ANTI-MYCOBACTERIAL EFFICACY OF SELECTED MEDICINAL PLANTS USED BY HERBAL PRACTITIONERS IN KISII TO TREAT TUBERCULOSIS

BY

AMBOGA ENOCK BICHANG'A, B.Ed, (Sci.)
I/56/5002/03

A thesis submitted in partial fulfillment of the requirements for the award of the Degree of Master of Science in the School of Pure and Applied Sciences of Kenyatta University, Nairobi, Kenya

MAY, 2009

DECLARATION

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

Signature ______________ Date 07/05/09

AMBOGA ENOCK BICHANG'A
Department of Plant and Microbial Sciences

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Signature ______________ Date 07/05/2009

Prof. Paul Okemo Owuor
Department of Plant and Microbial Sciences

Signature ______________ Date 07/05/09

Dr. Alex K. Machocho
Department of Chemistry

Signature ______________ Date 07/05/2009

Dr. Paul K. Mbugua
Department of Plant and Microbial Sciences

DEDICATION

This work has been dedicated to my dear Mum Yunuke, for her impeccable sacrifice for me, my loving wife, Mellen for her perseverance and sacrifice, and my lovely kids Kerubo, Matayo and Nyakora for their love and source of encouragement.

ACKNOWLEDGEMENT

I take this opportunity to appreciate the effort of the following people who made it possible to come up with this work.

Firstly, I would like to appreciate Prof. Okemo, Dr. Machocho and Dr. Mbugua who worked as a team to give me the necessary academic assistance. May God give them strength and good health to continue assisting the young aspiring academicians.

My thanks go to Laboratory Technologists Mr. Maina and Mr. Odhiambo of Chemistry Department for their technical support in extraction procedures, Mr. Nzau of Plant and Microbial Sciences Department for his great assistance in bioassays. I am also grateful to Mr. Onyango of ICIPE for assisting me in Freeze-drying of samples, Dr. Githui of KEMRI – Centre for Respiratory Diseases in assisting with relevant literature and technical support. I also acknowledge the two gentlemen who made it possible after a long struggle to have access to run my bioassays, Mr. Edupu and Mr. Njamunge Laboratory Assistants of Nairobi Hospital TB Laboratory, may God reward them abundantly.

My great thanks also go to all the herbalists in Kisii Central District who co-operated in the study, particularly John Mokandu of Eden Clinic, Omweri and Kennedy of East Africa Herbal Clinic for their great support.

However, this thesis could be wanting if I do not acknowledge my family for the moral, social and financial support. Among them my dear wife Mellen, daughter Kerubo, sons; Matayo and Nyakora for their love. Also my parents, brothers and sisters for encouraging me along the difficult academic pathway.

My thanks also go to the entire staff of the Departments of Plants and Microbial Sciences and Chemistry for their support. Specifically, I acknowledge Dr. Machocho for providing all reagents required for extraction process, may he be blessed richly. The Secretaries who typed my work also need a special mention, Mebic Computer Centre – Ogembo, Linet, Nyatichi, Naom and any other who contributed to the typing and editing of my work. I appreciate the role played by colleagues Nyarang'o, Nyanchong'i, Kerongo and Onkeo in reading and correcting errors in my manuscript. I give them hope and courage in this academic struggle.

Above all, I acknowledge my Lord and Saviour who has been my continuous source of strength, and courage and has made me prevail. For with Him I can scale a mountain.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

AIDS	–	Acquired Immune Deficiency Syndrome
ANOVA	–	Analysis of Variance
DOTS	–	Directly Observed Treatments
G.I	–	Growth Index
HCI	–	Hydrochloric acid
HIV	–	Human Immuno-Deficiency Virus
IPAR	–	Institute of Policy Analysis and Research
MBCS	–	Minimum Bactericidal Concentrations
MDR	–	Multi-Drug Resistant
MICS	–	Minimum Inhibitory Concentrations
TB	-	Tuberculosis
URD	–	Universal Resource Data (internet)
USA	–	United States of America
VAR	-	Varieties
WHO	-	World Health Organization

