,* 1967a; 1967b; Kioy *et al.,* 1990a, 1990b; Ying *et al.,* 1995). The ^{13}C NMR peaks at δ 170.52 and 169.56 were attributable to the lactone and acetyl carbonyl carbons, respectively while peaks at δ 135.56 and 127.00 were assignable to the olefinic carbons (Canonica et al., 1967a; 1967b; Kioy *et al.,* 1990a). Other siginificant peaks were at δ 79.92 assigned to the acetylated carbon at C-3; δ 66.74 assigned to lactone oxymethylene carbon at C-11; δ 27.58, 21.08, 15.84, 13.32 attributed to the methyl carbons at C-13, C-14, C-15 and MeCO (Kioy *et al.,* 1990a 1990b, Ying *et al.,* 1995).

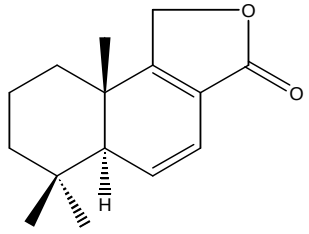
The ^{13}C NMR data were supported by ^{1}H NMR spectrum which confirmed the presence of a vinyl proton at δ 6.87 dt (J = 6.8, 3.5 Hz) and lactone oxymethylene protons at δ 4.39, t (J = 9.2 Hz) and 4.05 t (J = 9.2 Hz). The spectrum also showed the presence of an oxymethine proton (H-3) at δ 4.52 with two coupling constants, one of which (J = 11.5 Hz) indicating an axial configuration, thus confirming the acetyl group to be in equatorial (β) position. Other significant peaks in the ^{1}H NMR spectrum of **19** were at δ 2.07, 0.82, 0.92 and 0.98 each integrating for three protons and were assigned to methyl protons of MeCO and C_{13}, C_{14} and C_{15} methyls (Canonica *et al.,* 1967a; 1967b; Kioy *et al.,* 1990a, 1990b; Ying *et al.,* 1995). Based on the spectral data as well as comparison with literature data, compound **19** was identified as 3β-acetoxycinnamolide.

Table 15: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of 19 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{13}C (δ_c)*
1	36.64	CH_2	1.42 m, 1.64 m	36.7
2	23.33	CH_2	1.72 m	23.4
3	79.92	CH	4.52 dd (11.5, 5.0)	80.0
4	33.80	C	-	33.9
5	50.34	CH	1.46 m	50.5
6	24.44	CH_2	2.40 m, 2.23 m	36.7
7	135.56	CH	6.87 dt (6.8, 3.5)	135.6
8	127.00	C	-	127.1
9	49.06	CH	2.81 dd (4.9, 3.5)	49.2
10	37.43	C	-	37.5
11	66.74	CH_2	4.39 t (9.2), 4.05 t (9.2)	66.8
12	170.52	C	-	170.6
13	13.32	CH_3	0.92 s	13.3
14	15.84	CH_3	0.98 s	15.8
15	27.58	CH_3	0.82 s	27.6
MeCO	169.56	C	-	169.6
MeCO	21.08	CH_3	2.07	21.0

*^{13}**C** NMR (90 MHz, $CDCl_3$) data of 3β-acetoxylcinnamolide (Kioy *et al.,* 1990a)

Bemadienolide [102]



102

Compound **102** was isolated as white crystals from n-hexane- ethyl acetate mixture and had mp 124-126 °C, R_f value 0.22 (n-hexane: ethyl acetate, 3:2) and $[\alpha]_D$ -181° (MeOH, c, 0.35). Its EIMS spectrum exhibited a molecular ion peak at *m/z* 232 corresponding to molecular formula $C_{15}H_{20}O_2$, a fact that was supported by ^{13}C NMR and DEPT spectra (Appendix 13) which showed the presence of 15 carbon atoms consisting of three methyl, four methylene, three methine and five non-protonated carbon atoms. Its ^{1}H and ^{13}C NMR data (Table 16) were similar to those previously reported for bemadienolide (Canonica *et al.,* 1967a; 1967b; Kubo *et al.,* 1977). The ^{13}C NMR peaks at δ 171.66, 131.65, 117.56 and 122.33 cofirmed the presence of an additional carbon-carbon double bond which was assigned to be between C-6 and C-7 (Canonica *et al.,* 1967a; 1967b). Other ^{13}C NMR peaks at δ 170.21 and 67.67 were assigned lactone

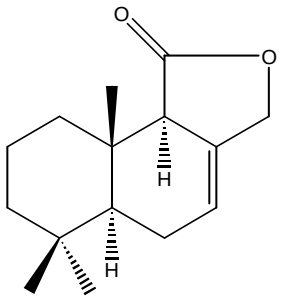
carbonyl and oxymethylene carbon atoms, respectively. Other diagnostic peaks observed at δ 32.25, 22.52 and 14.97 were assigned to the three tertiary methyl carbon atoms at C-13, C-14 and C-15, respectively.

The above data was supported by the ^{1}H NMR spectrum (Appendix 14) which gave peaks at δ 6.32 d (*J* = 10.0 Hz) and 6.01 d (*J* = 10.0 Hz) attributed to olefinic protons at C-6 and C-7; δ 4.83 d (*J* = 17.2 Hz) and 4.77 d (*J* = 17.2 Hz) attributed to lactone oxymethylene protons at C-11; δ 0.99, 1.01 and 1.04 attributed to tertiary the three methyl protons at C-13, C-14 and C-15 (Canonica *et al.,* 1967a; 1967b; Kubo *et al.*, 1977; Fukuyama *et al.,* 1982). Based on the spectral data as well as comparison with literature data, compound **102** was identified as bemadienolide.

Table 16: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of 102 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)
1	40.53	CH_2	1.68 m
2	17.93	CH_2	1.26 m
3	33.53	CH2	1.56 m
4	32.71	C	-
5	52.34	CH	2.22 m
6	117.56	CH	6.01 d (10.0)
7	131.65	CH	6.32 d (10.0)
8	122.33	C	-
9	171.66	C	-
10	36.75	C	-
11	67.67	CH_2	4.83 d (17.2), 4.77 d (17.2)
12	170.21	C	-
13	32.25	CH_3	1.01 s
14	22.52	CH_3	1.04 s
15	14.97	CH_3	0.99 s

Drimenin [103]



103

Compound **103** was isolated as white crystals from n-hexane - ethyl acetate mixture, mp 131-133 °C, R_f value 0.18 (n-hexane: ethyl acetate 3:2) and $[\alpha]_D$ -42° (MeOH, c, 0.76). The EIMS spectrum of **103** exhibited a molecular ion peak at *m/z* 235 $[M+1]^+$ suggesting a molecular formula $C_{15}H_{22}O_2$ and was supported by ^{13}C NMR spectrum (Appendix 14) which gave a total of 15 peaks attributed to three methyl, five methylene, three methine and four non protonated carbon atoms. The ^1H and ^{13}C NMR data (Table 17) were similar to those previously reported for drimenin (Suzuki *et al.,* 1970; Fukuyama *et al.,* 1985; Barrero *et al.,* 1995; Akita *et al.,* 2000). The ^{13}C NMR peaks at δ 175.31 was suggestive of lactone carbonyl presence while those at δ 121.13 and 129.77 indicated the carbon-carbon double bond at C-7 (Fukuyama *et al.,* 1985; Barrero *et al.,* 1995). The position of the double bond was supported by the presence of an olefinic proton signal (δ 5.74 d, J = 4.0 Hz) which demonstrated the carbon-carbon double bond to be tri-substituted (Fukuyama *et al.,* 1985; Barrero *et al.,* 1995). Other peaks in the ^{13}C NMR spectrum were observed at δ 69.79 assigned to oxymethylene carbon at C-12; δ 32.95, 21.32 and 13.89 assigned to tertiary methyl carbons at C_{13} –C_{15} (Suzuki *et al.,* 1970; Fukuyama *et al.,* 1985*;* Akita *et al.,* 2000). The more deshielded resonance for the lactone methylene carbon (δ 69.79) allowed its placement at C-12 as opposed to C-12 in cinnamolide (Kioy *et al.,* 1990; Fukuyama *et al.,* 1985; Barrero *et al.,* 1995).

Table 17: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of compound 103 in $CDCl_3$

Carbon	^{13}C (δ_C)	Multiplicity	^{1}H (δ_H, mult, *J* in Hz)	^{1}H (δ_H)*
1	38.37	CH_2	1.17 – 1.65 m	NR
2	18.17	CH_2	1.17 – 1.65 m	NR
3	23.25	CH_2	1.17 – 1.65 m	NR
4	34.30	C	-	
5	53.59	CH	1.98 m	NR
6	42.24	CH_2	1.78d (12.8), 2.48 d (18.1)	NR
7	121.13	CH	5.74 d (4.0)	5.62-5.84
8	129.77	C	-	
9	49.56	CH	2.78 s	NR
10	41.54	C	-	
11	175.31	C	-	
12	69.79	CH_2	4.66 s	4.57-4.27
13	32.95	CH_3	0.87 s	0.89-0.92
14	21.32	CH_3	0.92 s	
15	13.89	CH_3	0.89 s	

*^{1}H NMR (250 MHz, CDCl3) data of drimenin from literature (Wenkert and Strike, 1964).

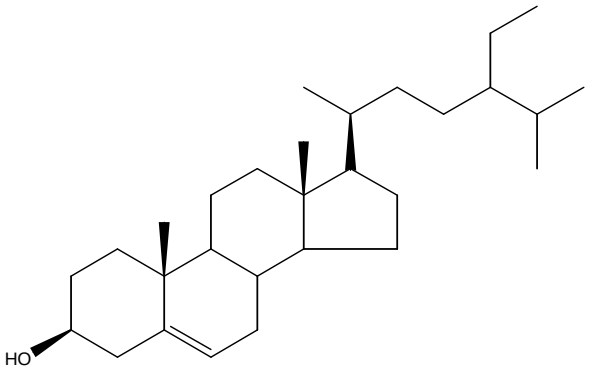
NR = Not reported.

Other diagnostic peaks in the ^{1}H NMR peak of **103** were observed at δ 4.66 s, attributed to lactone oxymethylene protons (-CO-OCH_2-); δ 0.87 s, 0.89 s and 0.92 s attributed to the methyl protons at C-13, C-14 and C-15 (Fukuyama *et al.,* 1985; Barrero *et al.,* 1995). Based on the spectral data as well as comparison with literature data, compound **103** was identified as drimenin.

4.3.2 Identification of compounds *from E. schweifurthianum*

Fractionation of extracts from *E. schweifurthianum* led to the isolation of nine known compounds which were identified as β-sitosterol [**63**], stigmasterol [**90**], lanosterol [**109**], 3-oxofriedooleanane [**44**], 3α-hydroxyfriedooleanane [**106**], 3-oxofriedoolean-29-ol [**108**], 3-oxofriedoolean-28-al [**45**], α-amyrin [**107]** and α-amyrin acetate [**105**]. Their structures were determined using spectroscopic methods as well as comparison with literature data.

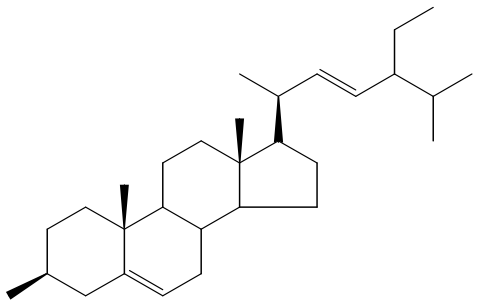
β-Sitosterol [63]



63

Compound **63** was obtained as white crystals from CH_2Cl_2-MeOH, (95:5), mp 134-135 °C, R_f value 0.42 (n-hexane: ethyl acetate 9:1) and $[\alpha]_D$ +49° (MeOH, c, 0.35). The The IR spectrum shwed rhe presence of OH (3404 cm^{-1}) and C=C (1635 cm^{-1}). ESI-MS spectrum of **63** gave a quasi-molecular ion peak at *m/z* 437 $[M+Na]^+$ which corresponded to molecular formula $C_{29}H_{50}O$ and was supported by ^{13}C NMR spectrum which showed the presence of 29 distinct carbon peaks resolved into six methyl, eleven methylene, nine methine and three non-protonated carbon atoms by DEPT. The ^1H and ^{13}C NMR data (Table 18) were in agreement with data previously reported for β-sitosterol (Shameel *et al.*, 1996; Hague *et al.*, 2008). The ^{13}C NMR spectrum (Appendix 15) showed the presence of two olefinic carbon atoms (δ 140.75, 121.70), an oxymethine carbon atom (δ 71.81) and six methyl carbon atoms δ 19.80, 19.39, 19.03, 17.77, 11.97 and 11.85 (Shameel *et al.*, 1996; Prachayasittikul *et al.*, 2010). The ^{13}C NMR data were supported by the ^1H NMR spectrum which showed the presence of one olefinic proton (δ 5.35 m), thus confirming the carbon-carbon double bond to be trisubstituted (Shameel *et al.*, 1996; Prachayasittikul *et al.*, 2010). The peak at δ 3.52 which was assigned to the proton at C-3 confirmed the presence of the hydroxyl group while those at δ 1.01(s), 0.92 (d, J = 6.2 Hz), 0.84 (t, J = 7.0 Hz), 0.82 (d, J = 6.5 Hz), 0.81 (d, J = 6.5 Hz) and 0.68 (s) confirmed the presence of two tertiary, three secondary and one primary methyl groups (Shameel *et al.*, 1996). Based on the spectral data as well as comparison with literature data, compound **63** was identified as β-sitosterol.

