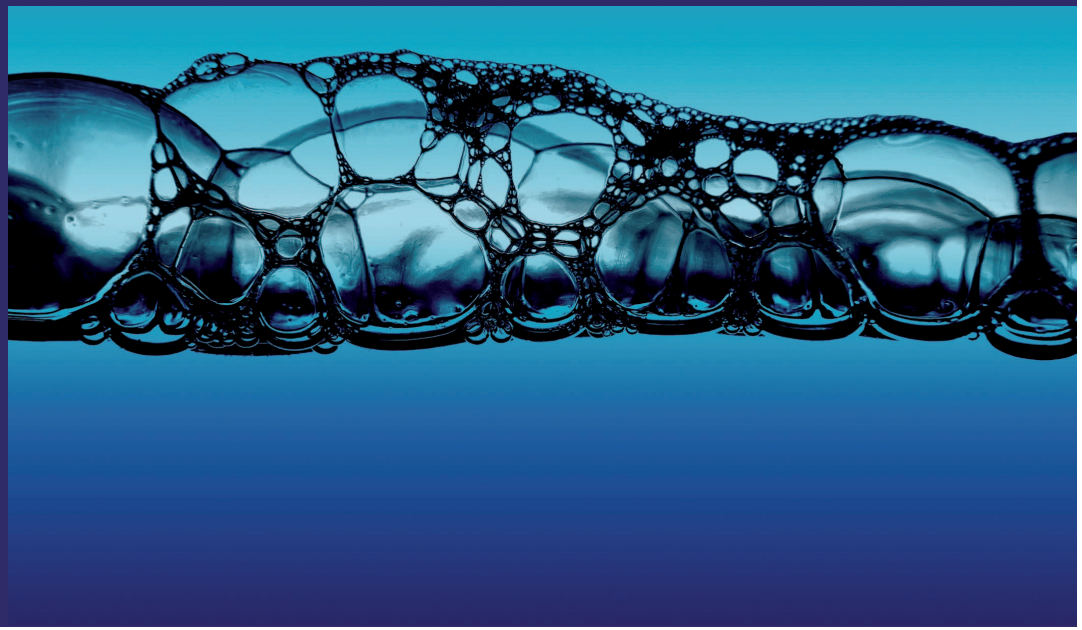
Sweetpotato is an important food crop since it is drought tolerant. However, its production is limited by viral, fungal and bacterial infections. Use of synthetic chemicals to manage such infections has not been effective because of pathogenic resistance, potential danger to both humans and environment, and the cost involved. In order to fully exploit the potential of the sweetpotato crop, there is need for rapid and sensitive virus detection techniques and affordable, readily available, sustainable, and environmentally friendly means of managing the infections.



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SWEETPOTATO VIRUS DETECTION PROTOCOL AND MICROBIAL INFECTIONS CONTROL

By

Dr. Sylvia Awino Opiyo

DEDICATION

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

ACKNOWLEDGEMENT

I would like to express special thanks to my supervisors, Prof. P.O. Owuor, Prof. L.A.O. Manguro and Dr. E.M. Ateka for supervising this research, and for their invaluable advice and encouragement during the time we worked together. I acknowledge Dr. B.A. Nyende, the Director of Institute of Biotechnology Research (IBR), Jomo Kenyatta University of Agriculture and Technology (JKUAT) for allowing part of the research to be done in his laboratory. The staff of the Department of Chemistry, Maseno University and research staff of the Department of Botany (Maseno University and JKUAT) are greatly acknowledged for their time and expertise generously given during the research period. Special appreciation is to my colleagues Mr. K. Bowa, Mr. C. Ochieng', Ms. D. Akinyi and Ms H.W. Karuri for their assistance, support and encouragement. Greatest thanks go to New Partnership for Africa's Development (NEPAD), Canadian International Development Agency (CIDA), Biosciences Eastern and Central Africa Network (BecANet) and Higher Education Loans Board (HELB) for financial support.

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Abstract

Sweetpotato 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 is not effective because apart from their potential danger to both humans and environment, they are unaffordable by most farmers. Moreover, because of pathogens resistance, most chemicals have become ineffective. In order to fully exploit the potential of the sweetpotato crop, there is a need to search for affordable, readily available, sustainable, and environmentally friendly means of managing the problems posed by these pathogens. Plants extracts have been reported to be safe, non-phytotoxic to humans, but effective against several plant pathogens. The aim of this study was to identify viruses infecting sweetpotato in western Kenya, develop a multiplex polymerase chain reaction (PCR) protocol for detection of major viruses of the crop, and evaluate efficacy of medicinal plant extracts for antimicrobial activity against the crop pathogens. Symptomatic sweetpotato vines from farmers' fields were tested for ten viruses using NCM-ELISA. mPCR protocol for detection of *Sweetpotato feathery monttle virus* (SPFMV) and *Sweetpotato chlorotic stunt virus* (SPCSV) was optimized through variation of test parameters under standard PCR conditions. Extracts from *Warburgia ugandensis,* traditionally used to manage microbial infections, were tested for antimicrobial activity against sweetpotato infection pathogens using paper disk diffusion assay. Five viruses namely SPFMV, SPCSV, *Sweetpotato mild mottle virus* (SPMMV), *Sweetpotato chlorotic fleck virus* (SPCFV) and *Cucumber mosaic virus* (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. A rapid multiplex PCR protocol that simultaneously detects SPFMV and SPCSV was developed. The best amplification was obtained with 2.0 µg of complementar cDNA template 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. From the antimicrobial assay, ethyl acetate extract exhibited the highest ($P \leq 0.05$) antifungal and antibacterial activities compared to n-hexane and methanol extracts. *Rhizopus stolonifer* was more susceptible to ethyl acetate extract (inhibition zone = 24.5 mm) than Blitox which was used as a positive control. The minimum inhibitory concentration (MIC) for the isolates ranged between 12.5 to 200 µg/ml with the lowest MIC value of 12.5 µg/ml being observed with polygodial against *Fusarium solani*; warburganal against *Aspergillus niger* and *F. solani*; and mukaadial against *Aspergillus niger*. This study provided a quantitative assessment of viruses infecting sweetpotato in western Kenya and a simplified mPCR protocol for routine rapid detection of SPFMV and SPCSV. Scientific proof for the efficacy of extracts and isolates of *W. ugandensis* as antimicrobial agents against sweetpotato 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
cDNA	Complementary deoxyribonucleic acid
CIP	International Potato Centre
CMV	*Cucumber Mosaic Virus*
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	2', 3'- Deoxyribonucleoside-5'- phosphate
ELISA	Enzyme-Linked Immunosorbent Assay
EtOAc	Ethyl acetate
F. oxysporum	*Fusarium oxysporum*
F. solani	*Fusafium solani*
FAO	Food Agriculture Organization
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
NBT	Nitroblue tetrazolium
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RNA	Ribonucleic acid
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SPCaLV	*Sweetpotato Caulimo-Like Virus*
SPCFV	*Sweetpotato Chlorotic Fleck Virus*
SPCSV	*Sweetpotato Chlorotic Stunt Virus*
SPFMV	*Sweetpotato Feathery Mottle Virus*
SPLCV	*Sweetpotato Leaf Curl Virus*
SPMMV	*Sweetpotato Mild Mottle Virus*
SPMSV	*Sweetpotato Mild Speckling Virus*