ABSTRACT

Tuberculosis (TB) is a global problem of staggering proportions. It is known to be a poverty related disease affecting millions of people in the world's poorest countries. Tuberculosis is difficult and expensive to control. Due to emerging resistance of the bacterium to available drugs, there is need for renewed strategies on treatment and prevention, hence development of new antimicrobials. The search for effective drugs for *Mycobacterium* is paramount. *Mycobacterium* infections especially those caused by *Mycobacterium fortuitum, M. avium* complex, *M. chelonae* and *M. tuberculosis* have increased in patients with Acquired Immunodeficiency Syndrome (AIDS). Infections caused by these bacteria, especially *Mycobacterium avium* complex are difficult to treat because the bacteria are resistant to multiple drugs. It is however, noted that a large proportion of the Kenyan population is using herbal medicine to combat several ailments including tuberculosis. Unfortunately scientific data to support the efficacy of herbal preparations is insufficient. This study surveyed plants used by Abagusii traditional medical practitioners to treat TB. Six plants from the survey reputed to be widely used in the treatment of TB were selected, botanically identified as *Spermacoce princeae, Warburgia ugandensis, Solanum aculeastrum, Carissa edulis, Synadenium glaucescens* and *Toddalia asiatica* and were tested for anti-mycobacterial activities. The various plant parts (roots, stems, leaves and flowers) were dried in the shade and ground to powder, which was stored in sterilized sample bottles. Extraction in Erlen Meyer flasks was done using cold water, hexane, chloroform and methanol. The extracts were tested against *Mycobacterium tuberculosis* strain provided by the Nairobi Hospital, known to be resistant to rifampicin, streptomycin and isoniazid. The plants found to have active antimycobactrial agents, were assayed further for identification of compounds to be used in treatment of tuberculosis. The Growth Index (G.I) of *Mycobacterium tuberculosis* in the various extracts were determined and used as a measure of efficacy. The plants with active antimycobacterial extracts were screened for phytochemical compounds with medicinal values used for treatment of TB. Extracts of *Synadenium glaucescens, Warburgia ugandensis, Carissa edulis* and *Toddalia asiatica* registered activity at 2 mg/ml. Methanolic extracts had a p value of 0.048 hence more effective in extracting the antimycobacterial compounds from plant materials. *Toddalia asiatica* methanolic crude extract was the most active extract against *Mycobacterium tuberculosis*. Since *Toddalia asiatica* registered impressive activity further research on cytotoxicity and *in vivo* activity should be carried out. It is also important to formulate capsules of tablets from the extracts. This type of identification of secondary metabolites produced by plants with antimycobacterial activities will enhance the search for efficacious anti – tuberculosis agents.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Tuberculosis, commonly known as TB, which was believed would disappear in our lifetime, has staged a frightening comeback. Despite medical progress in fighting tuberculosis, the disease remains a serious health problem, especially in developing countries (Sahims, 2005).

The World Health Organization (WHO) has identified tuberculosis as one of the most important emerging global health threats, with an estimated one-third of the world population infected with the causative agent (WHO, 2004). WHO states that at least 8 million people acquire tuberculosis, while two million deaths as a result of the disease are recorded every year worldwide (WHO, 2003). According to WHO (1999) someone is infected every second with *Mycobacterium tuberculosis* and it is present in about two billion people, although the vast majority do not develop the disease. The WHO has estimated 90 million new cases of tuberculosis in this decade (2000 - 2010) with approximately 30 million deaths worldwide (WHO, 1999).

The disease is back in developed countries which not too long ago believed themselves almost tuberculosis–free (Sahims, 2004). In the United States, the annual incidence of tuberculosis is considerably lower than in developing countries (Chaisson, 2004).

Nonetheless, tuberculosis remains an important problem in the U.S.A and the impact of tuberculosis has worsened in recent years (Frieden, 2004).

At present the tuberculosis problem seems insuperable in some situations and is one of the biggest frustrations of medical science. Across sub-Saharan Africa, rates of TB have more than trebled and this is attributed to AIDS epidemic (WHO, 2003). In the past 10 - 15 years TB case numbers have increased by 300 – 400% in high prevalence countries of Africa like Nigeria, D.R. Congo, South Africa, Ethiopia, Uganda, Tanzania, Sudan, Botswana and Kenya (WHO, 2004). The highest mortality rate per capita in the African countries is quite alarming with 350 cases per 100,000 populations (WHO, 2004). Estimated TB, incidence and mortality in various regions of the world in 2002 are given in table 1.

Table 1.1: TB Incidence and Mortality Rate in 2002 (WHO, 2004)

	Number of cases (thousands)		Cases per 100 000 population		Deaths from TB	
WHO regions	**All forms (%)**	**Smear - positive**	**All forms**	**Smear - positive**	**Number (thousands)**	**Per 100,00[0] populatio[n]**
Africa	2354 (26)	1000	350	149	556	83
The Americas	370 (4)	165	43	19	53	6
Eastern Mediterranean	622 (7)	279	124	55	143	28
Europe	472 (5)	211	54	24	73	8
South – East Asia	2890 (33)	1294	182	81	625	39
Western Pacific	2090 (24)	939	122	55	373	22
Global	8797 (100)	3887	141	63	1823	29

The survey carried out by the Institute of Policy Analysis and Research (IPAR) indicates that the Kenyan situation is more alarming with more than 50% of the public hospital beds occupied by patients with TB related complications (Obonyo and Owino, 1997). Kenya ranks 12th among the world's 22 countries with a high tuberculosis burden according to the World Health Organization's Global Tuberculosis Control (WHO, 2004). In 2002 Kenya had more than 170,000 TB cases and an incidence rate of 223 new sputum smear positive (SS+) cases per 100,000 people.