Stigmasterol [90]



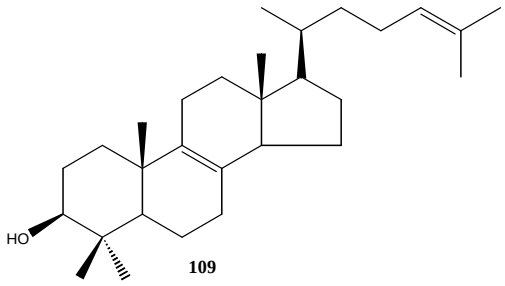
90

Compound **90** was isolated as white crystals from CH_2Cl_2 – MeOH (95:5), mp 169-170 °C, R_f value 0.16 (n-hexane: ethyl acetate 9:1) and $[\alpha]_D$ +51° (MeOH, c, 2.0). IR spectrum of **23** showed peaks at 3356 and 1647 cm^{-1} indicating the presence of hydroxyl group and carbon-carbon double bond, respectively. The EIMS spectrum afforded a molecular ion peak at *m/z* 412 which corresponded to a molecular formula $C_{29}H_{50}O$. The EIMS data were supported by ^{13}C NMR and DEPT spectra (Appendix 16) which shows the presence of 29 carbon atoms consisting of six methyl, nine methylene, eleven methine and three quaternary carbon atoms. The ^{1}H and ^{13}C NMR data (Table 18) were similar to those previously reported for stigmasterol (Sukurai *et al.,* 1972; Shameel *et al.,* 1996; Reginatto *et al.,* 2001; Forgo and Kover, 2003). The EIMS spectrum further showed daughter ions at *m/z* 369 $[M\text{-}C_3H_7]^+$, 300 $[M\text{-}C_8H_{17}]^+$ and 271 $[M\text{-}C_{10}H_{21}]^+$ which were similar to those of stigmasterol (Sukurai *et al.,* 1972; Shameel *et al.,* 1996; Reginatto *et al.,* 2001). The ^{13}C NMR olefinic peaks at δ 139.56, 138.09, 127.65 and 117.32 confirmed the presence of two carbon-carbon double bonds (tri- and di-substituted) at C-5 and C-22 while the peak at δ 71.04 confirmed the presence of oxymethine carbon atom at C-3 (Shameel *et al.,* 1996; Reginatto *et al.,* 2001; Forgo and Kover, 2003). Other peaks in the ^{13}C NMR spectrum were at δ 12.06, 21.99, 21.38, 12.16, 19.05 and 12.9 which confirmed the presence of six methyl groups and were assigned as shown in Table 18.

The ^{13}C NMR data were supported by ^{1}H NMR spectrum (Table 18) which showed the presence of three olefinic protons at δ 5.18 m, 5.15 d (J = 15.3 Hz) and 5.03 dd (J = 15.3, 8.1 Hz); a proton attached to a oxymethine carbon atom (δ 3.54 m) and six methyl groups at δ 0.54 s, 0.84 s, 0.78 d (J = 7.1 Hz), 0.82 d (J = 6.5 Hz), 1.00 d (J = 6.5 Hz) and 0.79 t (J = 8.0 Hz) corresponding to two tertiary, three secondary and one primary methyl groups (Sukurai *et al.,* 1972; Shameel *et al.,* 1996; Reginatto *et al.,* 2001). Based on the spectral data as well at comparison with literature information, compound **90** was identified as stigmasterol.

Lanosterol [109]

Compound **109** was isolated as white crystals from CH_2Cl_2 – MeOH (95:5) mp 144-145 °C, R_f value 0.46 (n-hexane: ethyl acetate 4:1) and $[\alpha]_D$ +62° (MeOH, c, 1.12). the IR spectrum showed the presence of OH (3462 cm^{-1}) and C=C (1644cm^{-1}. The compound was shown to have a molecular formula of $C_{30}H_{50}O$ as determined by ^{13}C NMR, DEPT and ESI-MS spectrum (Appendix 17) which gave a quasi-molecular ion peak at *m/z* 449 $[M+Na]^+$ suggesting the molecular weight of the compound to be *m/z* 426. The ^1H and ^{13}C NMR data (Table 18) were similar to those previously reported for lanosterol (Müller, 2002). ^{13}C NMR and DEPT spectra showed the presence of four olefinic peaks corresponding to three quaternary (δ 134.05, 134.05, 130.56) and one methine (δ 124.91) carbon atom, a fact which was supported by the presence of an olefinic peak at δ 5.08 t (*J* = 6.6 Hz) in the ^1H NMR spectrum (Müller, 2002). The HMBC peak at δ 78.68 correlating with a proton at δ 3.24 dd (*J* = 10.7, 4.4 Hz) suggested the OH group at C-3 to be in equatorial conformation (Mohanlall and Odhiv, 2009; Nagwa, 2000; Reginatto *et al.,* 2001). Other characteristic peaks in the ^{13}C NMR spectrum were the methyl signals (δ 15.52, 15.52, 17.80, 19.27, 21.25, 21.90 25.93 and 28.00) which confirmed the presence of the eight methyl groups in lanosterol (Müller, 2002), a fact which was further supported by ^1H NMR peaks at δ 0.69 s, 0.81 s, 0.87 s, 0.98 s, 1.01 s, 1.68 s, 1.60 s, and 0.95 d (*J* = 6.3 Hz). Based on the spectral data as well as comparison with literature data, compound **109** was identified to be lanosterol.



109

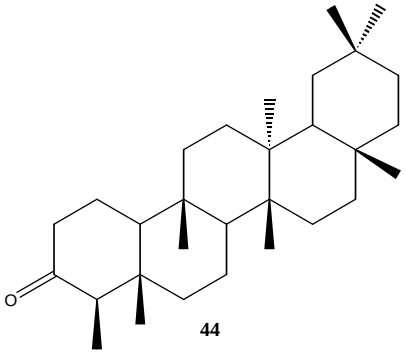
Table 18: ^{13}C (90 MHz) and ^{1}H (360 MHz) NMR data of 63, 90 and 109 in $CDCl_3$

Carbon	63 (δ_C)	63* (δ_C)	90 (δ_C)	90** (δ_C)	109 (δ_C)	63 (δ_H)	90 (δ_H)	109 (δ_H)
1	37.25	37.31	37.28	37.0	36.02		1.76	
2	31.66	31.81	31.87	31.9	27.82		1.74, 1.34	
3	71.81	71.90	71.04	72.0	78.68	3.52	3.54	3.24
4	42.30	42.40	40.69	42.5	38.53		1.66	-
5	140.75	140.90	139.56	140.8	50.06		-	
6	121.70	121.87	117.32	121.8	18.25	5.35	5.18	
7	33.71	32.07	29.75	32.1	28.25		1.67	
8	31.91	32.00	30.12	32.2	134.05		2.01	-
9	50.14	50.81	49.64	50.5	134.05		1.62	-
10	36.50	36.61	34.32	36.5	35.25		-	-
11	21.08	21.12	21.63	21.2	21.00		1.54	
12	39.78	40.30	39.57	40.0	39.18		1.88 1.22	
13	42.32	42.61	43.37	42.2	44.14		-	-
14	56.77	56.78	56.14	57.1	49.47		1.26	-
15	24.29	24.32	23.08	24.5	24.25		1.45 1.36	
16	28.24	28.24	28.40	28.9	35.93		1.75, 1.25	
17	56.06	56.20	55.22	56.3	36.68		1.80	-
18	11.97	11.90	12.06	12.2	15.83	0.68	0.54	0.69
19	19.39	19.44	21.99	19.5	19.27	1.01	0.84	0.98
20	36.14	36.26	40.43	40.4	36.68		2.20	
21	19.03	10.09	21.38	21.4	18.90	0.92	1.00	0.95
22	33.95	34.00	138.09	138.3	36.02		5.15	
23	29.16	29.31	127.65	129.7	26.49		5.03	
24	45.82	45.80	51.28	51.5	124.91		1.53	5.08
25	26.09	26.21	31.57	32.2	130.56		1.52	-
26	18.77	18.80	12.16	12.2	28.00	0.82	0.78	1.68
27	19.80	19.80	19.05	19.2	17.80	0.81	0.82	1.60
28	23.07	23.10	25.35	25.4	25.93		1.39	0.81
29	12.85	11.92	12.99	12.2	15.5	0.84	0.79	1.01
30					21.25			0.87

*^{13}**C** NMR (75 MHz, $CDCl_3$) data of β-sitosterol (Shameel *et al.*, 1996)

^{13}C** NMR (75 MHz, $CDCl_3$) data stigmasterol (Forgo and Kover, 1996)

3-Oxofriedooleanane [44]

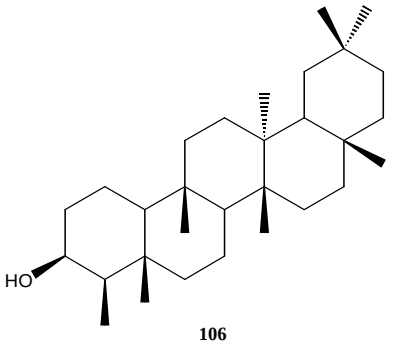


44

Compound **44** was isolated as white crystals, R_f value 0.33 (n-hexane: ethyl acetate 9:1), mp 260-261 °C and $[\alpha]_D$ -28° (MeOH, c, 1.12). The EIMS spectrum of **44** gave a molecular ion peak at *m/z* 426 corresponding to a molecular formula $C_{30}H_{50}O$ and was supported by the ^{13}C NMR and DEPT spectra (Appendix 18) which displayed a total of 30 carbon atoms attributed to eight methyl, eleven methylene, four methine and seven quaternary carbon atoms. The ^{1}H and ^{13}C NMR data (Table 19 and 20) were similar to data previously reported for 3-oxofriedooleanane (Anjaneyulu and Rao, 1980a; Weeratunga *et al.,* 1983; Mahato and Kundu, 1994; De Lampasona *et al.,* 1998; Costa and Carvalho, 2003) and were supported by the IR spectrum which exhibited a peak at 1706 cm^{-1} thus confirming the presence of a carbonyl carbon (Anjaneyulu and Rao, 1980a; Weeratunga et *al.,* 1983). The ^{13}C NMR spectrum gave a peak at δ 213.24 which further confirmed the presence of a carbonyl carbon atom at C-3. Other characteristic peaks in the ^{13}C NMR and DEPT spectra were the methyl peaks at δ 6.81, 14.59, 17.91, 18.63, 20.21, 32.05, 31.73 and 34.94 which confirmed the presence of the eight methyl groups in 3-oxofriedooleanane (Anjaneyulu and Rao, 1980a; Weeratunga *et al.,* 1983; Mahato and Kundu, 1994; Costa and Carvalho, 2003).

The ^{13}C NMR data were supported by ^{1}H NMR spectrum (Table 20) which showed the presence of eight methyl signals attributed to seven tertiary (δ 0.70 s, 0.84 s, 0.92 s, 0.97 s, 0.98 s, 1.04 s and 1.16 s) and one secondary (δ 0.86 d, *J* =7.0 Hz) methyl groups (Anjaneyulu and Rao, 1980a; Weeratunga *et al.,* 1983; Costa and Carvalho, 2003). Further examination of EIMS spectrum of **44** revealed fragmentation pattern typical of a saturated triterpene (Costa and Carvalho, 2003; Budzikiewics *et al.,* 1963; Anjaneyulu and Rao, 1980a; Weeratunga *et al.,* 1983) as evidenced by characteristic daughter ions at *m/z* 411$[M-15]^+$, 344, 273, 205 and 123. Based on the spectral data as well as comparison with literature data, compound **44** was identified as 3-oxofriedooleanane.