SPVD	Sweetpotato Virus Disease
SPVG	*Sweetpotato Virus* G
SwPLV	*Sweetpotato Latent Virus*
Taq	*Thermophilus acquaticus*
TBS	Tris buffered saline
T-TBS	Tris Buffered Saline supplemented with Tween-20
TLC	Thin Layer Chromatography

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Sweetpotato

Sweetpotato (*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 sweetpotato 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). Sweetpotato 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).

Sweetpotato 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 sweetpotato 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. Sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato yields to meet the demand for the crop. The main sweetpotato producing regions of Kenya are western, eastern, central and coastal areas (MOA, 1999).

Despite its high potential for food security, production of sweetpotato 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. *Sweetpotato 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 sweetpotato (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). A greater damage is realized when SPFMV infects sweetpotato in the presence of *Sweetpotato chlorotic stunt virus* (SPCSV), leading to sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato infections are shown in Fig. 1 and 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 sweetpotato 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 sweetpotato viruses.

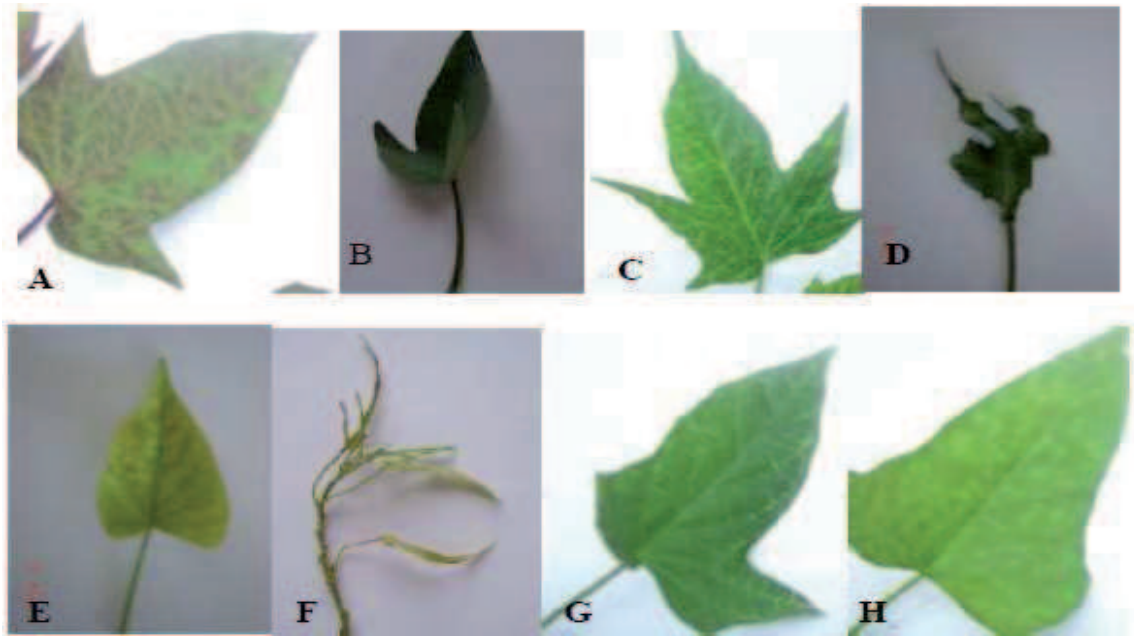

Fig. 1: Some virus symptoms observed on sweetpotato plants (A) purpling of leaves in plants infected with SPCSV, (B) symptomless leaves of plants infected with SPFMV, (C) vein clearing in leaves of plants infected with SPMMV, (D) deformed leaves of plants infected with SPFMV and SPCSV, (E) interveinal 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).