Table 1.2: Summary of TB Incidence in Kenya (2002)

Country Population	31,540,420
Global rank out of 22 high-burden TB countries	12
Estimated Number of TB cases in 2002	170,213
Estimated TB incidence (all cases per 100,000 pop)	540
Rate of new sputum smear-positive cases (per 100,000 pop)	223

Source: WHO, 2004

HIV/AIDS pandemic has magnified the problem of TB (WHO, 2004). HIV and TB form a lethal combination, each speeding the other's progress. HIV weakens the immune system increasing chances of TB infection, which accounts for about 13% of AIDs deaths worldwide. In Africa, HIV is the single most important factor determining the increased incidence of TB in the past 10 years (Sahims, 2004).

Drug resistant tuberculosis is increasing in prevalence globally (Chaisson, 2004). Resistance to anti-tuberculosis drugs has been exacerbated by the HIV epidemic. Multi-drug resistant *Mycobacterium tuberculosis* (MDR-TB) has recently been defined as resistance to isoniazid and rifampicin, but currently it includes all other first line drugs (Viskum and Kok –Jensen, 1997). MDR-TB has become a rising problem in the industrialized world in the last five to ten years (Frieden, 2004). Treatment of multi-drug resistant tuberculosis is frequently unsuccessful, even in immuno-competent patients (Chaisson, 2004) resistance has been reported to all the seven used drugs (Isoniazid, rifampicin, ethambutol, streptomycin, para-amino salisylic acid, pyrazinamide and flouroquinolones (Porter and McAdam, 1994).

In Kenya about 400 species of plants have been recorded to be used by traditional herbalists in the treatment of various ailments including TB (Kokwaro, 1976). Inhabitants of rural areas rely on traditional medicine greatly due to economic and cultural factors. The high cost of improved conventional drugs and inaccessibility of western health care facilities implies traditional mode of health care is the only form of health care that is affordable and available to the rural people (Matu and Staden, 2003). On the other hand even when western health facilities are available, traditional medicine is viewed as an efficient and acceptable form of treatment from a cultural perspective and treatment with plant products have also managed incurable diseases (Munguti, 1997). Therefore in most places traditional medicine usually exist side by side with western form of health care (Sindiga, 1994).

In the recent past anti-tuberculosis drugs have been rendered less effective as a result of microbial resistance. Coupled with the resistance are the adverse reactions shown by some drugs in HIV positive patients increasing their mortality rate (WHO, 2004). The search for more effective anti-tuberculosis drugs is therefore paramount. Plants have been used as a source of compounds used in manufacturing drugs (natural blue print for the development of new drugs) or as phytomedicine used directly for the treatment of diseases (Sofowora, 1982). Medicinal plants, which form the backbone of traditional medicine, have in the last few decades been the subject of very intense pharmacological studies as potential sources of new compounds of therapeutic value and as sources of lead compounds in drug development (Matu and Staden, 2003).

It is estimated that today plant materials are present in or have provided the models for over 50% of western drugs (Baker, *et al*, 1995). The use of plant-derived medicines has proved effective in the treatment of many infections and reducing many side effects often associated with synthetic anti-microbials. Herbal plants have been in use for treatment of tuberculosis for many years in different parts of the World (Kokwaro, 1976).

Different communities in Kenya use concoctions from various plants to treat respiratory infections (Ochora, 2001). The Kisii community over the years has used some plants traditionally to treat respiratory infections (Gisesa, 2004). The efficacy of the plants used has not been scientifically determined and documented, and it is this gap this research intends to fill.

1.2 Problem statement

The resurgence of TB in Africa with 300-400% increase rate in high prevalent countries such as Kenya, South Africa and other South and Central African countries is a problem, if not checked that will be a disaster (WHO, 2004). Across sub-Saharan Africa, it is documented that TB has more than trebled (WHO, 2003) in the past 10 years. Drugs used to treat TB have adverse effects, which induce cutaneous reactions. These drugs developed 40 – 60 years ago are now outdated and need to be replaced with new, more effective and less risky drugs. The increase in number of the causative agents of tuberculosis due to immune suppression such as *M. bovis, M. africanum, M. microti* and *M. chelonae* has complicated TB treatment. The organisms are multi-drug resistant and currently used drugs like rifampicin and isoniazid are less effective. This is why there is need to explore herbal sources, for useful antimycobacterial properties and possible identification of compounds, which can be used to develop new TB drug.

1.3 Justification

Tuberculosis has re-emerged as an epidemic possibly because of the existing HIV/AIDS related immuno-suppression. *Mycobacterium tuberculosis,* which has been the primary causative agent, has now other former commensals such as *M. Chelonae* causing tuberculosis. These organisms are resistant to previously useful drugs such as rifampicin, isoniazid, streptomycin and ethambutol. The search for new effective drugs which can treat tuberculosis is therefore very significant. This research was intended to aid in identification of compounds, which might be used to develop new drugs.