3α-Hydroxyfriedooleanane [106]



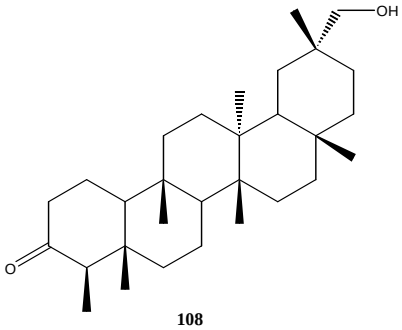
106

Compound **106** was obtained as white crystals from CH_2Cl_2 – MeOH (9:1), R_f value 0.28 (n-hexane ethyl acetate 4:1) mp 290-292 °C and $[\alpha]_D$ +18° (MeOH, c, 1.12). The IR spectrum of **106** showed the presence of a hydroxyl group at 3476 cm^{-1} (Silverstain *et al.*, 1981). The EIMS spectrum showed a molecular ion peak at *m/z* 428, corresponding to $C_{30}H_{52}O$ formula and was supported by ^{13}C NMR spectrum (Appendix 19) which showed 30 distinct carbon peaks attributed to eight methyl, eleven methylene, five methine and six quaternary carbon atoms. Other significant peaks were at *m/z* 413 $[M\text{-}Me]^+$, 395 $[M\text{-}Me\text{-}H_2O]^+$, 206 $[C_{14}H_{22}O]^+$ and 220 $[C_{16}H_{28}]^+$ which are characteristic peaks commonly found in the spectra of oleanane type of triterpenes with OH at C-3 (Anjaneyulu and Rao, 1980b; Siddiqui *et al.*, 1989; Mahato and Kundu, 1994).

The ^1H and ^{13}C NMR data of **106** (Table 19) were in agreement with those previously reported for 3α-hydroxyfriedooleanane (Anjaneyulu and Rao, 1980a; Mahato and Kundu, 1994) and closely resembled those of 3-oxofriedooleanane [**44**] except for the presence of an oxymethine peak at δ 72.72 with corresponding oxymethine proton signal at δ 3.73 d (J = 2.3 Hz) in the ^1H NMR spectrum, thus confirming the presence of OH group at C-3 (Anjaneyulu and Rao, 198a; Siddiqui *et al.*, 1989; Mahato and Kundu, 1994). The small coupling constant (2.3 Hz) allowed the assignment of α- orientation of the OH group (Anjaneyulu and Rao, 1980a; Gupta and Sing, 1989; Mahato and Kundu, 1994; El-Lahlou *et al.*, 1999). Other characteristic peaks in the ^{13}C NMR spectrum were observed at δ 11.61, 16.38, 18.23, 18.64, 20.11, 31.78, 32.08 and 35.02 which confirmed the presence of the eight methyl groups in friedooleanane type of triterpenes (Anjaneyulu and Rao, 1980a; Mahato and Kundu, 1994).

The ^{13}C NMR data were further supported by the ^1H NMR spectrum which displayed seven tertiary methyl singlets (δ 0.88, 0.96, 0.98, 0.99, 1.00, 1.01 and 1.17) and a doublet at δ 0.94 (J = 7.0 Hz) assigned to methyl protons at C-23 (Anjaneyulu and Rao, 1980a; Anjaneyulu and Rao 1980b). Based on the spectral data as well as comparison with literature data, compound **106** was identified as 3α-hydroxyfriedooleanane.

3-Oxofriedoolean-29-ol [108]



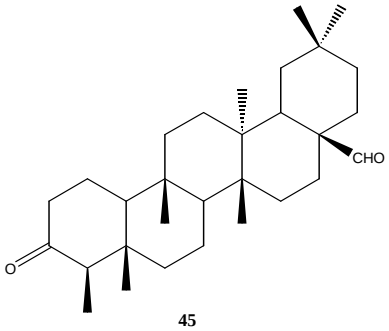
108

The compound was isolated as white crystals from n-hexane- ethyl acetate mixture, R_f value 0.38 (n-hexane: ethyl acetate 4:1), mp 250-252 °C and $[\alpha]_D$ +87° (MeOH, c, 1.0). The ESI-MS spectrum of **108** gave a quasi-molecular ion peak at *m/z* 465 $[M+Na]^+$ corresponding to a molecular weight of 442 and formula $C_{30}H_{50}O_2$ and was supported by ^{13}C and DEPT spectra (Appendix 20) which showed the presence of 30 carbon atoms consisting of seven methyl, twelve methylene, four methine and seven non-protonated carbon atoms.

The ^1H and ^{13}C NMR data (Table 19 & 20) were similar to those reported for 3-oxofriedoolean-29-ol (Weeratunga and Kumar, 1985; Mahato and Kundu, 1994) and closely resembled those of 3-oxofriedooleanane [**44**] except for the presence of an oxymethine peak at δ 74.74 in the ^{13}C NMR spectrum which suggested the presence of a hydroxyl group at C-28 (Weeratunga and Kumar, 1985; Mahato and Kundu, 1994). Other significant peaks in the ^{13}C NMR were at δ 213.19 (carbonyl carbon at C-3) and the seven methyl peaks at δ 6.81, 14.64, 17.68, 18.43, 20.74, 25.79 and 32.06 (Weeratunga and Kumar, 1985; Mahato and Kundu, 1994).

The ^1H NMR spectrum (Appendix 20) peak at δ 3.26 s, further confirmed the presence of –OH at C-29 (Weeratunga and Kumar, 1985). ^1H NMR spectrum singlets at δ 0.72, 0.86, 1.02, 1.04, 1.21, 1.25 further confirmed the presence of six tertiary methyl groups while a doublet at δ 0.87 (J = 6.5 Hz) confirmed the presence of secondary methyl protons at C-23 (Weeratunga and Kumar, 1985; Mahato and Kundu, 1994). Peaks at δ 2.36 (m) and 2.40 (m) were assigned to protons at C-4 and C-2, repectively. Based on the spectral data as well as comparison with literature data, compound **108** was identified as 3-oxofriedoolean-29-ol.

3-Oxofriedoolean-28-al [45]



45

Compound **45** was isolated as white needles from CH_2Cl_2-MeOH (95:5), R_f value, 0.22 (n-hexane- ethyl acetate 4:1), mp 263-265 °C and $[\alpha]_D$ +65° (MeOH, c, 1.0). The ESI-MS of the **45** showed a quasi-molecular ion peak at *m/z* 463 $[M+Na]^+$, corresponding to a molecular weight of 440 and molecular formula $C_{30}H_{48}O_2$. The ESI-MS data were supported by ^{13}C NMR and DEPT experiments (Appendix 21) which showed the presence of 30 distinct carbon peaks consisting of seven methyl, eleven methylene, five methine and seven non-protonated carbon atoms. The ^1H and ^{13}C NMR data of **45** (Table 19 and 20) closely resembled those of 3-oxofriedooleanane [**44**] except for the presence of a peak at δ 205.47, which suggested **45** to be 3-oxofriedooleanane with an aldehyde group attached to it (Mahato and Kundu, 1994). The ^{13}C NMR data (Table 19) were similar to those previously reported for 3-oxofriedoolean-28-al, canophyllal (Mahato and Kundu, 1994; Abbas *et al.,* 2007)

and gave carbonyl carbon peaks at δ 213.01 and 205.47 which were assigned to the ketone and aldehyde carbonyl carbon atoms at C-3 and C-28, respectively (Mahato and Kundu, 1994; Abbas *et al.*, 2007; Olmedo *et al.,* 2008). Other characteristic peaks were at δ 6.77, 13.74, 14.46, 16.12, 18.58, 18.92, 26.80 which were assigned to the seven methyl carbon atoms at C-23, C-27, C-29 and C-30 (Mahato and Kundu, 1994).

^1H NMR spectrum of **45** which gave a peak at δ 9.39 (-CHO) also displayed seven methyl peaks consisting of six singlets (δ 0.70, 0.85, 0.87, 0.89, 0.97 and 1.01) and a doublet (δ 0.88 J = 6.6), thus further supporting the ^{13}C data (Mahato and Kundu, 1994; Abbas *et al.,* 2007; Olmedo *et al.,* 2008). Based on the spectral data as well as comparison with literature data, compound **45** was identified as 3-oxofriedoolean-28-al (canophyllal).

Table 19: ^{13}C NMR (90 MHz, $CDCl_3$) of compounds 44, 106, 108 and 45

Carbon	44	44*	106	106**	108	108***	45	45***
1	22.42	22.3	17.50	16.43	22.26	22.3	22.25	22.3
2	41.50	41.5	35.17	35.46	41.50	41.6	41.41	41.5
3	213.24	213.2	72.76	72.79	213.19	213.2	213.01	214.0
4	58.17	58.2	49.17	49.39	58.30	58.3	58.12	58.2
5	42.14	42.1	37.10	37.99	42.17	42.3	42.02	42.0
6	41.23	41.3	41.73	41.92	41.27	41.4	41.15	41.0
7	18.20	18.2	15.79	17.65	18.22	18.3	18.15	18.1
8	53.05	53.1	53.19	53.34	53.38	53.5	50.41	52.8
9	37.38	37.4	37.82	37.27	37.42	37.5	37.12	37.1
10	59.43	59.4	61.35	61.58	59.44	59.6	59.45	59.2
11	35.54	35.6	35.55	35.71	35.62	35.7	36.30	35.4
12	30.43	30.5	30.63	30.73	29.74	29.8	28.49	30.6
13	39.67	39.7	39.69	38.41	39.93	40.0	39.39	38.7
14	38.25	38.3	38.37	39.81	38.22	38.3	37.47	37.7
15	32.27	32.4	32.33	32.48	32.21	32.8	30.07	32.4
16	35.90	36.0	36.08	36.25	35.85	36.0	35.12	34.9
17	29.95	30.0	30.02	30.13	30.50	29.8	43.91	47.7
18	42.78	42.8	42.82	43.05	41.84	42.0	44.53	36.4
19	35.28	35.3	35.36	35.39	30.57	30.6	36.20	35.4
20	28.13	28.1	28.17	28.22	33.09	33.2	26.80	28.3
21	32.73	32.7	32.81	33.00	27.77	29.9	28.96	32.4
22	39.23	37.2	39.28	39.36	39.48	39.6	26.19	28.8
23	6.81	6.8	11.61	11.59	6.81	6.8	6.77	6.9
24	14.59	14.4	16.38	15.90	14.64	14.7	14.64	14.6
25	17.91	17.9	18.23	18.29	17.86	17.9	16.12	17.2
26	18.63	18.6	18.64	18.62	18.43	18.4	18.92	20.0
27	20.21	20.1	20.11	20.13	20.74	20.8	18.58	18.8
28	32.05	32.1	32.08	32.16	32.06	32.1	205.47	210.0
29	31.73	31.8	31.78	31.84	74.79	74.8	32.74	34.5
30	34.98	35.0	35.02	35.01	25.79	25.9	26.80	29.4

*^{13}**C** NMR (75 MHz, $CDCl_3$) data of 3-oxofriedooleanane (Mahato and Kundu, 1994)

^{13}C** NMR (75 MHz, $CDCl_3$) data of 3α-hydroxyfriedooleanane (Mahato and Kundu, 1994)

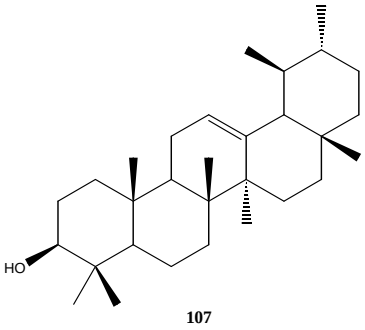
***^{13}**C** NMR (75 MHz, $CDCl_3$) data of 3-oxofriedoolean-29-ol (Olmedo *et al.,* 2008)

****^{13}**C** NMR (75 MHz, $CDCl_3$) data of 3-oxofriedoolean-28-al (Abbas *et al.,* 2007)

Table 20: ^{1}HNMR (360 MHz) of compounds 44, 106, 108 and 45 (δ_H, mult, *J* in Hz)

Carbon	CPD 44	CPD 106	CPD 108	CPD 45
2	1.34 dd (3.6, 1.8), 2.25 m	1.45 m, 0.96 m	2.40 m	2.26 m, 2.38 m
3	-	3.73 d (2.2)	-	-
4	2.22 d (3.6)	1.26 m	2.36 m	2.23 q (6.7)
23	0.86 d (7.0)	0.94 d (7.0)	0.87 d (6.6)	0.88 d (6.5)
24	0.70 s	0.94 s	0.72 s	0.70 s
25	0.84 s	0.88 s	0.86 s	0.85 s
26	1.04 s	0.99 s	1.02 s	0.87 s
27	0.98 s	1.00 s	1.25 s	0.91
28	0.97 s	1.01 s	1.04 s	9.39 s
29	1.16 s	1.17 s	1.21 s	1.09 s
30	0.92 s	0.96 s	3.26 s	0.89 s

α-Amyrin [107]



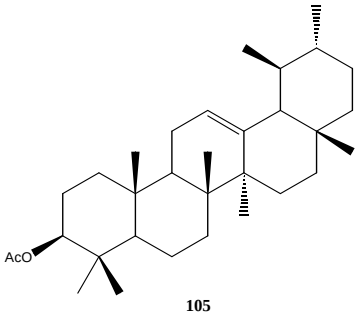
107

Compound **107** was isolated as white crystals from 5% MeOH in CH_2Cl_2 and had R_f value of 0.32 (n-hexane: ethyl acetate 4:1), mp 180-181 °C and $[\alpha]_D$ +83° (MeOH, c, 1.12). The ESI-MS spectrum of **107** gave a quasi-molecular ion peak at *m/z* 449 $[M+Na]^+$ corresponding to a molecular weight of 426 and molecular formula $C_{30}H_{50}O$ and was supported by ^{13}C NMR and DEPT spectra (Appendix 22) which showed a total of 30 carbon peaks attributed to eight methyl, nine methylene, seven methine and six quaternary carbon atoms.