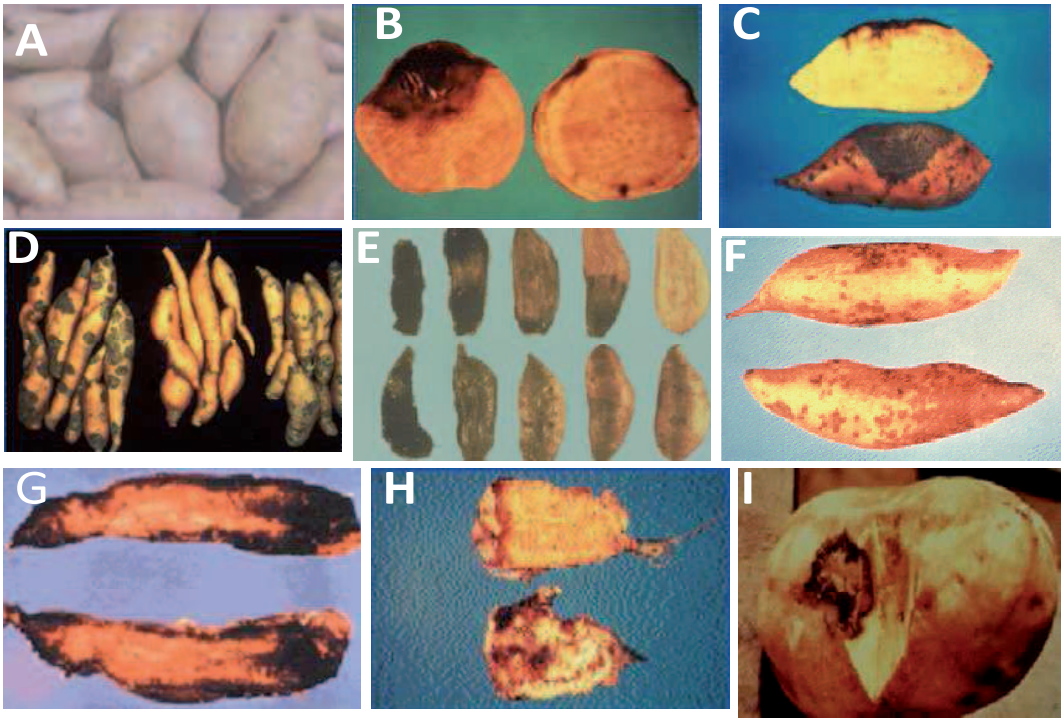

Fig. 2: Some of the symptoms exhibited by microbial infections in sweetpotato; (**A**) Healthy roots, (**B**) Cross section of root with symptoms of Fusarium root rot (left) and Fusarium surface rot (right), (**C**) Internal and external views of roots infected with black rot (*Ceratocystis fimbriata*), (**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**) Sweetpotatoes 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**) Sweetpotato 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 (Lozoya *et al.,* 1984; Sivropou *et al.,* 1997; Kim *et al.,* 1998; Perez *et al.,* 1999; Udo *et al.,* 2001; Amadioha, 2003; Okigbo and Emoghene, 2004; Okigbo and Nmeka, 2005). Extracts of *Warburgia ugandensis* are used in ethnomedicine to manage viral, fungal and bacterial infections (Kokwaro, 2009). Phytochemical studies of *Warburgia* species have lead to isolation of different compounds some of which have antimicrobial activities (Kioy *et al.,* 1090a; 1990b; Rabe and Staden, 2000; Olila *et al.,* 2001; 2002; Rugutt, *et al.,* 2006). It is necessary to evaluate the extracts of these plants for their possible use in managing infections of sweetpotato.

1.2 Statement of the problem

Sweetpotato 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. In most cases the infecting viruses co-infect the crop leading to more severe infections. However, effective methods of multiple virus infections are lacking. Synthetic chemical currently used to manage fungal and bacterial infections pose health danger not only to the users and the environment but are also unaffordable by small scale farmers. Environmentally friendly methods of managing the infections are lacking.

1.3 Null hypotheses

i. No more viruses rather than the reported ones infect sweetpotato in western Kenya with possible multiple interactions.

ii. Multiplex PCR-based technique may not enable simultaneous detection of several viruses.

iii. *Warburgia ugandensis* extracts and isolates do not contain secondary metabolites which exhibit antimicrobial activity against fungi and bacteria that infect sweetpotato.

1.4 Justification of the study

Sweetpotato 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. However, the production of sweetpotato 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 sweetpotato viruses in western Kenya, which is the main sweetpotato 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 methods are not applicable unless the antibody specific to the virus studied is available. Since the viruses co-infect sweetpotato 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 sweetpotato 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 (Reiner, 1982; Cameron and Julian 1984; Zhonghua and Michailides, 2005). In order to fully exploit the potential of the sweetpotato crop, there is a need to search for affordable, readily available, sustainable and environmentally friendly means of managing the problems posed by these pathogens.

1.5 Objectives of the study

The aim of this study was to identify the viruses infecting ordinary sweetpotato grown by rural folk in western Kenya, develop a protocol for simultaneous detection of major viral infections and investigate the efficacy of 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 sweetpotato in western Kenya.
ii. Develop a mPCR protocol for simultaneous detection of SPFMV and SPCSV, the most economically important viruses of sweetpotato.
iii. Evaluate *Warburgia ugandensis* extracts and isolates for antifungal and antibacterial activities against pathogens which infect the crop .

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Origin and distribution of sweetpotato

Sweetpotato (*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 sweetpotato

Staple crops such as maize, rice, sorghum and millet do not match the yield per unit area of sweetpotato that also has higher food value (FAO, 2002). On a worldwide scale, the importance of sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato 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 (Woolfe, 1987; www.recipes4us.co.uk, 12th July, 2010). Besides simple starches, sweetpotato is rich in carbohydrates, dietary fiber, beta carotene (a vitamin A equivalent nutrient), vitamin C, and vitamin B_6 (Woolfe, 1987; www.recipes4us.co.uk, 12th July, 2010). Important minerals found in the crop include calcium, phosphorous, iron, sodium and potassium (Woolfe, 1992). Young sweetpotato 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). Sweetpotato 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 sweetpotato, there is a need to improve its production in order to meet its demand.