1.4 Hypotheses

(i) Plants used to treat bacterial infections are a potential source of natural products with useful anti-mycobacterial agents.

(ii) The anti-mycobacterial agents are stable compounds which can be extracted and identified.

(iii) The compounds remain bioactive even when separated into individual principles.

1.5 Objectives

1.5.1 General Objective

To extract, characterize and identify compounds from plants used for the treatment of respiratory infections and test the extracts against *Mycobacteria.*

1.5.2 Specific Objectives

(i) To identify and collect plants used for treatment of tuberculosis in Kisii Central District.

(ii) To prepare crude extracts and test the extracts against *Mycobacterium tuberculosis.*

(iii) To identify the class of active compounds in the plant extracts.

1.6 Significance

The treatment of tuberculosis currently is a health problem due to re-emerging of multi-drug resistant strains of *Mycobacterium.* The isolation, characterization and identification of the active compounds in herbal plants used in treatment of tuberculosis will be useful in giving direction to finding alternative, effective and less risky drugs against *Mycobacteria.*

CHAPTER TWO

LITERATURE REVIEW

2.1 *Mycobacterium Species*

The genus *Mycobacterium* contains members with thick, complex, lipid-rich, waxy cell walls that are Gram-positive, non-sporing, non-capsulated, non-motile, pleomorphic rods, related to the Actinomyces (Greenwood, *et al,* 2002). They are also acid-fast or resist decolorization by a dilute mineral acid (or alcohol) after staining with hot carbol Fuchsin or other aryl methane dyes (Greenwood, *et al.*, 2002).

Mycobacterium tuberculosis, along with *M. bovis, M. africanum* and *M. microti* all cause the disease known as tuberculosis (TB) and are members of the *Mycobacterium* species complex. *M. bovis* was causing TB in animals (bovines) long before invading humans. After domestication of cattle between 8000 – 4000 B.C., there is archaeological evidence of human infection by *M. bovis* probably through milk consumption (Dormandy, 2004). *M. tuberculosis* is probably a human specialized form of *M. bovis* developed among milk drinking Indo-Europeans who then spread the disease during their migration into Western Europe and Eurasia. By 1000 B.C., *Mycobacterium tuberculosis* and pulmonary TB had spread throughout the known world (Lee, *et al.*, 2004).

Mycobacterium tuberculosis is able to grow on a wide range of enriched culture media but Lowenstein Jensen (LJ) medium is the most widely used in clinical practice (Greenwood *et al.,* 2002). In this medium (LJ), the human bacteria produce visible growth in about 2 weeks forming colonies white in colour with a dry crumb-like appearance. The bacterial cell wall contains complex lipids, amino acids and polysaccharides (Burdon and Williams, 2001). The active ingredients in the plant extracts should target the formation of the wax C in the cell wall (the cord factor), which appears to be one of the significant materials responsible for pathogenecity (Burdon and Williams, 2001).

2.2 Tuberculosis

In the 18th century, tuberculosis was not only common in England. but was one of the leading causes of death in U.S.A (Dubos and Dubos, 1987). In the 1950s introduction of effective chemotherapy made tuberculosis a curable disease and contributed to average declines in TB incidence rates of 6% per year until the 1980s. The decline in TB incidence rates was reversed by HIV/AIDS epidemic after 1980 (Porter and McAdam, 1994). In the early 1990s, sanatoria were built to isolate infectious patients from society and provide supportive care. Homelessness, immigration, intravenous drug use, poverty and poor access to primary health care have also contributed to the resurgence of TB in developing countries (Snider and Roper, 1992).

Tuberculosis is a leading single infectious cause of deaths in the world, killing over one million women every year (Countenary, 1996). The disease accounts for more than 2,700 women deaths each day. Women are more at risk during their productive years than men of similar age because of the hormone and nutritional stress of pregnancy which weakens their immune system making them susceptible to TB infections (Countenary, 1996).

The WHO has estimated that in 2000s there will be approximately 90 million new cases of tuberculosis worldwide, with approximately 30 million deaths (WHO, 1999). In 2002, it is estimated that there were 28 million deaths globally from AIDS and related cases like TB (WHO, 2003). HIV infection has fanned epidemics of TB in some African countries, increasing the risks to the whole population, regardless of sero-status.

There were 8.8 million cases of TB in 2002, of which 3.9 million had sputum smear-positive pulmonary TB (the most infectious type of TB) (WHO, 2004). Between 1995 and 2002 the global incidence rate of TB (per capita) was growing at approximately 1.1% per year, and the number of new cases at 2.4% per year. The incidence of TB in the WHO African region is growing at approximately 4% per year and at 6% per year in Eastern and Southern Africa. This is faster than any continent and considerably faster than the 1.2% per year global increase.