The ^{1}H and ^{13}C NMR data (Table 21) were similar to those previously reported for α-amyrin (Seo *et al.*, 1975; Morris and Mansor, 1991; Mahato and Kundu, 1994). The ^{13}C NMR spectrum showed the presence of a carbon-carbon double bond (δ 139.84 and 124.42), an oxymethine carbon (δ 79.06) and eight methyl carbon atoms consisting of six tertiary (15.61, 15.67, 16.86, 23.26, 28.10, 28.73) and two secondary (δ 17.46, 21.35) methyl carbon atoms (Seo *et al.*, 1975; Morris and Mansor, 1991; Mahato and Kundu, 1994). The ^{1}H NMR spectrum (Appendix 22) gave olefinic peak at δ 5.12 t (*J* = 3.5 Hz) and an oxymethine peak at δ 3.22 dd (*J* = 11.5, 4.5 Hz) thus further confirming the presence of the carbon-carbon double bond at C-12

and OH at C-3. The large coupling constant (J = 11.5 Hz) observed for H-3 showed the OH to be in equatorial position (Seo *et al.,* 1975; Morris and Mansor, 1991). Other characteristic peaks were at δ 0.79, 0.80, 0.87, 0.93, 0.95, 0.99, 1.01 and 1.07 which confirmed the presence of eight methyl groups (Seo *et al.,* 1975; Morris and Mansor, 1991; Mahato and Kundu, 1994). Based on the spectral the data as well as comparison with literature data, compound **107** was identified as α-amyrin.

α-Amyrin acetate [105]



105

The compound was isolated as white crystals fromn-hexane - ethyl acetate mixture and had R_f value 0.23 (n-hexane: ethyl acetate 9:1) mp 214-215 °C and $[\alpha]_D$ +77° (MeOH, c, 1.22). ESI-MS spectrum of **105** showed a quasi-molecular ion peak at *m/z* 491 $[M+Na]^+$ corresponding to a molecular formula $C_{32}H_{52}O_2$. The ^1H and ^{13}C NMR spectra (Appendix 23) closely resembled those of α-amyrin [**107**], except for the presence of two extra peaks at δ 170.04 and 21.36 in the ^{13}C NMR spectrum which suggested the compound to be acetylated α-amyrin (Morris and Mansor, 1991; 1992; Mahato and Kundu, 1994; Reginatto *at al.*, 2001). The ^{13}C NMR spectrum gave 32 carbon signals attributed to nine methy, nine methylene, seven methine and seven non-protonated carbon atoms. Peaks at δ 139.62 and 124.31 confirmed the presence of the carbon-carbon double bond at C-12 and was further supported by the ^1H NMR peak δ 5.12 t (J = 3.6 Hz) which was attributed H-12 (Morris and Mansor, 1991; Mahato and Kundu, 1994). Further examination of the ^{13}C NMR spectrum revealed the presence of nine methyl carbon peaks which were assigned as shown in Table 21.

The ^1H NMR spectrum of **105** further showed the presence of an oxymethine proton at C-3 (δ 4.51 m) and nine methyl groups consisting of seven tertiary (δ 2.05, 0.80, 1.57, 0.98, 1.07, 0.91, 1.01) and two secondary at δ 0.87 d *(J* = 5.5 Hz) methyl groups. Based on the spectral data as well as comparison with literature data, compound **105** was identified as α-amyrin acetate.

Table 21: ^{13}C (90 MHz, $CDCl_3$) compounds 107 and 105

Carbon	107	107*	105	105**
1	38.77	38.7	33.88	33.4
2	27.23	27.2	23.24	23.6
3	79.06	78.3	80.95	80.7
4	38.77	38.7	37.70	37.6
5	55.19	55.2	55.25	55.3
6	18.35	18.3	18.13	18.3
7	32.94	32.9	32.69	32.8
8	40.04	40.0	40.01	40.1
9	47.72	47.7	47.63	47.1
10	36.90	36.9	36.78	36.8
11	23.3	23.3	22.74	17.5
12	124.42	124.3	124.31	124.1
13	139.58	139.3	139.62	139.4
14	42.08	42.0	42.06	42.1
15	27.23	28.7	28.09	28.7
16	26.61	26.6	26.58	26.7
17	33.75	33.7	33.88	33.8
18	59.07	58.7	59.04	59.0
19	39.66	39.6	39.64	39.7
20	39.60	39.6	39.60	39.7
21	31.35	31.2	31.24	31.3
22	41.53	41.5	41.53	41.5
23	28.73	28.1	28.73	28.1
24	15.67	15.6	18.13	16.8
25	15.61	15.5	15.44	15.7
26	16.86	16.8	16.84	16.8
27	23.26	23.3	23.40	23.2
28	28.10	28.1	27.83	28.1
29	17.46	17.4	21.97	23.2
30	21.35	21.3	21.42	21.4
MeCO			171.04	170.4
MeCO			21.36	21.2

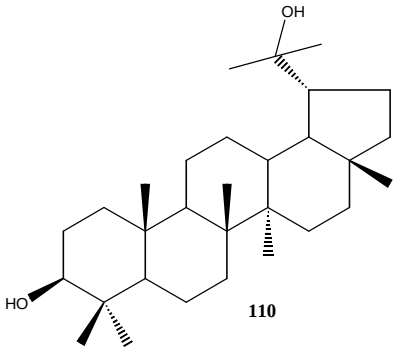
*^{13}**C** NMR (75 MHz, $CDCl_3$) data of α-amyrin (Mahato and Kundu, 1994)

^{13}C** NMR (75 MHz, $CDCl_3$) data of α-Amyrin acetate (Morris and Mansor, 1991)

4.3.3 Identification of compounds from *T. brownii*

Fractionation of extracts from *T. brownii* led to the isolation of five known compounds consisting of two steroids and three pentacyclic triterpenoids which were identified as β-sitosterol [**63**], stigmasterol [**90**], monogynol A [**110**], betulinic acid [**88**] and arjungenin [**83**]. The structures of the compounds were determined using spectroscopic methods as well as comparison with literature data.

Monogynol A [110]



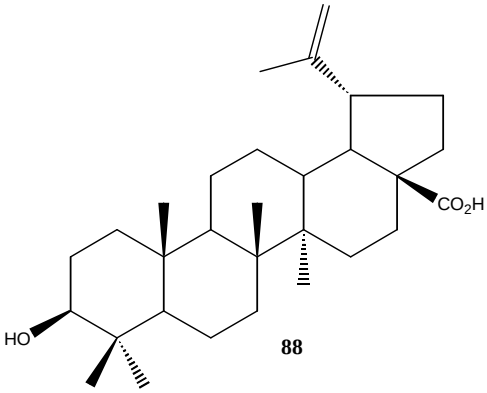
110

Compound **110** was isolated as white crystals from ethyl acetate with R_f value of 5.8 (n-hexane: ethyl acetate 9:1), mp 237-238 °C and $[\alpha]_D$ +39° (MeOH, c, 1.28). The ESI-MS spectrum (Appendix 24) afforded a positive quasi molecular ion peak at *m/z* 467 $[M+Na]^+$ corresponding to the molecular formula $C_{30}H_{52}O_2$ (molar mass 444). The ESI-MS data were supported by the ^{13}C NMR spectrum (Appendix 24) which exhibited the presence of 30 carbon atoms consisting of a carbon bearing a tertiary OH and another carbon bearing a secondary OH at δ 73.59 and 78.77, respectively. The ^{13}C NMR data also showed the presence of eight methyl carbon atoms at δ 30.02, 27.73, 17.90, 17.77, 16.22, 16.16, 15.40 and 14.78. The rest of ^{13}C NMR signals (Table 22) corresponded to six quaternary carbons (δ 73.59, 43.17, 43.17, 41.39, 38.18, 35.15), six methine carbons (δ 78.77, 54.87, 49.37, 47.19, 38.78, 38.55) and ten methylene carbon atoms (δ 38.41, 37.93, 37.50, 35.21, 29.11, 27.12, 26.37, 21.31, 18.07) as evidenced by the DEPT and HMQC spectra (Appendix 24).

Comparison of the above spectral data with those available in the literature indicated that the compound is a derivative of lupanol (Cole e*t al.*, 1991; Mahato and Kundu, 1994; Ulubelen *et al.*, 1994). The large coupling constant (*J*= 7.9 Hz) in the ^1H NMR spectrum allowed the assignment of β and equitorial orientation of the OH group at C-3 (Cole *et al.*, 1991; Ulubelen *et al.*, 1994). The ^1H NMR spectrum (Appendix 24) further afforded an oximethine signal centered at δ 3.30 thus confirming the presence of the OH group at C-3. The presence of the eightmethyl group was further evidenced by the eight singlets (δ 0.76, 0.84, 0.85, 0.95, 0.97, 1.07, 1.09, 1.18), each integrating for three protons in the ^1H NMR. Based on the above results as well

as comparison with literature data, compound **110** was identified as lupan-3β, 20-diol (monogynol A).

Betulinic acid [88]



88

Compound **88,** R_f value 0.18 (n-hexane: ethyl acetate 7:3) was isolated as white crystals from methanol and had mp 280-282 °C and $[\alpha]_D$ +10° (MeOH, c, 1.0). The IR spectrum showed the presence of OH (3455 cm^{-1}), an exomethylene (3072 cm^{-1}) and a carbonyl (1687 cm^{-1}). The EIMS spectrum (Appendix 25) gave a molecular ion peak at *m/z* 456 corresponding to the molecular formula $C_{30}H_{48}O_3$ and was supported by the ^{13}C NMR spectrum (Appendix 25) which exhibited 30 non-equivalent carbon atoms and among them were the carbonyl carbon (δ 180.0), a quaternary vinyl carbon (δ 150.40), a terminal methylene carbon (δ 109.67) and a carbon holding a secondary alcohol (δ 79.11). The other ^{13}C NMR signals corresponded to five quaternary carbons (δ 56.95, 42.59, 40.89, 38.56, 37.36), ten methylene carbons (δ 39.89, 37.09, 34.52, 32.28, 30.73, 29.81, 27.54, 25.68, 21.00, 18.40), five methine carbons (δ 55.56, 50.72, 49.51, 46.99, 38.95) and six methyl carbons (δ 28.06, 19.45, 16.14,16.14, 15.35, 14.77) as evidenced by DEPT and HMQC spectrum (Appendix 25).