2.3 Constraints to sweetpotato production

Sweetpotato 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 sweetpotato 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 sweetpotato

Among the biological factors, virus diseases rank second to weevil in causing yield reduction in sweetpotato (Karyeija *et al.,* 1998; Gibson and Aritua, 2002). Several viruses have been reported

infecting sweetpotato including *Sweetpotato feathery mottle virus* (SPFMV), *Sweetpotato chlorotic stunt virus* (SPCSV), *Sweetpotato mild mottle virus* (SPMMV), *Sweetpotato chlorotic fleck virus* (SPCFV), Sweetpotato *latent virus* (SPLV), Sweetpotato caulimo-like virus (SPCa-LV), *Cucumber mosaic virus* (CMV), Sweetpotato virus Y (SPVY), C-6, Sweetpotato virus G (SPVG), *Sweetpotato mild speckling virus* (SPMSV) and *Sweetpotato 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 sweetpotato viruses in western Kenya, which is the main sweetpotato producing region in the country.

Sweetpotato feathery mottle virus (SPFMV) is the most prevalent and widespread in many parts of the world where sweetpotato is cultivated (Moyer and Salazar, 1989; Nishiguchi *et al*., 1995; Sakai *et al.,* 1997). When infecting alone, SPFMV seem insignificant in its effects on sweetpotato (Gibson *et al.,* 1998; Karyeija *et al.,* 2000). A greater economic impact is realized when SPFMV infects sweetpotato in the presence of *Sweetpotato chlorotic stunt virus* (SPCSV), leading to sweetpotato 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 sweetpotato not yet identified. There is need for further tests to identify the unknown infections.

2.3.2 Fungal and bacterial infection in sweetpotato

Several fungi are reported to be associated with reduction of yield and rotting of sweetpotato 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 Sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato viruses (Nishiguchi *et al.,* 1995; Ki and Sun, 2002; Kokkinos and Clark, 2006). Since sweetpotato 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 sweetpotato.

Synthetic chemicals such as virazole [**1**], pyrazofurin [**2**], azaguanine [**3**], carbaryl [**4**], dichloronitroaniline [**5**] and blitox [**6**] (Fig. 3) have been 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).

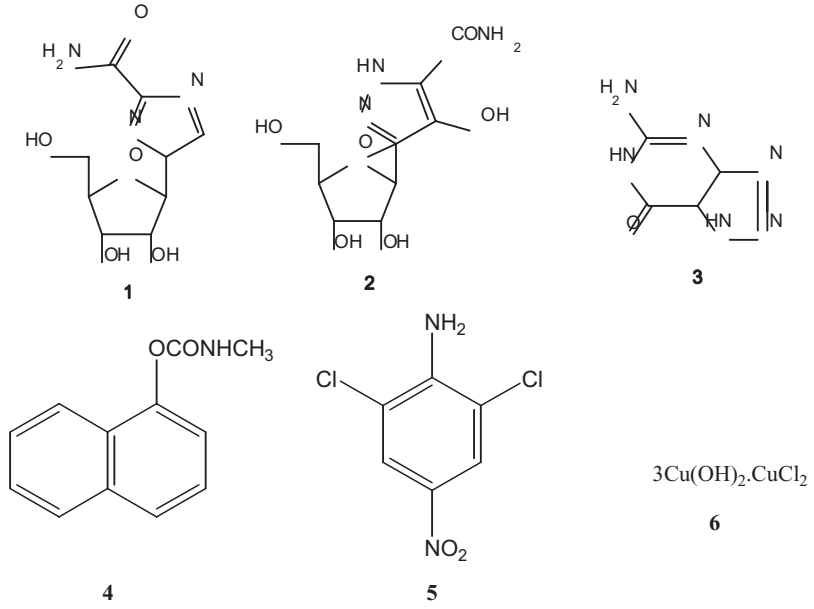

Fig.3: Some synthetic chemicals used to manage microbial infections.

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.

2.4 The use of plant extracts in disease management

The use of medicinal plants in managing diseases is widespread and the production of medicines for treatment of diseases began with the use of herbs (Tyler, 1997; Farnsworth, 1984). 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 (Farnsworth *et al.,* 1986). Apart from being used directly to manage infections, , 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 (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). 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). In another study, 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); essential oil from carrot and *Tagetes minuta* were active against *tobacco mosaic virus* (TMV), *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). In another study (Mekbib *et al.,* 2007), extracts of *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). Further such for effective and environmentally friendly means of controlling plant pathogen is necessary.

2.5 *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. 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).

A number of drimane-type sesquiterpenes (Kubo *et al.,* 1976; Brooks and Draffan, 1969a; 1969b; Kioy *et al.,* 1989; 1990a; 1990b; Mashimbye *et al.,* 1999; Wube *et al.,* 2005; Xu *et al.,* 2009) and flavonoids (Manguro *et al.,* 2003a; 2003b) have been reported from various parts of the plant. In vitro pharmacological studies on this plant have also confirmed the presence of insect antifeedant (Kubo *et al.,* 1977), molluscicidal (Kubo *et al.,* 1983), antimicrobial (Lee *et al*., 1998; Mbwambo *et al.,* 2009; Wube *et al.,* 2005; Madikane *et al.,* 2007) and antileishmanial (Ngure *et al.,* 2009) active sesquiterpenes. 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.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Establishment of the incidence and identity of sweetpotato viruses in western Kenya

3.1.1 Survey of sweetpotato viruses

In western Kenya, sweetpotato 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 A). A survey was conducted in sweetpotato fields in the ten districts in April 2009 as previously described (Ateka, 2004; Tairo *et al.,* 2004). Sweetpotato 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 sweetpotato 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 fertilizer 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 sweetpotato samples

Symptomatic sweetpotato 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 sweetpotato plant (one month old) were ground in 1 ml of extraction buffer (Appendix B) 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 B) 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 B) 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 sweetpotato 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 sweetpotato 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 sweetpotato 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 sweetpotato 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*

3.3.1 Collection of plant material

The stem bark of *W. ugandensis* was collected near St Mary's Hospital along the Nakuru- Gilgil highway (latitude 0° 24' 42.49" S and longitude 36° 15' 10.59" E) in August 2008 and voucher specimen (2008/08/01/SAO/CHEMMK) was identified at the Kenya National Museum herbarium after comparison with authentic samples. The plant materials were chopped into smaller pieces, air-dried in the open and reduced to a powder using a mill.