Drugs used to treat TB have been shown to have adverse effects, which induce cutaneous reactions particularly in HIV patients and are less effective. It is not surprising that HIV infected patients have a much higher mortality rate during and after TB treatment compared with HIV-negative patients (WHO, 2004). This has complicated TB treatment and has put an immense burden on its control. Baroness Chalker in 1993 while addressing TB congress in London School of Hygiene said, "Tuberculosis is a world health problem of staggering proportions. Like malaria it has returned to haunt us, and seems to be getting many millions of people in the world's poorest countries also it is difficult and expensive to control" (Porter and McAdam, 1994).

Outbreaks of multi-drug resistant tuberculosis (MDR). have been reported from both industrialized and developing countries in patients with HIV infection (Frieden, 2004). The overall objective of tuberculosis control is to reduce mortality, morbidity and transmission of the disease. Nevertheless, as long as there is a rise in HIV prevalence TB rates will generally rise too.

The Kenyan situation seem to be more alarming with over 50% of the beds in public hospitals occupied by patients with AIDS-related tuberculosis which accounts for three times the number of deaths due to all other diseases combined (Obonyo and Owino, 1997).

2.3 The History of Herbal Medicine used against TB

Human beings have always had to rely on plants for their food, shelter, ornamentals, industrial purposes, fodder and other necessities including their medicines. Traditional medicine, based on plants, originally the only healing system known to humans, has never entirely disappeared and in some places in the world is the system available to people.

Plants have served as the basis of sophisticated traditional medicine for thousands of years in countries such as India and China (Okemo, 1996). These plant-based medicines continue to play an essential role in health care in those countries and many other third world countries (Gisesa, 2004).

It has been estimated by the world health organization (WHO, 1978) that about 80 percent of the inhabitants of the world, especially in the developing countries, rely mainly on traditional medicine and medicinal plants for their primary health care. Plant products also play an important role in the health care systems of the remaining 20 percent of the population who are mainly in the developed countries (Sofowora, 1982).

Several herbal plants have been in use for treatment of TB for many years in different parts of the world (Kokwaro, 1993). The North American Indians for several years used *Ptelea trifoliata* L. (Okemo, 1996). The use of herbal medicines from plants like Iceland moss, Licorice, Elecampane, Lungwort and Echinacea to treat TB is not new (Lewis, 1977; Kuria, 1995).

Previous surveys indicate that concoctions of *Warburgia* species (Nagendran and Ochira, 1994) and *Melia Volkensii* Var. *Keniae* (Ochora, 2001) are used by different communities in Kenya to treat TB and other respiratory diseases.

A survey carried out by Kokwaro (1993) indicates that a number of plants are used by different communities in the treatment of tuberculosis; and such treatments include infusion of the bark of *Aporrhiza paniculata which* is drunk to treat TB by mixing with a concoction of the leaves of *Croton sylvaticus* by the Digo community. The Sukuma and Swahili communities use the roots and leaves of *Datura metel* for the same purpose. Roots of *Harrisonia abyssinica* and *Entada leptostachya* are crushed to make a decoction and used by the Akamba community to treat respiratory infections. Leaves of *Pterolobium stellatum* are boiled and drunk by Meru and Luo communities as a cure of respiratory infections, while the Nyamwezi community uses the roots of *Trichoderma zeylanicum* for treatment of the same disease.

Natural product extracts embracing terrestrial and marine plants, animals and microbial cultures are screened for biological activities in many industrial, governmental and University laboratories all over the world. The list of drugs from higher plants alone includes the widely used agents like digoxin, digitoxin, reserpine, codeine, atropine, hyoscyamine, scopolamine, quinine, quinidine pilocarpine, artemisinin and phytostigmine. Plants are still used in western medicine in the form of potent pharmacologically active extracts as exemplified by the official drug atropine, Ipecac, Opium, Rauwolfia and Digitalis.

Herbal or traditional medicine has greater advantage to the individual patient than conventional medicine (Olawayo, 1990). It is cheap and more readily available, natural and does not have the toxic preservatives, binders and dyes that are part and parcel of conventional medicine. Herbal medicine is also wholesome, in that besides controlling or curing illness it compliments the body's efforts in the generation of carbohydrates, proteins, minerals, vitamins and hormones vitally required in times of sickness to speed up recovery.

Many plants are credited with curative attributes some of which have been found to be useful and authentic while others are of unproven status. There is need to further investigate more plant drugs because only about 20% of the registered medicinal plants have so far been investigated (Nkunya, 1990). Moreover, there are various side effects associated with synthetic drugs and therefore the world is turning back to natural substances (Huang *et al.*, 1992; Oketch-Rahab, 1996). It is becoming very common in many countries to produce unrefined drugs for export.

Pharmaceutical companies are already marketing preparations of tablets and capsules made directly from appropriate plant extracts for treatment of specific diseases in India and China such as the best known pain killers, morphine from opium poppy, cocaine a stimulant from coca leaves, steroids with medicinal values, gossypol from cotton seeds and sterols from Soya beans used as sex hormones.