The ^1H NMR spectrum of **88** (Appendix 25) afforded two singlets at δ 4.73 and 4.60 corresponding to two protons of a terminal vinyl methylene group while the while the vinyl methyl group was centered at δ 1.69. Comparison of these data with those available in the literature indicated that **88** is either α- or β-betulinic acid (Mahato and Kundu, 1994; Hague *et al.*, 2006) Table 22. The large coupling constant (*J*= 8.1, 6.0 Hz) in the ^1H NMR spectrum allowed the assignment of β and equatorial orientation of the OH group at C-3 (Peng *et al.*, 1989).The ^1H NMR data together the EIMS diagnostic peaks at *m/z* 456 $[M]^+$, 438 $[M\text{-}H_2O]^+$, 423 $[M\text{-}Me\text{-}H_2O]^+$, 411 $[M\text{-}45]^+$, 248 $[A]^+$, 207 $[B]^+$, 219 $[C]^+$, 203 $[D]^+$ and 189 $[E]^+$ (Fig. 5**)** confirmed the compound to be lup-20(29)-ene type and the position of the carbonyl group was in

ring D/E at C-17 (Budzikiewics *et al.*, 1963; Kwon *et al.*, 2003; Chaiyade, 2004; Haque *et al.*, 2006). Based on these results compound **88** was concluded to be *β*-betulinic acid.

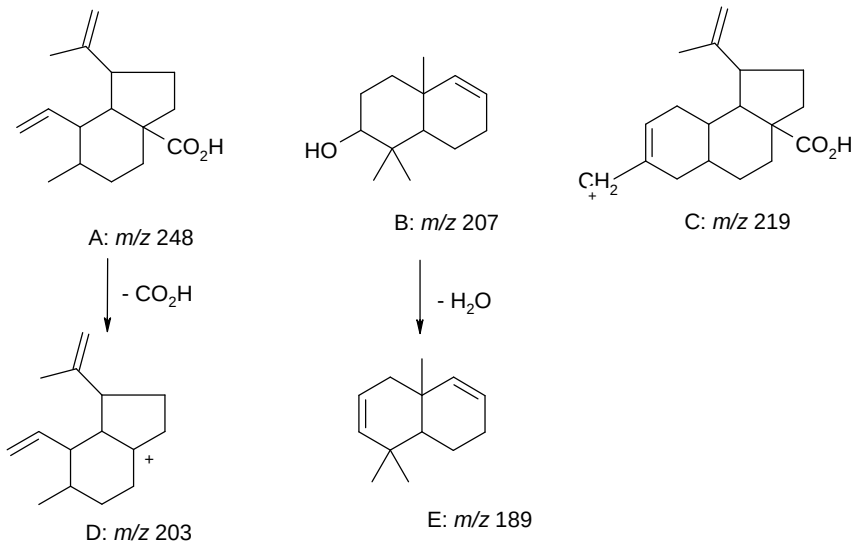
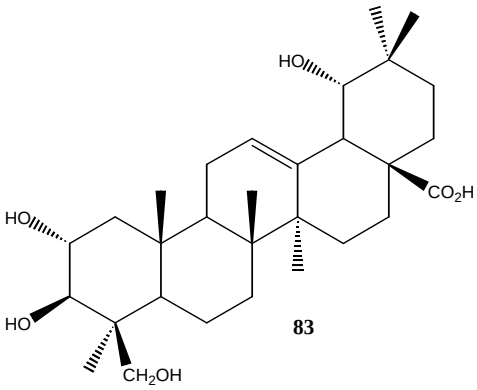


Fig. 5: Proposed EIMS fragmentation pattern of compound **88**

Arjungenin [83]



Compound **83** R_f value of 0.32 (n-hexane: ethyl acetate 1:1), mp. 293-295 °C, $[\alpha]_D$ +29° (MeOH, c, 2.0), was isolated as white amorphous solid from methanol. The IR spectrum of the compound showed the presence of OH (3404 cm^{-1}), carbonyl (1705 cm^{-1}) and C-C double bond (1640 cm^{-1}). The ESI-MS spectrum gave a positive quasi-molecular ion peak at *m/z* 527 $[M+Na]^+$ corresponding to the molecular formula $C_{30}H_{48}O_6$.

The ^{13}C NMR spectrum of **83** (Appendix 26) exhibited 30 non-equivalent carbon atoms which indicated the presence of a carbonyl carbon (δ 179.72), three carbon atoms bearing secondary OH groups (δ 84.52, 79.76, 67.40), a carbon atom bearing a primary OH group (δ 63.82), a quaternary vinyl carbon (δ 144.13) and a methine vinyl carbon (δ 122.06) (Mahato and Kundu, 1994; Reddy *et al.*, 1994; Singh *et al.*, 2004; Garcez *et al.*, 2006; Xu *et al.*, 2009b). The rest of the ^{13}C NMR signals (Table 22) corresponded to six quaternary carbons (δ 43.78, 41.69,

39.37, 39.24, 38.22, 35.49), three methine carbons (δ 43.13, 47.37, 55.48), eight methylene carbons (δ 18.87, 23.39, 27.15, 27.86, 28.41, 32.29, 32.80, 46.80) and six methyl carbon atoms (δ 16.37, 16.59, 23.33, 24.03, 24.67, 28.08) (Mahato and Kundu, 1994; Xu *et al.*, 2009b).

Table 22: ^{13}C NMR (90 MHz, CDCl3) data of 110, 88 and 83

Carbon	**110**	**88**	**88***	**83**	**83****
1	37.50	38.89	39.0	46.80	47.4
2	26.63	27.54	27.6	67.40	68.9
3	78.77	79.11	78.2	70.76	78.4
4	37.93	38.56	39.0	39.37	43.6
5	54.87	55.56	55.5	55.48	48.2
6	18.32	18.40	18.4	18.87	18.8
7	35.14	34.52	34.5	32.80	33.6
8	41.39	40.89	40.8	39.24	40.1
9	49.37	50.72	50.7	47.37	48.5
10	38.18	37.36	37.3	38.22	38.6
11	21.56	21.00	21.0	27.86	28.8
12	27.37	25.68	25.6	122.06	123.5
13	38.55	38.95	38.2	144.13	144.9
14	38.72	42.57	42.5	41.69	42.2
15	29.36	29.81	30.4	28.41	29.2
16	35.21	32.28	32.6	23.39	24.3
17	43.17	56.95	56.3	43.78	46.0
18	43.17	46.99	47.1	43.13	44.8
19	47.19	49.51	49.4	84.52	81.3
20	73.59	150.40	150	35.49	35.7
21	34.48	30.73	29.9	27.15	28.4
22	38.41	37.07	27.3	32.29	33.1
23	17.90	28.06	27.9	63.82	66.8
24	17.77	15.35	15.4	16.37	14.2
25	16.16	16.14	16.2	23.33	17.7
26	16.22	16.14	16.3	16.59	17.3
27	15.40	14.77	14.6	24.67	24.9
28	14.78	180.00	180.6	179.72	180.8
29	27.73	109.67	108.8	28.08	29.2
30	30.02	19.45	19.6	24.03	24.9

*^{13}C NMR data (75 MHz, $CDCl_3$) of betulinic acid (Haque *et al.*, 2006)

**^{13}C NMR data (75 MHz, $CDCl_3$) of arjungenin (Mahato and Kundu, 1994)

The ^{1}H NMR spectrum of compound **83** (Appendix 26) showed the presence of a vinyl proton (δ 5.22 *br*. S), five protons on carbon atoms bearing hydroxyl groups at δ 4.48 d (*J* = 8.1 Hz), 4.01 m, 3.75 d (*J* = 10.1 Hz), 3.36 d (*J* = 10.0 Hz) and 3.33 m (Reddy *et al.*, 1994; Singh *et al.*, 2004; Xu *et al.*, 2009b). Other diagnostic peaks in the ^{1}H NMR spectrum were the six singlets, each integrating for three protons observed at δ 1.27, 1.08, 0.89, 0.87, 0.83 and 0.64. Based on the spectral data as well as comparison with literature data, compound **83** was concluded to be 2α, 3β, 19α, 23-tetrahydroxyolean-12-ene-28-oic acid (arjungenin).

4.4 Results from antifungal and antibacterial assays

4.4.1 Antifungal activity of crude extracts and isolates

Methanol, ethyl acetate and n-hexane extracts of *W. ugandensis*, *E. schweinfurthianum* and *T. brownii* were tested for antifungal activity against *Alternaria spp, A. niger, F. oxysporum, F. solanum* and *R. stolonifer* which infect sweet potato. Results from the *in vitro* antifungal assay (Table 23) revealed that all the extracts exhibited inhibitory effect against at least one or more of the fungi tested. Methanol, ethyl acetate and n-hexane extracts of *W. ugandensis* and *E. schweifurthianum* were active against all the five fungi. Methanol and ethyl acetate extracts of *T. brownii* inhibited the growth of *Alternaria* spp, *A. niger* and *F. oxysporum* while n-hexane extract of the plant was actve against *A. niger and F. solani* but had no effect on the growth of *F. oxysporum* and *R. stolonifer*. Methanol extract of *W. ugandensis* showed the highest inhibitory effects against *Alternaria* spp*, A. niger, F. oxysporum* and *R. stolonifer* followed by methanol extracts of *E. schweifurthianu*. Methanol extract of *E. schweifurthianum* was the most active against *F. solani,* followed by methanol extract of *W. ugandensis.* Inhibition of growth of test fungi varied with plant species and extraction solvent. For example, in the antifungal test against *Alternaria* spp, extracts from *W. ugandensis* caused the highest inhibition to the growth of the fungi followed by extracts from *E. schweifurthianum* (Table 23 and Fig. 6). Methanol extracts of the three medicinal plants exhibited significantly ($P \leq 0.05$) high inhibitory effects against the tested fingi, followed by ethyl acetate extracts while n-hexane extracts showed the least inhibitory effect. Crude extracts from the three plants exhibited lower antifungal activity compared to Blitox and Dithane M-5 which were used as standards (Fig. 7).

Table 23: Antifungal activity of crude extracts against sweet potato fungi

Fungi	Plant	Diameter of zone of growth inhibition in mm*					
		Extracts**				Controls	
		MeOH	EtOAc	n-Hex	Mean	Blitox	Dithane M-5
Alternaria spp	*W.ugandensis*	17.10	14.07	5.43	12.20	22.1	19.6
	E. schweinfurthianum	12.07	8.43	2.10	7.53		
	T. brownii	8.07	4.03	0.00	4.03		
	Mean extract	12.41	8.40	2.51			
	CV (%)		1.56				
	LSD, P≤ 0.05		0.25		0.25		
A. niger	*W.ugandensis*	21.07	11.53	3.07	11.89	28.0	24.3
	E. Schweinfurthianum	16.43	13.43	6.00	11.96		
	T. brownii	10.53	5.08	1.47	5.68		
	Mean extract	16.01	10.00	3.51			
	CV (%)		0.93				
	LSD, P≤ 0.05		0.18		0.18		
F. oxysporum	*W.ugandensis*	13.43	8.07	1.00	7.50	16.9	19.1
	E. Schweinfurthianum	8.07	3.43	0.63	4.04		
	T. brownii	0.00	0.00	0.00	0.00		
	Mean extract	7.17	3.88	0.54			
	CV (%)		2.98				
	LSD, P≤ 0.05		0.23		0.23		
F. solani	*W.ugandensis*	9.43	4.00	2.52	5.34	25.1	14.8
	E. Schweinfurthianum	10.43	6.10	0.87	5.80		
	T. brownii	5.80	2.20	1.17	3.06		
	Mean extract	8.56	4.12	1.52			
	CV (%)		2.83				
	LSD, P≤ 0.05		0..27		0.27		
R. stolonifer	*W.ugandensis*	24.50	13.73	5.63	14.62	18.3	16.3
	E. Schweinfuthianum	9.33	4.37	3.10	5.60		
	T. brownii	0.00	0.00	0.00	0.00		
	Mean extract	11.28	6.03	9.91			
	CV (%)		1.41				
	LSD, P≤ 0.05		0.19		0.19		

*Values are means of three replicates; ** Conc. = 5 mg/ml.

Fig. 6: Comparative activity of methanol extracts of *W. ugandensis* (A), *E. schweifurthianum* (B) and *T. brownii* (C) at 5 mg/ml against *Alternaria* spp. Tests were done in triplicates.