3.3.2 Extraction and isolation of compounds from *W. ugandensis*

Plant materials were cut into small pieces and air-dried for one month under a shadeand ground into fine powder. The powdered material (2 kg) was sequentially extracted with n-hexane, EtOAc and MeOH in the cold for seven days each, with occasional shaking. The macerate was filtered and the filtrate concentrated under vacuum using rotary evaporator to afford 20 g, 105 g and 215 g of n-hexane, ethyl acetate and methanol extracts, respectively. Extracts were stored at 4 ^{0}C for phytochemical and antimicrobial activity studies against sweetpotato fungi and bacteria.

Crude extracts were subjected to chromatography over silica gel column and eluting with organic solvents to yield 14 pure compounds (Fig. 4) which were identified as polygodial [**7**], warburganal [**8**], mukaadial [**9**], ugandensidial [**10**], muzigadial [**11**], 6α-hydroxymuzigadial [**12**], 9-deoxymuzigadial [**13**], ugandensolide [**14**], 7α-acetoxyugandensolide [**15**], deacetoxyugandensolide [**16**], cinnamolide [**17**], 3β-acetoxycinnamolide [**18**], bemadienolide [**19**] and drimenin [**20**]. (Kioy *et al.,* 1990a).

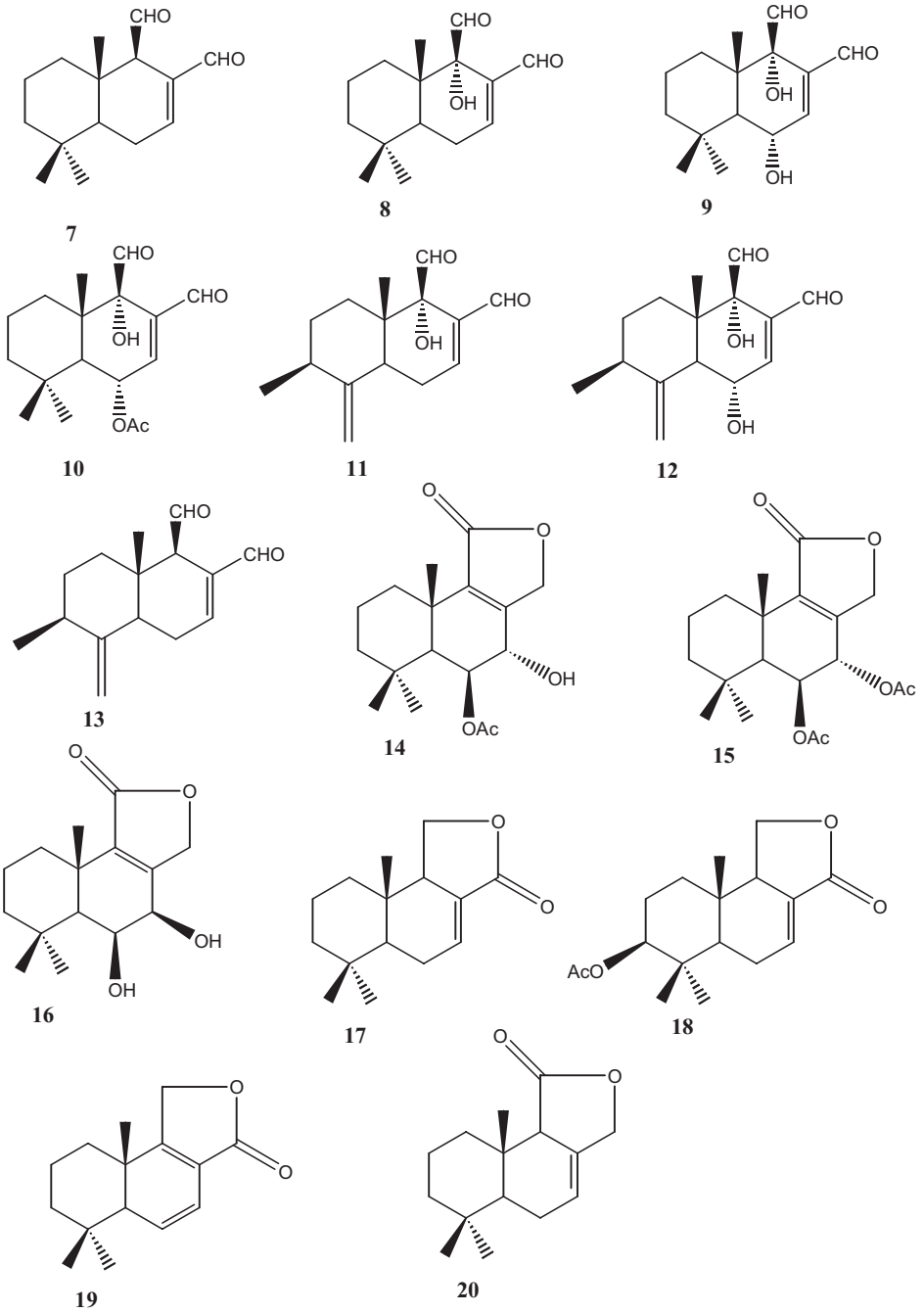

Fig. 4: Structures of compounds isolated from *W. ugandensis*

3.4 Evaluation of antifungal and antibacterial activity of plant extracts

3.4.1 Isolation of spoilage fungi and bacteria from decayed sweetpotato

Five fungi and two bacteria isolated from infected sweetpotato roots were used in this study. Sterilized pieces of infected sweetpotato were incubated in nutrient agar (NA) and potato dextrose agar (PDA) at room temperature for up to 5 days and fungal and bacterial growth associated with rot affected tissues were identified with the aid of the appropriate taxonomic keys (Ainsworth *et al.,* 1973). The isolates were maintained on NA and PDA slants.