Insecticides, fungicides (from pyrethrum plant) and industrial raw materials are some of the useful active compounds, which are provided by plants (Oketch-Rahab, 1996). Moreover 40% of all pharmaceuticals presently in use are derived from natural sources (plants, fungi and other micro-organisms) and either used directly as such or with some modifications (Nkunya *et al.*, 1990).

In general medicinal plants are used in two forms; they could be administered as complex mixtures containing abroad range of constituents such as infusions, essential oils, tinctures and extracts, for example, the drug "aloes" obtained from the leaves of several

Aloe species, used as purgative containing a mixture of anthroquinone C-glycosides and resins. Medicinal plants could also be used as pure chemically defined active principle in the plant, exhibits a strong specific activity or has a small therapeutic index requiring accurate and reproducible dosage, for example poppy alkaloids, morphine, codeine and papavarine which are used in pure form (Hamburger, *et al.*, 1991).

There is ample evidence to show that the plant kingdom is a vast storehouse of chemical substances manufactured and used by the plants in their defense mechanism against attacks by bacteria (Kokwaro, 1993). Herbal plants are known to be associated with some basic compounds with medicinal values. These compounds include the oils and fats such as resins, and glycosides like tannins, alkaloids, toxalbumins and anthraquinone cathartics. Alkaloids and sterols which are known to be effective as anti-microbial compounds have been isolated from Rutaceae family and *Prunus africana* respectively (Nkunya, *et al.*, 1990).

About 3000 alkaloids and 3000 glycosides have been isolated from plants and new ones are being reported daily (Miller, 1973). In addition to the amino acids essential for the necessary proteins, over 200 species amino acids are known, similarly over 100 non-essential sugars have been isolated. About 9000 essential oils are known. Also a number of compounds including rubber, special pigments, phenolic compounds, lignins, tannins, fibres, gums, resins, cellulose products and others have been isolated from various plant species.

It is important to continue scientifically evaluating herbal practices and to provide direction for future herbal medicinal applications (Okemo, 1996). The wealth of knowledge accumulated for millennia by traditional medicine has developed into the modern discipline of ethno-pharmacology, the critical study of traditional plant medicines, which has only recently come into its own. The use of plant extracts without any specific evaluation could lead to serious complications. Ineffective drugs could be used as a matter of belief or tradition, under/over doses could be taken of highly toxic drugs consumed within a short time. There is need therefore to evaluate an d establish a scientific protocol for use of traditional medicinal plants through chemical, pharmacological, toxicological and microbial studies (Nkunya, *et al.*, 1990).

2.4 Development of Traditional Medicine

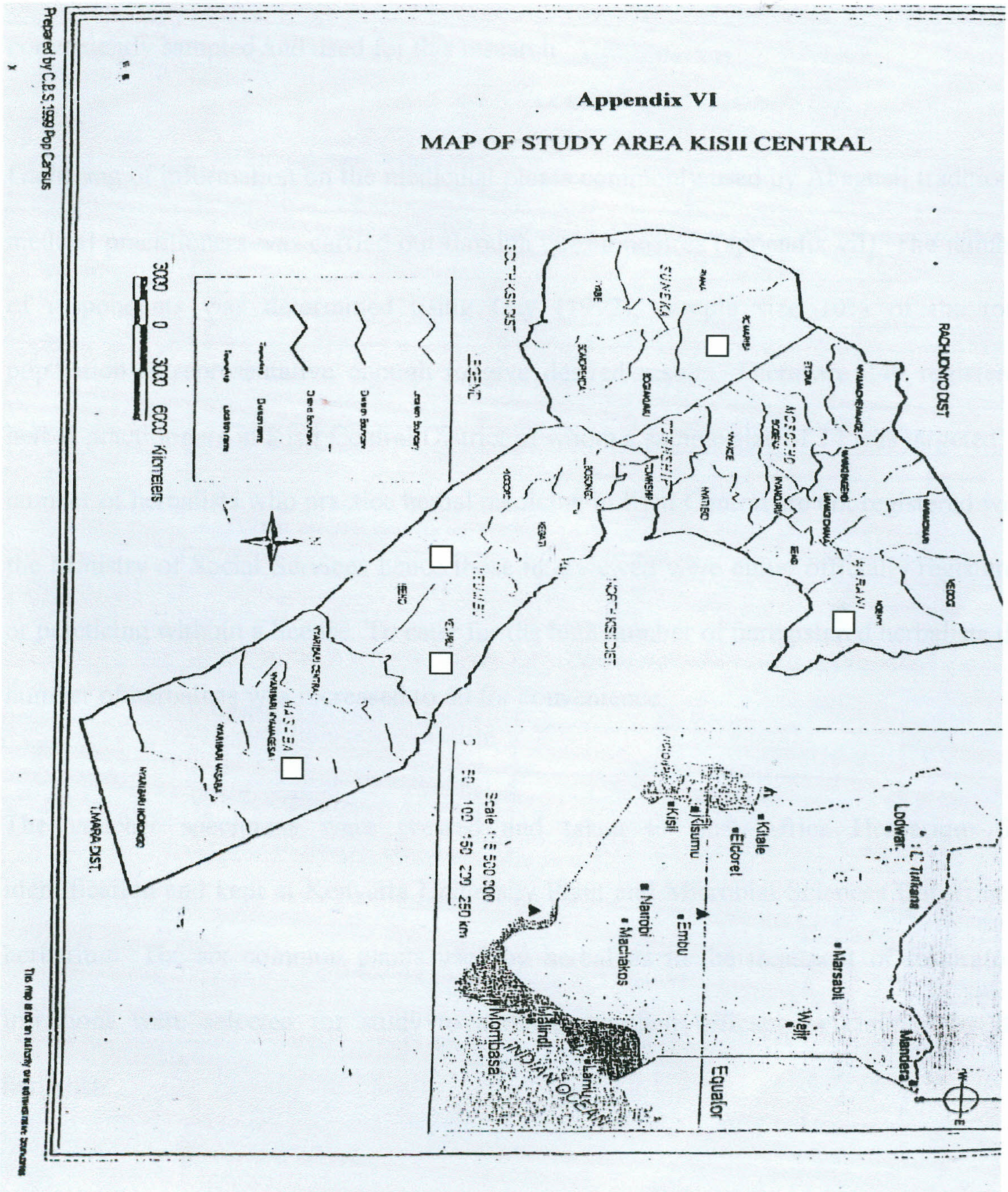
Traditional medical practice is as old as human history and comprises the beliefs and practices that have been in existence before the development of modern scientific medicine (Gisesa, 2004). The knowledge that certain plants possess medicinal value and have been used as a source of drugs by mankind for the treatment of diseases is not new. Historical records show that some plants were used mainly in preparation of crude medicaments that were administered to produce relief from numerous ailments suffered by human beings and their livestock.