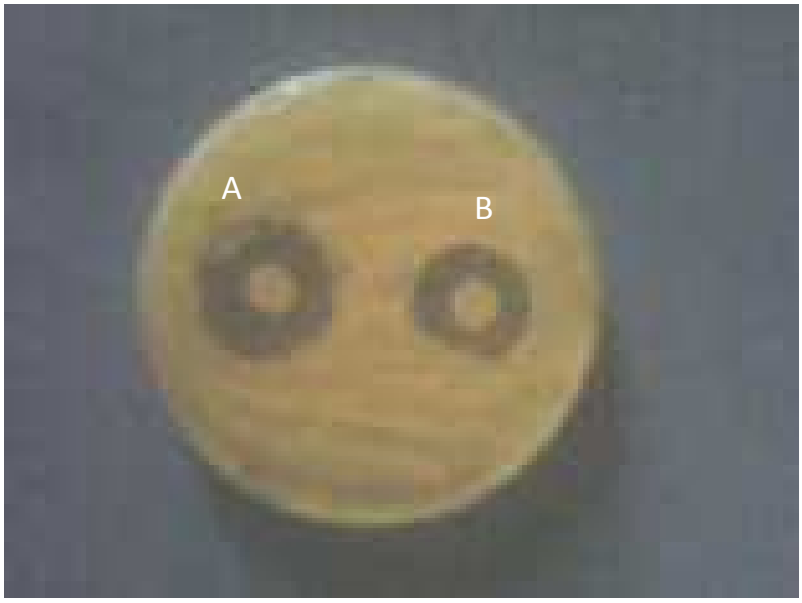


Fig. 7: Comparative activity of Blitox (A) and methanol extract of *W. ugandensis* (B) against *Alternaria* spp.

Compounds isolated from *W. ugandensis*, *E. scweinfurthianum* and *T. brownii* which were identified as polygodial [**13**], warburganal [**14**], mukaadial [**15**], ugandensidial [**16**], muzigadial [**22**], 6α-hydroxymuzigadial [**23**], 9-deoxymuzigadial [**101**], ugandensolide [**20**], 7α-acetoxyugandensolide [**104**], deacetoxyugandensolide [**21]**, cinnamolide [**17**], 3β-acetoxycinnamolide [**19**], bemadienolide [**102**], drimenin [**103**], β-sitosterol [**63**], stigmasterol [**90**], lanosterol [**109**], 3-oxofriedooleanane [**44**], 3α-hydroxyfriedooleanane [**106**], 3-oxofriedoolean-29-ol [**108**], 3-oxofriedoolean-28-al [**45**], α-amyrin [**107]**, α-amyrin acetate [**105**] monogynol A [**110**], betulinic acid [**88**] and arjungenin [**83**] were subjected to antifungal activity studies against *Alternaria spp, A. niger, F. oxysporum, F. solanum* and *R. stolonifer* at varying concentrations. Sixteen out of the 26 compounds (**13-16, 22, 23, 20, 21, 17, 63, 90, 44, 106, 107, 88 and 83)** were active against one or more of the fungi tested at concentrations ≤ 200 µg/ml while nine were not (Table 24).

Compound **13 – 16, 20** and **21** exhibited comparable antifungal activity against all or most of the fungi tested (MIC ≤50 µg/ml) with reference to blitox and dithane M-5 which are standard fungicides while the rest of the active compounds had MIC values ≤200 µg/ml against one or more of the tested fingi. Compounds **13** and **15** were the most effective against *Alternaria* spp (MIC = 25 µg/m) while compound **14** and **15** were the most effective against *A. niger* (MIC = 12.5 µg/ml). Compounds **14** and **22** were the most effective against *F. oxysporum* (MIC = 25 µg/m) while compound **13** and **14** were the most effective against *F. solani* (MIC = 12.5 µg/ml). *R. stolonifer* was most susceptible to compounds **13** and **14** (MIC = 12.5 µg/ml).

Table 24: MIC (µg/ml) of isolated compounds against sweet potato fungi

Compound	MIC, µg/ml of isolated compounds				
	Alternaria spp	*A. niger*	*F. oxysporum*	*F. solani*	*R. stolonifer*
Polygodial [**13**]	25	50	50	12.5	50
Warburganal [**14**]	50	12.5	25	12.5	25
Mukaadial [**15**]	25	12.5	100	25	50
Ugandensidial [**16**]	50	25	50	100	25
Muzigadial [**22**]	50	50	25	100	50
6α-Hydroxymuzigadial [**23**]	200	>200	100	>200	>200
9-Deoxymuzigadial[**101**]	>200	>200	>200	>200	>200
Ugandensolide [**20**]	50	50	100	200	100
7α-Acetoxyugandensolide [**104**]	>200	>200	>200	>200	>200
Deacetoxyugandensolide [**21**]	50	>200	>200	100	200
Cinnamolide [**17**]	100	100	>200	200	>200
3β-Acetoxycinnamolide [**19**]	>200	>200	>200	>200	>200
Bemadienolide [**102**]	>200	>200	>200	>200	>200
Drimenin [**103**]	>200	>200	>200	>200	>200
β-Sitosterol [**63**]	100	>200	>200	100	200
Stigmasterol [**90**]	100	>200	200	200	>200
Lanosterol [**109**]	>200	>200	>200	>200	>200
3-Oxofriedooleanane [**44**]	100	100	200	200	>200
3α-Hydroxyfriedooleanane [**106**]	200	200	200	>200	200
3-Oxofriedoolean-29-ol [**108**]	>200	>200	>200	>200	>200
3-Oxofriedoolean-28-al [**45**]	>200	>200	>200	>200	>200
α-Amyrin [**107**]	200	200	200	>200	200
α-Amyrin acetate [**105**]	>200	>200	>200	>200	200
Monogynol A [**110**]	>200	>200	>200	>200	>200
Betulinic acid [**88**]	100	>200	>200	100	100
Arjungenin [**83**]	100	100	>200	200	>200
Blitox	50	6.25	12.5	6.25	12.5
Dithan M-5	25	12.5	6.25	6.25	25

4.4.2 Antibacterial activity of crude extracts and isolates

Methanol, ethyl acetate and n-hexane extracts of *W. ugandensis, E. schweinfurthianum* and *T. brownii* were tested for antibacterial activity against *Ralstonia solanacearum* and *Stereptomyces ipomoeae* which infect sweet potato. The *in vitro* antibacterial activity (Table 25) showed that all the extracts were active against all the bacteria tested. The antibacterial activity varied with extraction solvent and the plant species. Methanol extracts exhibited the highest inhibition (P≤ 0.05) against the two bacteria followed by ethyl acetate extracts while n-hexane extracts had the lowest inhibition (Fig. 8). *Warburgia ugandensis* extracts were the most active against *R. solanacearum* followed by *E. schweinfurthianum* while *T. brownii* was the least active. The susceptibility of *S. ipomoeae* to the plant extracts were in the order of *W. ugandensis > T. brownii > E. schweinfurthianum.*

Table 25: Antibacterial activity of crude extracts (5 mg/ml)

		Diameter of zone of growth inhibition in mm*				
		Extracts**				Control
Bacteria	Plant	MeOH	EtOAc	n-Hex	Mean	Streptomycin
R. solanacearum	*W.ugandensis*	16.23	12.27	5.00	11.20	
	E. Schweinfurthianum	14.10	10.23	3.27	9.20	20.3
	T. brownii	10.03	7.37	4.14	7.18	
	Mean extract	13.46	9.96	4.17		
	CV (%)		1.27			
	LSD, P≤ 0.05		0.23		0.23	
S. ipomoeae	*W.ugandensis*	12.33	10.23	6.18	9.58	18.4
	E. Schweinfurthianum	8.50	5.18	2.08	5.24	
	T. brownii	13.63	8.20	4.83	8.89	
	Mean extract	11.49	7.87	4.36		
	CV (%)		1.22			
	LSD, P≤ 0.05		0.19		0.19	

*Values are means of three replicates

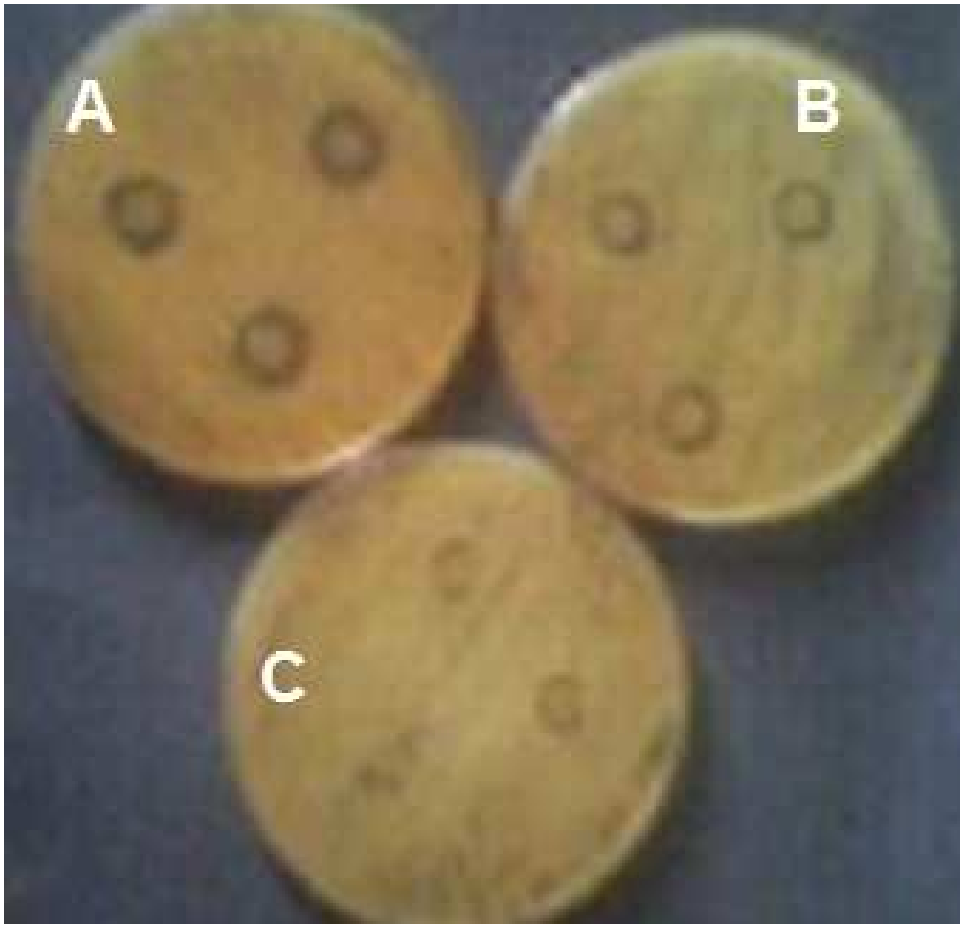


Fig. 8: Comparative antibacterial activity of *E. schweinfurthianum* methanol (A), ethyl acetate (B) and n-hexane (C) extracts at 5 mg/ml against *Streptomyces ipomoea.*

The compounds isolated from *W. ugandensis, E. schweifurthianum* and *T. brownii* were subjected to antibacterial activity against *R. solanacearum* and *S. ipomoeae.* Out of the 26 compounds tested, 17 inhibited the growth of one or both bacteria at concentrations ≤ 200 µg/ml while nine did not (Table 26). Compounds **13, 14, 15, 16, 22, 23, 104, 21, 63, 109, 45** and **88** were active against *R. solanacearum* while compounds **13, 14, 15, 16, 22, 20, 21, 102, 44, 105** and **88** were active against *S. ipomoeae* at concentrations ≤ 200 µg/ml **.** *R. solanacearum* was most susceptible to compounds **13**, **15** and **22** while *S. ipomoeae* was most susceptible to compound **16** (MIC = 25 µg/ml). Antibacterial activities of compounds **13 -16, 22 and 23** were comparable to those of streptomycin sulphate which is a standard antimicrobial drug (MIC ≤ 50 µg/ml).

Several fungal and bacteria cause losses in sweet potato production (Clark, 1987; Clarence and Leaton, 1990; Skuglund *et al.,* 1990; Ristaino, 1993; Clark and Hoy, 1994; Skuglund and Smith, 1994; Carey *et al.,* 1996; Clark *et al.,* 1998; Onuegbu, 2002; Oywale, 2006). In this study, susceptibility of similar fungi and bacteria were identified from decayed sweet potato were tested. In most cases, these pathogens gain entry into sweet potato roots through natural openings and wounds created during harvesting, transportation and handling. However, at times sweet potato roots may already be infected by pathogens derived from disease foliage, roots or mother roots (Okigbo and Nmeka, 2005).

The World Health Organization (WHO) banned many agriculturally important pesticides due to their wide range of toxicity against non-targeted organisms and their persistence in the environment which leads to pollution (Barnard *et al.,* 1997). Plants produce economically important organic compounds, pharmaceuticals and pesticides but little work has been done to investigate the use of natural plant products as pesticides for control of storage rot diseases of sweet potato.