3.4.2 Antifungal and antibacterial assays of crude extracts

Antimicrobial activities of the methanol, ethyl acetate and n-hexane extracts of *W. ugandensis* were evaluated by the agar diffusion method (Barry *et al.* 1979). The tests were performed in sterile petri-dishes (90 mm diameter) containing 20 ml PDA and NA for fungi and bacteria, respectively. The PDA and NA media were prepared by suspending 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 cfu/ 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 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 and streptocycline (10µg/ml) were used as positive controls while DMSO without plant extract was used as a negative control. All tests were done in triplicates for statistical purposes. The presence of zones of inhibition around the disc was interpreted as an indication of antimicrobial activity.

3.4.3 Antifungal and antibacterial assay of pure isolates and MIC determination

The minimum inhibitory concentrations (MIC) of pure isolates were determined as outlined by Kariba *et al.* (2001). The compounds were dissolved in DMSO and different concentrations ranging between 200 and 1 and µg/ml prepared. Sterile paper discs were impregnated with 100 µl of the reconstituted samples in DMSO. The dried discs were transferred aseptically into PDA

an NA plates previously inoculated with test fungi and bacteria, respectively and MIC was regarded as the lowest concentration that produced a visible zone of inhibition.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Viruses infecting sweetpotato in western Kenya

Symptomatic sweetpotato 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%).

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**

Previously, SPFMV, SPCSV, SPMMV, SPCFV and SPMSV were reported to infect sweetpotato 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 sweetpotato 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 sweetpotato 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 3: Proportion (%) of sweetpotato 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 = Sweetpotato feathery mottle virus; SPCSV = Sweetpotato chlorotic stunt virus; SPMMV = Sweetpotato mild mottle virus; SPCFV = Sweetpotato 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 sweetpotato virus disease (SPVD) which is economically the most important disease of sweetpotato (Gibson *et al*., 1998; Mukasa *et al*., 2003). The viral mixtures of infections lead to synergistic effect which results in severer damage to sweetpotato 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. 5). 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 four 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 sweetpotato 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 sweetpotato in Kenya.

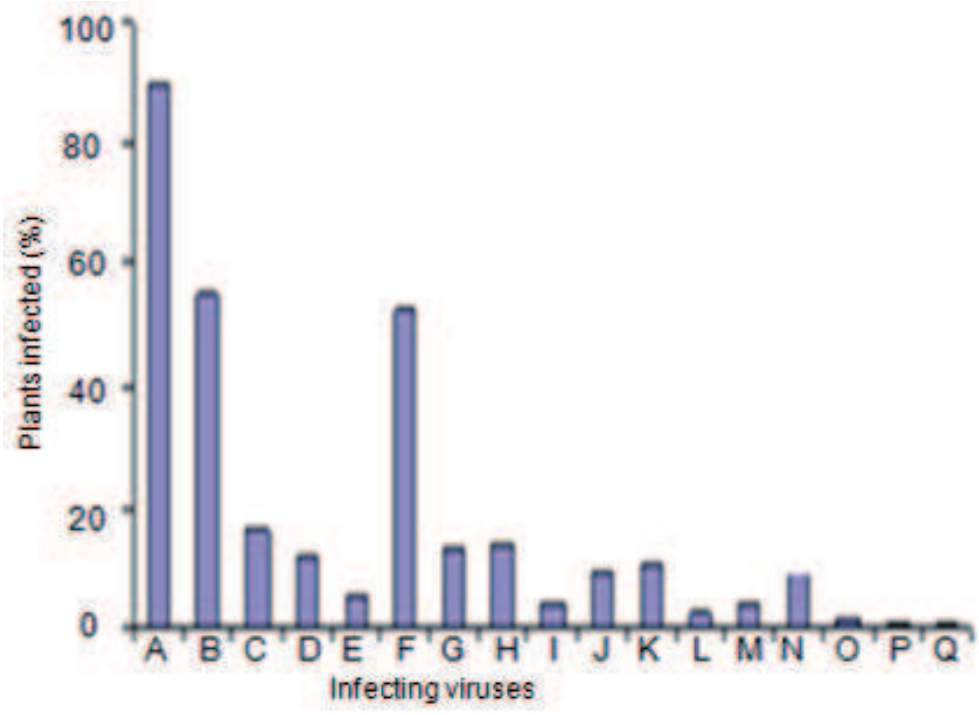

Fig. 5: Proportion (%) of single and mixed virus infections detected by NCM-ELISA in symptomatic sweetpotato plants in western Kenya. SPFMV (**A**), SPCSV (**B**), SPMMV (**C**), SPCFV (**D**), CMV (**E**), SPFMV + SPCSV (**F**), SPFMV + SPMMV (**G**), SPFMV + SPCFV (**H**), SPFMV + CMV (**I**), SPCSV + 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 sweetpotato leaf samples infected with SPFMV and SPCSV by both sPCR and mPCR (Fig. 6) while no amplification products were obtained from healthy plants.

Thirteen sweetpotato samples showing sweetpotato 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 sweetpotato 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 sweetpotato 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.