Through trial and error man learned how to use nature's healing powers for himself and particularly by observing the behaviour of animals. This inherited, intuitive knowledge of which plant is the right remedy for a given ailments is one of the fascinating aspects of nature. This accumulated knowledge about plants and their medicinal uses was usually passed down from generation to generation verbally. It was customary to pass on this cumulative knowledge to the first son, but occasionally to a trustworthy person (Chhabra *et al.*, 1981).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Site



Kisii Central district is within Nyanza province

☐ Areas sampled for specimen collection at random

3.2 Plant Samples

Medicinal plant species used by traditional medical practitioners to treat TB were collected based on ethno-medical data. The most commonly used plants were conveniently sampled and used for this research.

Gathering of information on the medicinal plants commonly used by Abagusii traditional medical practitioners was carried out through questionnaires (appendix vii). The number of respondents was determined using Gay (1992); sample size 10% of the total population is representative enough to give desired results. There are 340 registered herbal practitioners in Kisii Central District of whom a sample size of 34 was targeted. A number of herbalists who practice herbal medicine in Kisii Central are not registered with the Ministry of Social Services hence those interviewed were either officially registered or practicing without a license. To cater for the high number of unregistered herbalists the number of herbalists was increased to 60 for convenience.

The voucher specimens were pressed and taken to East Africa Herbarium for identification and kept at Kenyatta University Plant and Microbial Sciences Department herbarium. The six common plants used by herbalists in the treatment of respiratory infections were selected for study to authenticate their efficacy as claimed by the herbalists.

3.3 Mycobacterium strains

The strains in this study were obtained from Nairobi Hospital TB laboratory. They were cultured in Broth medium at 37 ^{0}C for 2 – 3 weeks. The medium was prepared according to the Media – manufacturer's instructions.

3.4 Preparation of Plants Specimens

The plant parts collected (depending on the report of herbal medical practitioners to be of medicinal value) were dried in the shade and shredded using laboratory warring blender (for leaves) and electrical mill (for roots and barks). The powder was kept in sterilized sample bottles for extraction.

3.5 Extraction

3.5.1 Cold Water Extraction

About 50 g of the powdered plant material of each plant part collected was placed in 500 ml, Erlenmeyer flasks and soaked in distilled water to a .depth of about 2 cm above the surface. The flasks were plugged with cotton wool to stop contaminants from the environment and shaken in the dark for 24 h on a janke and Kunkel shaker set at 188 strokes per minute. The resulting infusion was filtered using Whatman filter paper No. 1 aided by an aspirator pump. The filtrate was freeze-dried and used for bioassay.

3.5.2 Extraction with Organic Solvents

Three organic solvents were used sequentially in this process in the following order; hexane chloroform and methanol. Each solvent was redistilled twice to obtain the purified form before use. 50 g of each powdered plant material were placed in the sample thimble of the soxhlet apparatus and the solvent added to a level of 2 cm above the powder. Each solvent extraction was subjected to heat and left for 10 h at or until the extract was clear.