Table 26: MIC (µg/ml) of isolated compounds against sweet potato bacteria

	MIC, µg/ml of isolated compounds	
Isolated compound	*R. solanacearum*	*S. ipomoeae*
Polygodial [**13**]	25	50
Warburganal [**14**]	50	50
Mukaadial [**15**]	25	50
Ugandensidial [**16**]	100	25
Muzigadial [**22**]	25	50
6α-Hydroxymuzigadial [**23**]	100	>200
9-Deoxymuzigadial[**101**]	>200	>200
Ugandensolide [**20**]	>200	100
7α-Acetoxyugandensolide [**104**]	200	>200
Deacetoxyugandensolide [**21**]	100	200
Cinnamolide [**17**]	>200	>200
3β-Acetoxycinnamolide [**19**]	>200	>200
Bemadienolide [**102**]	>200	100
Drimenin [**103**]	>200	>200
β-Sitosterol [**63**]	100	>200
Stigmasterol [**90**]	>200	>200
Lanosterol [**109**]	200	>200
3-Oxofriedooleanane [**44**]	>200	200
3α-Hydroxyfriedooleanane [**106**]	>200	>200
3-Oxofriedoolean-29-ol [**108**]	>200	>200
3-Oxofriedoolean-28-al [**45**]	200	>200
α-Amyrin [**107**]	>200	>200
α-Amyrin acetate [**105**]	>200	200
Monogynol A [**110**]	>200	>200
Betulinic acid [**88**]	100	100
Arjungenin [**83**]	200	>200
Steptromycin sulphate	25	12.5

This study revealed that extracts of *W. ugandensis, E. schweifurthianum and T. brownii* have antifungal activity against *F. oxysporum, F. solani, Alternaria spp, R. stolonifer. A. niger* (fungi), *R. solanacearum* and *S. ipomoeae* (bacteria) which cause disease in sweet potato. This was in agreement with previous reports that pathogens which infect crop can be managed using herbal extract (Avgelis and Morios, 1989; Khanna *et al.,* 1989; Udo *et al.,* 2001; Okigbo and Nmeka, 2005). Methanol extracts of the plants were the most effective inhibitors of growth of all the fungi and bacteria followed by ethyl acetate extracts. This observation suggests that the antifungal principles from the plants are polar compounds and could be extracted using polar solvents such as water, methanol, ethanol and acetone.

Extracts from *W. ugandensis, E. schweifurthianum* and *T. brownii* were active against most of the sweet potato fungi and bacterial (Gram positive and gram negative) tested, thus suggesting that the antimicrobial principles in the three plants have broad antifungal activity. The antimicrobial activity of *W. ugandensis* was previously demonstrated (Kubo and Nakanishi, 1979; Olila *et al.,* 2001; 2002; Wube *et al.,* 2005; Rugutt *et al.,* 2006; Clarkson *et al.,* 2007; Mbwambo *et al.,* 2009). The antimicrobial activity of *T. brownii* reported in this study is in agreement with earlier findings where methanol and water extracts of the plant inhibited the growth of *Candida albicans* and *Cryptococcus neoformans* (*Mbwambo et al.,* 2007).

The antimicrobial compounds from *W. ugandensis* are polygodial [**13**], warbuganal [**14**], mukaadial [**15**], ugandensidial [**16**], cinnamolide [**17**], ugandensolide [**20**], deacetoxyugandensolide [**21**], muzigadial [**22**] and 6α-hydroxymuzigadial [**23**]; those from *E. schweifurthianum* are stigmasterol [**90**], β-sitosterol [**63**], 3-oxofriedooleanane [**44**] 3α-hydroxyfriedooleanane [**106**] and α-amyrin [**107**] while those from *T. brownii* are stigmasterol [**90**], β-sitosterol [**63**], arjungenin [**83**] and betulinic acid [**88**] at concentrations ≤200 µg/ml.

CHAPTER FIVE

5.0 SUMMARY, CONCLUSIONS AND RECOMMENDATIONS

5.1 Summary

- SPFMV, SPCSV, SPMMV, SPCFV and CMV were detected in symptomatic sweet potato in western Kenya. Out of the 327 samples tested, 89% were infected while 11% were not. In single infections, SPFMV had the highest frequency of detection while a combination of SPFMV and SPCSV was the most frequently detected dual infection. SPFMV, SPCSV, SPMMV and SPCFV were previously reported (Ateka *et al.,* 2004; Miano *et al.,* 2006; Nyaboga *et al.,* 2008). CMV was detected in sweet potato in Kenya for the first time.
- A rapid and efficient multiplex PCR protocol that simultaneously detects SPFMV and SPCSV was developed. The cDNA from sweet potato samples with dual infection of the two viruses were amplified using two sets of primers specific to viruses using the following parameters: one cycle at 95 ºC for 5 min followed by 35 cycles 96 ºC, 5 sec for denaturation; 63 ºC, 5 sec for primer annealing and 72 ºC, 30 sec for extension and a final extension at 72 ºC for 10 min.

- n-Hexane, ethyl acetate and methanol extracts from *W. ugandensis, E. schweifurthianum and T. brownii*, were tested for antimicrobial activity against sweet potato fungal and bacterial infections caused by *F. oxysporum, F. solani, Alternaria spp, R. stolonifer, A. niger, R. solanacearum* and *S. ipomoeae*. All the extracts were active against one or more of the microorganisms. The activities varied with plant species and the extraction solvent. Of the three medicinal plants, extracts from *W. ugandensis* exhibited the highest activity while methanol extracts gave the best activities compared to ethyl acetate and n-hexane extracts.
- *W. ugandensis, E. scweinfurthianum* and *T. brownii* afforded fourteen, nine and five compounds, respectively The compounds were identified as polygodial [**13**], warburganal [**14**], mukaadial [**15**], ugandensidial [**16**], muzigadial [**22**], 6α-hydroxymuzigadial [**23**], 9-deoxymuzigadial [**101**], ugandensolide [**20**], 7α-acetoxyugandensolide [**104**], deacetoxyugandensolide [**21**], cinnamolide [**17**], 3β-acetoxycinnamolide [**19**],

bemadienolide [**102**], drimenin [**103**], β-sitosterol [**63**], stigmasterol [**90**], lanosterol [**109**], 3-oxofriedooleanane [**44**], 3α-hydroxyfriedooleanane [**106**], 3-oxofriedoolean-29-ol [**108**], 3-oxofriedoolean-28-al [**45**], α-amyrin [**107**], α-amyrin acetate [**105**] monogynol A [**110**], betulinic acid [**88**] and arjungenin [**83**]. Compound **104** is new while compound **103** was isolated from *W. ugandensis* for the first time.

- Out of the twenty six isolates from the three plants, eighteen isolates were active against one or more of the fungi and bacteria tested (MIC≤ 200 µg/ml). Polygodial [**13**], warburganal [**14**], mukaadial [**15**], ugandensidial [**16**], ugandensolide [**20**], deacetoxyugandensolide [**21**] and muzigadial [**22**] were the most active isolates (MIC≤ 50 µg/ml).

5.2 Conclusion

- Five viruses namely SPFMV, SPCSV, SPMMV, SPCFV and CMV infect sweet potato in western Kenya. SPFMV is the most widespread followed by SPCSV. Viruses infect sweet potato in multiples. The most common dual viral infection is SPCSV+SPCSV. This is the first report of CMV detection in Kenya.

- An mPCR protocol was successfully developed for the simultaneously detection of SPFMV and SPCSV. The best amplification was obtained with 2.0 µg of cDNA template with 35 cycles at 63°C (annealing temperature).
- Extracts from *W. ugandensis, E. schweifurthianum and T. brownii*, have the potential for managing sweet potato fungal and bacterial infections caused by *F. oxysporum, F. solani, Alternaria spp, R. stolonifer, A. niger, R. solanacearum* and *S. ipomoeae*. Methanol extracts from the three plants are more active than ethyl acetate and n-hexane extracts.
- Phytochemical analysis of *W. ugandensis, E. schweinfurthianum* and *T. brownii*, yielded 26 compounds consisting of 25 known and one reported for the first time from *W. ugandensis*. The new compound was named as 7α-acetylugandensolide [**104**]. This is the first report of drimenin [**103**] from *W. ugandensis*.
- The antimicrobial principles from *W. ugandensis* are polygodial [**13**], warbuganal [**14**], mukaadial [**15**], ugandensidial [**16**], muzigadial [**22**], 6α-hydroxymuzigadial [**23**], ugandensolide [**20**], deacetoxyugandensolide [**21**] and cinnamolide [**17**]; those from *E. schweifurthianum* are β-sitosterol [**63**], stigmasterol [**90**], 3-oxofriedooleanane [**44**] 3α-hydroxyfriedooleanane [**106**] and α-amyrin [**107**] while those from *T. brownii* are β-sitosterol [**63**], stigmasterol [**90**], betulinic acid [**88**] and arjungenin [**83**].

5.3 Recommendation

- Due to the high frequency of viruses infection in sweet potato there is a need to develop a virus-free seed production program in Kenya, from which farmers could obtain clean planting materials. To prevent introduction of new viruses other than the ones already reported in the country, it is necessary to establish quarantine stations at the borders to ensure that only clean sweet potato planting materials get in.
- Since the developed protocol is rapid and enables simultaneously detection of SPFMV and SPCSV (the most common and economically important viruses), we recommend it for employment in screening sweet potato germplasm and cultivars for freedom of virus.
- Since extracts from *W. ugandensis, E. chweinfurthianum* and *T. brownii* have antifungal activity against sweet potato pathogens, they may provide an alternative way of controlling infections by farmers as it is less expensive and environmentally safe.
- Polygodial [**13**], warburganal [**14**], mukaadial [15], ugandensidial [**16**], ugandensolide [**20**], deacetoxyugandensolide [**21**] and muzigadial [**22**] exhibited low MIC comparable to the positive standards in the antimicrobial tests (MIC $\leq$ 50 µg/ml) and are recommended as antimicrobial agents.

5.4 Suggestions for further studies

- Since some of the symptomatic sweet potato samples did not test positive to any of the ten viruses tested in this study, there is a need for further investigation to identify the unknown viral infections. In order to find a lasting solution to viral infections in sweet potato, which often occur in multiples, there is a need to search for antiviral agents which can manage multiple viral infections of the crop.
- Further search into methods of multiple PCR detection techniques which can simultaneously detect SPFMV, SPCSV, SPMMV, SPCFV and CMV which were detected in this study are necessary.
- Methanol extracts from the medicinal plants exhibited the highest activity against the fungi abd bacteria hat were tested in this study. However, attampts to isolate antimicrobial compounds from the extracts failed. Further phytochemical work on the extracts is necessary.
- Further *in vivo* antibacterial and antifungal assays with the crude extracts and pure isolates from *W. ugandensis, E. chweinfurthianum* and *T. brownii* are nessessary to establish doses that can effectively protect sweet potato from the pathogens.
- It is necessary to carry out synergistic and antagonist studies of the pure isolates in order to determine which combinations have the best antimicrobial activity. The active pure isolates are recommended as candidates for further search for safe antimicrobial agents.