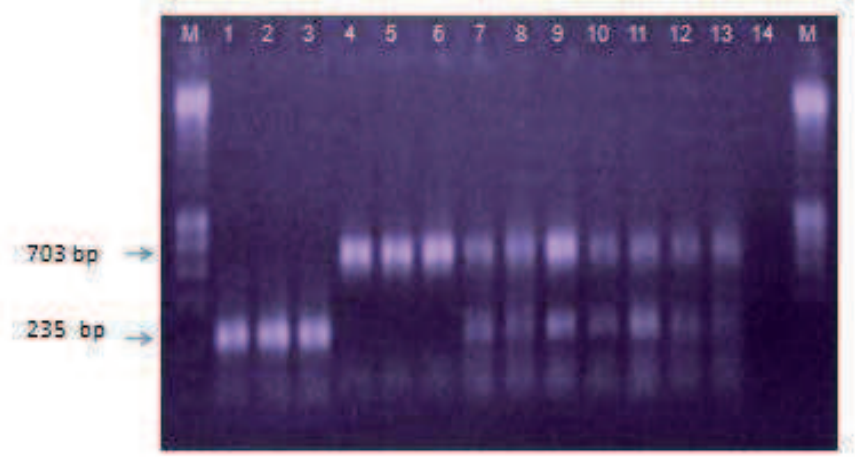

Fig. 6: Electrophoresis profile of DNA amplified products from total RNA obtained from 13 symptomatic and 1 healthy sweetpotato samples by singlex and multiplex PCR. Lane M: DNA size marker (200 bp); Lanes 1-3: singlex PCR using primers specific to SPCSV; Lanes 4-6: singlex PCR using primers specific 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 antifungal and antibacterial assays

4.3.1 Activity of crude extracts

Results of the antimicrobial activities of MeOH, EtOAc and n-hexane extracts of *W. ugandensis* bark are shown in Table 4. The activity of extract were tested against fungi (*Alternaria spp, Aspegillus niger, Fusarium oxysporum, F. solani* and *Rhizopus stolonifer*) and bacteria (*Ralstonia solanacearum* and *Steptomyces ipomoeae*). The extracts showed activity against the fungi and bacteria tested with ethyl acetate extract exhibiting the highest ($P \leq 0.05$) inhibitory effects against the microorganisms. Methanol extract exhibited the lowest activity. In our observation, the fungus *R. stolonifer* were found to be the most susceptible to the ethyl acetate extract with inhibition zone of 24.5 mm, respectively, while *F. solani* was the least susceptible (inhibition zone, 9.4 mm). The antifungal activity of ethyl acetate extract against *R. stolonifer* was significantly ($P \leq 0.05$) higher than that of Blitox which was used as a positive control. Similarly, the bacteria *R. solanacearum* used in this study showed moderate susceptibility to EtOAc extract with inhibition zones of 16.2 mm.

4.3.2 Activity of isolated compounds

Fouteen (14) pure compounds isolated from *Warburgia ugandensis* (Kioy *et al.,* 1990a) tested for antifungal and antibacterial activities and the result are shown in Table 5 and 6 . Ten (10) of the isolated compounds showed activity against one or more of the tested microorganisms while four compounds (9-deoxymuzigadial [**13**], 7α-acetoxyugandensolide [**15**], 3β-acetoxycinnamolide [**18**] and drimenin [**20**]) were not. Polygodial has been previously reported to exhibit antimicrobial activity (Taniguchi *et al.,* 1978; 1988). In the present work, the compound inhibited the growth of *F. solani* (MIC = 12.5 µg/ml) while with both *Alternaria spp* and *R. solanacearum* a MIC = 25 µg/ml was afforded. Mukaadial was another isolate with promising antimicrobial results.

Table 4: Antimicrobial activity of crude extracts against sweetpotato pathogens

	*Zone of growth inhibition in mm (5 mm diameter)				
	Extracts			Standard drugs	
Microorganism	EtOAc	n-hexane	Methanol	Blitox	Streptocycline
Fungi					
Alternaria spp.	17.1	14.1	5.4	22.1	ND
A. niger	21.1	11.5	3.1	28.0	ND
F. oxysporum	13.4	8.1	1.0	16.9	ND
F. solani	9.4	4.0	2.5	25.1	ND
R. stolonifer	24.5	13.7	5.6	18.3	ND
Bacteria					
R. solanacearum	16.2	12.3	5.0	ND	18.8
S. ipomoeae	12.3	10.2	6.2	ND	14.4
Mean	**16.3**	**10.2**	**6.2**	**22.1**	**16.6**

*Values are means of three replicates; ND = Not done.

The compound was observed to inhibit the growth of *A.niger* with MIC=12.5 µg/ml. With this compound, the microorganisms *Alternaria spp, F. solani* and *R. solanacearum* afforded a MIC value of 25 µg/ml. Antimicrobial activity of drimane sesquiterpene dialdehydes has been attributed to α- and -β-unsaturated aldehydes (Taniguchi *et al.,* 1983), it is therefore worth noting that warburganal, which possesses this structural feature appeared to be the most active principle. The compound inhibited the growth of of *A. niger* and *F. solani* with MIC value of 12.5 µg/ml and both *F. oxysporum* and *R. stolonifer* with MIC = 25 µg/ml. Another dialdehyde, ugandensidial was found to be effective against *A. niger, R. stolonifer and S. ipomoeae* with MIC value 25 µg/ml.

Table 5: MIC (µg/ml) of isolates from *W. gandensis* against sweetpotato fungi

	MIC, µg/ml				
Isolates	*Alter spp*	*A. niger*	*F. oxysporum*	*F. solani*	*R. stolonifer*
Polygodial [**7**]	25	50	50	12.5	50
Warburganal [**8**]	50	12.5	25	12.5	25
Mukaadial [**9**]	25	12.5	100	25	50
Ugandensidial [**10**]	50	25	50	100	25
Muzigadial [**11**]	50	50	25	100	50
6α-Hydroxymuzigadial [**12**]	200	>200	100	>200	>200
9-Deoxymuzigadial [**13**]	>200	>200	>200	>200	>200
Ugandensolide [**14**]	50	50	100	200	100
7α-Acetoxyugandensolide [**15**]	>200	>200	>200	>200	>200
Deacetoxyugandensolide [**19**]	50	>200	>200	100	200
Cinnamolide [**17**]	100	100	>200	200	>200
3β-Acetoxycinnamolide [**18**]	>200	>200	>200	>200	>200
Bemadienolide [**19**]	>200	>200	>200	>200	>200
Drimenin [**20**]	>200	>200	>200	>200	>200
Blitox	50	6.25	12.5	6.25	12.5
Streptocycline	ND	ND	ND	ND	ND

ND = Not done

Within the range of activity of drimane sesquiterpenes, it is noted that a structure in which one of the methyls at C-4 shifts to C-3 leaving exocyclic methylene group as in the case of muzigadial does not enhance the antimicrobial activity. Muzigadial was found to inhibited the growth of both *F. oxysporium* and *R. solanocearum* with MIC=25 µg/ml.

Compounds polygodial, warbuganal and mukaadial exhibited comparable antimicrobial activity against one or more of the tested microorganisms (MIC ≤12.5 µg/ml) with reference to Blitox and Sterptocycline which were standard antibiotics. The rest of the active compounds gave MIC values ≤200 µg/ml.

Table 6: MIC (µg/ml) of isolates from *W. gandensis* against sweetpotato bacteria

	MIC, µg/ml	
Isolated compounds	*R. solanacearum*	*S. ipomoeae*
Polygodial [**7**]	25	50
Warburganal [**8**]	50	50
Mukaadial [**9**]	25	50
Ugandensidial [**10**]	100	25
Muzigadial [**11**]	25	50
6α-Hydroxymuzigadial [**12**]	100	>200
9-Deoxymuzigadial [**13**]	>200	>200
Ugandensolide [**14**]	>200	100
7α-Acetoxyugandensolide [**15**]	200	>200
Deacetoxyugandensolide [**16**]	100	200
Cinnamolide [**17**]	>200	>200
3β-Acetoxycinnamolide [**18**]	>200	>200
Bemadienolide [**19**]	>200	100
Drimenin [**20**]	>200	>200
Blitox	ND	ND
Streptocycline	25	12.5

ND = Not done

This study revealed that extracts of *W. ugandensis* have antimicrobial activity against *F. oxysporum, F. solani, Alternaria spp, R. stolonifer, A. niger R. solanacearum* and *S. ipomoeae* which are soil pathogens associated with rotting of sweetpotato and other root crops (Ristaino, 1993). This suggests that the pathogens can be managed using herbal extracts as had also been observed in other studies (Okigbo and Nmeka, 2005). The herbal extracts are more environmentally safe compared to the synthetic antimicrobial drugs currently used (Masuduzzaman *et al.,* 2008; Siva *et al.,* 2008). Extracts from *W. ugandensis* are not only active against fungi and bacteria that cause disease in animals/man (Kubo and Nakanishi, 1979; Mbwambo *et al.,* 2009) but are also active against plant pathogens thus suggesting that the antimicrobial principles in the plant have broad spectrum activity. Most of the compounds isolated from the plant exhibited lower antimicrobial activity compared to the extracts. This observation suggests that the metabolites possibly exert synergistic effects or that there could be other more active minor compounds that were not isolated in this study.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusion

1. Five viruses namely SPFMV, SPCSV, SPMMV, SPCFV and CMV infect sweetpotato in western Kenya. SPFMV is the most widespread followed by SPCSV. In most cases viruses infect sweetpotato in multiples leading to more severe damage. The most common dual viral infection is SPCSV+SPCSV. This is the first report of CMV detection in in sweetpotato Kenya.
2. A rapid and efficient multiplex PCR protocol that simultaneously detects SPFMV and SPCSV was developed. The best amplification was obtained with 2.0 µg of cDNA template 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.
3. Extracts from *W. ugandensis* have the potential for managing sweetpotato fungal and bacterial infections caused by *F. oxysporum, F. solani, Alternaria spp, R. stolonifer, A. niger, R. solanacearum* and *S. ipomoeae*. Ethyl acetate from the the plant was the most active compared to methanol and n-hexane extracts.
4. Phytochemical analysis of *W. ugandensis,* 14 drimane sesquiterpenes which were also subjected to antimicrobial assays. The antimicrobial principles from the were identified to be polygodial [**7**], warbuganal [**8**], mukaadial [**9**], ugandensidial [**10**], muzigadial [**11**], 6α-hydroxymuzigadial [**12**], ugandensolide [**14**], deacetoxyugandensolide [**16**], cinnamolide [**17**] and bemadienolide [**19**].

5.2 Recommendation

1. Due to the high frequency of viruses infection in sweetpotato 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 sweetpotato planting materials get in.
2. Since some of the symptomatic sweetpotato 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 sweetpotato, which often occur in multiples, there is a need to search for antiviral agents which can manage multiple viral infections of the crop.
3. 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 sweetpotato germplasm and cultivars for freedom of virus.
4. Since extracts from *W. ugandensis* have antifungal activity against sweetpotato pathogens, they may provide an alternative way of controlling infections by farmers as it is less expensive and environmentally safe.
5. Polygodial [10], warburganal [11], mukaadial [12], ugandensidial [13], muzigadial [14], ugandensolide [17] and deacetoxyugandensolide [18] exhibited low MIC against comparable to the positive standards in the antimicrobial tests (MIC $\leq$ 50 μg/ml. We recommend them as candidates for further research for safe antimicrobial agents.
6. 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.

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APPENDIX A: A MAP SHOWING THE SAMPLING AREAS IN WESTERN KENYA

APPENDIX B: 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