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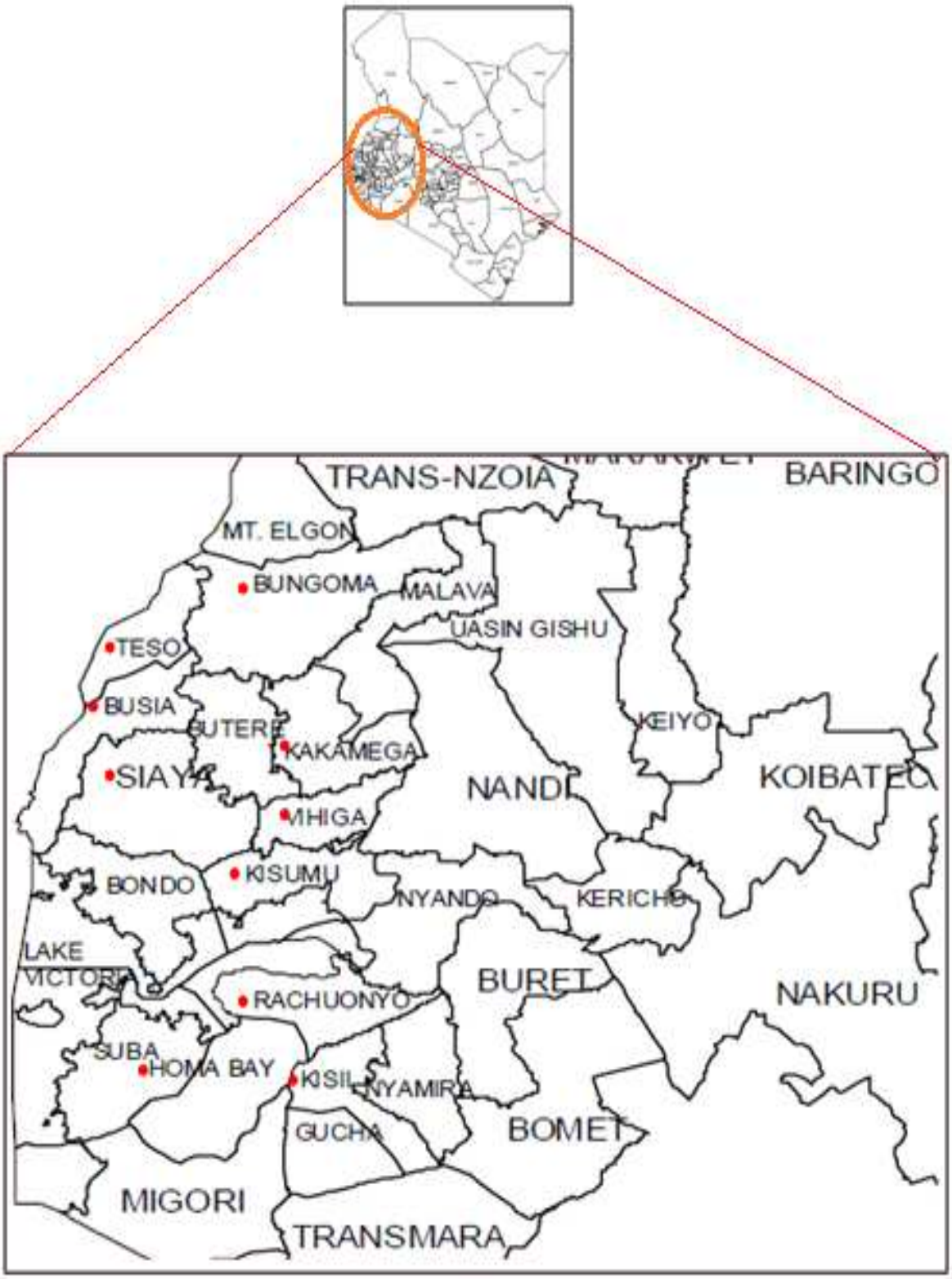
APPENDIX A: Publications for this study

1. **Opiyo S.A.,** Ateka E.M., Owuor P.O., Manguro L.O.A., Miano D.W. (2010). Development of a multiplex PCR technique for simultaneous detection of *Sweet potato feathery mottle virus* and *Sweet potato chlorotic stunt virus. Plant Pathology*, 92(2) 353-356.

2. **Opiyo S.A.,** Ateka E.M., Owuor P.O., Manguro L.O.A., Karuri H.W. (2010). Survey of sweet potato viruses and detection of *Cucumber mosaic virus* in western Kenya. *Plant Pathology*, 92(3) 795-799.

3. **Opiyo S.A.,** Manguro L.O.A., Owuor P.O., Ateka E.M., Lemmen P. (2011). 7α-Acetylugandensolide and antimicrobial properties of *Warburgia ugandensis* extracts and isolates against sweet potato pathogens. *Phytochemistry letters*, doi:10.1016/j.phytol.2011.02.007.

APPENDIX B: A map showing the sampling areas in western Kenya



APPENDIX C: BUFFERS FOR NCM-ELISA

1. TBS pH 7.5 (2 L)

0.02 M Tris base = 4.84 g

0.50 M Nacl = 58.44 g

5.0 ml HCl (18.5%)

2. Extraction buffer (500 ml)

500 ml TBS pH 7.5

1.0 g Sodium sulfite

3. Antibody buffer

600 ml TBS pH 7.5

12.0 g Powdered cow milk

4. Blocking solution

300 ml Antibody buffer

6.0 ml Triton

5. T-TBS (2 L)

2Litres TBS pH 7.5

2 ml Tween-20

6. Conjugate buffer (300 ml)

300 ml TBS pH 7.5

Powdered cow milk 6.0 g

7. Substrate buffer pH 9.5 (250 ml)

3.03 g Tris base

1.45 g NaCl

0.25 g $MgCl.6H_2O$

0.5 ml HCl

250 ml Distilled water

8. Substrate solution

(a) NBT stock solution

NBT 25 mg

N,N-dimethyl formaldehyde (70%) 1 ml

Mix well and store at 4°C protect from light

(b) BCIP stock solution

BCIP 12.5 mg

N,N-dimethyl formaldehyde (100%) 1 ml

Mix well and store at 4°C protect from light

(c) Preparation of substrate solution

250 ml substrate buffer

1 ml NBT stock solution

1.0 ml BCIP stock solution

APPENDIX D: Statistical analysis of antimicrobial activity

Data file: SYLVIA
Title: Antifungal
Function: FACTOR
Experiment Model Number 8:
Two Factor Randomized Complete Block Design
Data case no. 1 to 27.
Factorial ANOVA for the factors:
Replication (Var 1: Replication 1= Rep One, 2= Two, 3= Three) with values from 1 to 3
Factor A (Var 2: Plant 1= W. ugandensis, 2= E. scweifurthianum, 3= T.brownii) with values from 1 to 3
Factor B (Var 3: Extract 1= Methanol, 2= Ethyl acetate, 3= n-Hexane) with values from 1 to 3
Variable 4: Alternaria spp
Grand Mean = 7.922 Grand Sum = 213.900 Total Count = 27

T A B L E O F M E A N S

1	2	3	4	Total
1	*	*	7.889	71.000
2	*	*	7.933	71.400
3	*	*	7.944	71.500
*	1	*	12.200	109.800
*	2	*	7.533	67.800
*	3	*	4.033	36.300
*	*	1	12.411	111.700
*	*	2	8.844	79.600
*	*	3	2.511	22.600
*	1	1	17.100	51.300
*	1	2	14.067	42.200
*	1	3	5.433	16.300
*	2	1	12.067	36.200
*	2	2	8.433	25.300
*	2	3	2.100	6.300
*	3	1	8.067	24.200
*	3	2	4.033	12.100
*	3	3	0.000	0.000

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.016	0.008	0.5091	
2	Factor A	2	302.167	151.083	9889.1128	0.0000
4	Factor B	2	452.527	226.263	14809.9963	0.0000
6	AB	4	17.573	4.393	287.5643	0.0000

-7 Error 16 0.244 0.015

Total 26 772.527

Coefficient of Variation: 1.56%

s_ for means group 1: 0.0412 Number of Observations: 9
y
s_ for means group 2: 0.0412 Number of Observations: 9
y
s_ for means group 4: 0.0412 Number of Observations: 9
y
s_ for means group 6: 0.0714 Number of Observations: 3
y

==========

Variable 5: A. niger
Grand Mean = 9.841 Grand Sum = 265.700 Total Count = 27

T A B L E O F M E A N S

1	2	3	5	Total
1	*	*	9.844	88.600
2	*	*	9.833	88.500
3	*	*	9.844	88.600
*	1	*	11.889	107.000
*	2	*	11.956	107.600
*	3	*	5.678	51.100
*	*	1	16.011	144.100
*	*	2	10.000	90.000
*	*	3	3.511	31.600
*	1	1	21.067	63.200
*	1	2	11.533	34.600
*	1	3	3.067	9.200
*	2	1	16.433	49.300
*	2	2	13.433	40.300
*	2	3	6.000	18.000
*	3	1	10.533	31.600
*	3	2	5.033	15.100
*	3	3	1.467	4.400

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.001	0.000	0.0447	

2	Factor A	2	233.979	116.989	14117.0913	0.0000
4	Factor B	2	703.467	351.734	42443.6984	0.0000
6	AB	4	81.386	20.346	2455.2096	0.0000
-7	Error	16	0.133	0.008		
	Total	26	1018.965			

Coefficient of Variation: 0.93%

s_ for means group 1: 0.0303 Number of Observations: 9
y
s_ for means group 2: 0.0303 Number of Observations: 9
y
s_ for means group 4: 0.0303 Number of Observations: 9
y
s_ for means group 6: 0.0526 Number of Observations: 3

==========

Variable 6: F. oxysporum
Grand Mean = 3.848 Grand Sum = 103.900 Total Count = 27

T A B L E O F M E A N S

1	2	3	6	Total
1	*	*	3.833	34.500
2	*	*	3.856	34.700
3	*	*	3.856	34.700
*	1	*	7.500	67.500
*	2	*	4.044	36.400
*	3	*	0.000	0.000
*	*	1	7.167	64.500
*	*	2	3.833	34.500
*	*	3	0.544	4.900
*	1	1	13.433	40.300
*	1	2	8.067	24.200
*	1	3	1.000	3.000
*	2	1	8.067	24.200
*	2	2	3.433	10.300
*	2	3	0.633	1.900
*	3	1	0.000	0.000
*	3	2	0.000	0.000
*	3	3	0.000	0.000

A N A L Y S I S O F V A R I A N C E T A B L E

K	Degrees of	Sum of	Mean	F

Value	Source	Freedom	Squares	Square	Value	Prob
1	Replication	2	0.003	0.001	0.1127	
2	Factor A	2	253.645	126.823	9645.6688	0.0000
4	Factor B	2	197.345	98.673	7504.6814	0.0000
6	AB	4	120.544	30.136	2292.0298	0.0000
-7	Error	16	0.210	0.013		
	Total	26	571.747			

Coefficient of Variation: 2.98%

s_ for means group 1: 0.0382 Number of Observations: 9
y
s_ for means group 2: 0.0382 Number of Observations: 9
y
s_ for means group 4: 0.0382 Number of Observations: 9
y
s_ for means group 6: 0.0662 Number of Observations: 3
y

==========

Variable 7: F. solani
Grand Mean = 4.733 Grand Sum = 127.800 Total Count = 27

T A B L E O F M E A N S

1	2	3	7	Total
1	*	*	4.711	42.400
2	*	*	4.722	42.500
3	*	*	4.767	42.900
*	1	*	5.344	48.100
*	2	*	5.800	52.200
*	3	*	3.056	27.500
*	*	1	8.556	77.000
*	*	2	4.122	37.100
*	*	3	1.522	13.700
*	1	1	9.433	28.300
*	1	2	4.067	12.200
*	1	3	2.533	7.600
*	2	1	10.433	31.300
*	2	2	6.100	18.300
*	2	3	0.867	2.600
*	3	1	5.800	17.400
*	3	2	2.200	6.600
*	3	3	1.167	3.500

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.016	0.008	0.4375	
2	Factor A	2	38.936	19.468	1095.0633	0.0000
4	Factor B	2	227.647	113.823	6402.5675	0.0000
6	AB	4	24.298	6.074	341.6877	0.0000
-7	Error	16	0.284	0.018		
	Total	26	291.180			

Coefficient of Variation: 2.82%

s_ for means group 1: 0.0444 Number of Observations: 9
y
s_ for means group 2: 0.0444 Number of Observations: 9
y
s_ for means group 4: 0.0444 Number of Observations: 9
y
s_ for means group 6: 0.0770 Number of Observations: 3
y

==========

Variable 8: R. stolonifer
Grand Mean = 6.741 Grand Sum = 182.000 Total Count = 27

T A B L E O F M E A N S

1	2	3	8	Total
1	*	*	6.756	60.800
2	*	*	6.733	60.600
3	*	*	6.733	60.600
*	1	*	14.622	131.600
*	2	*	5.600	50.400
*	3	*	0.000	0.000
*	*	1	11.278	101.500
*	*	2	6.033	54.300
*	*	3	2.911	26.200
*	1	1	24.500	73.500
*	1	2	13.733	41.200
*	1	3	5.633	16.900
*	2	1	9.333	28.000
*	2	2	4.367	13.100
*	2	3	3.100	9.300
*	3	1	0.000	0.000

*	3	2	0.000	0.000
*	3	3	0.000	0.000

A N A L Y S I S O F V A R I A N C E T A B L E

K Value	Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	Prob
1	Replication	2	0.003	0.001	0.1649	
2	Factor A	2	979.710	489.855	54540.3875	0.0000
4	Factor B	2	321.761	160.880	17912.4046	0.0000
6	AB	4	280.848	70.212	7817.4012	0.0000
-7	Error	16	0.144	0.009		
	Total	26	1582.465			

Coefficient of Variation: 1.41%

s_ for means group 1: 0.0316 Number of Observations: 9
y
s_ for means group 2: 0.0316 Number of Observations: 9
y
s_ for means group 4: 0.0316 Number of Observations: 9
y
s_ for means group 6: 0.0547 Number of Observations: 3
y

APPENDICES 1-26

SPECTRA OF ISOLLATED COMPOUNDS