Concentration of the extract was done using a rotary evaporator at 50 ^{0}C and transferred to a clean sterile sample bottle.

3.5.3 Bioassay

For each plant extract, doubling dilutions were made in the Broth medium, which was prepared according to the manufacturer's instructions. The medium and extract dilutions put in tubes containing 3.5 ml of the above medium with final concentration of 0.5, 1 and 2 mg / ml were used according to the growth proportion method (Chhabra *et al.,* 1984).

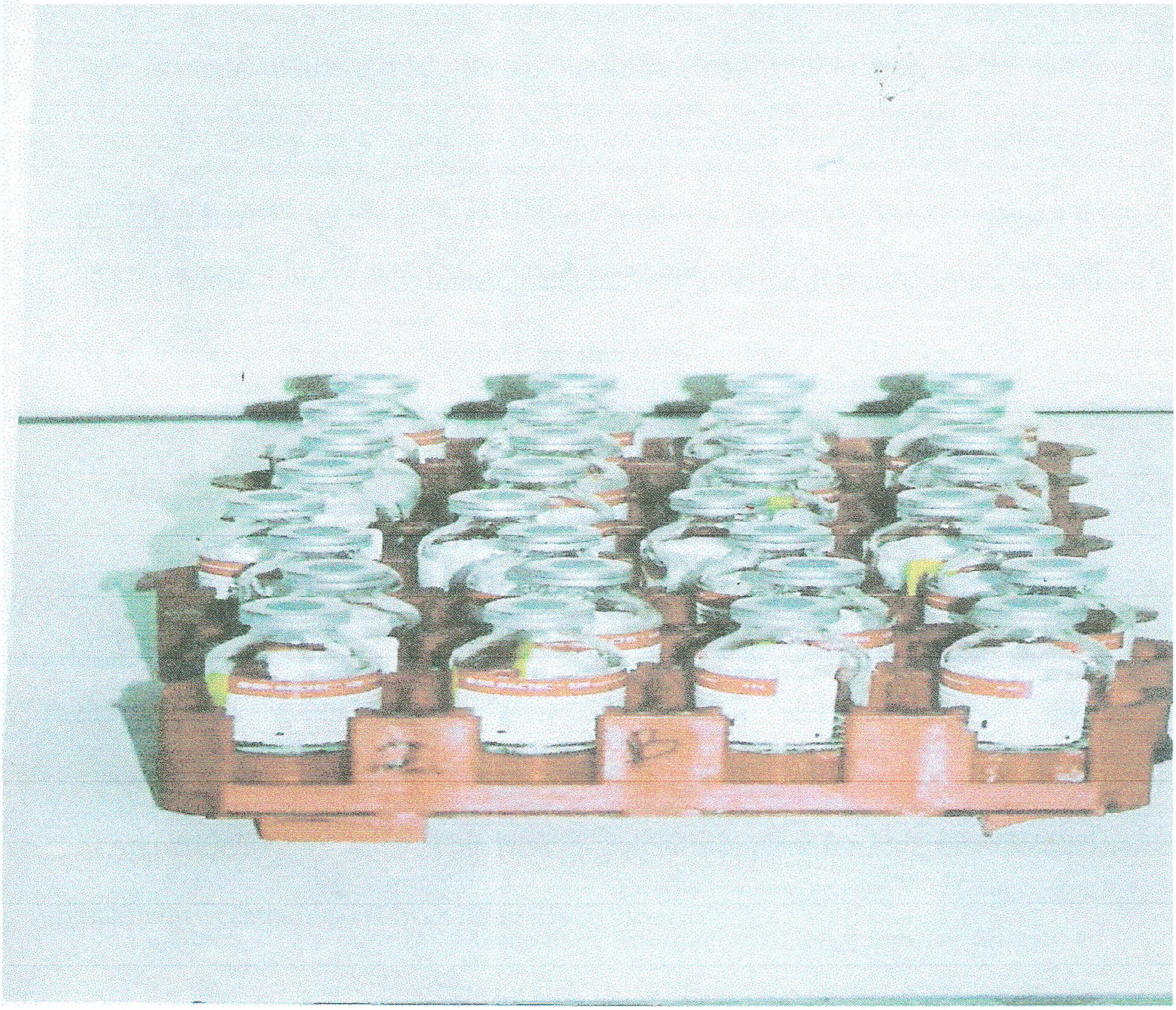
Extracts of the six plants were tested for growth inhibition of selected *Mycobacterium tuberculosis*. Tubes with broth medium without extracts were used for growth control. The Growth Index (G.I) was used to determine the efficacy of the extracts for the inhibition of Mycobacterial growth. The loading of the machine was done as illustrated in the TB BACTEC machine (plates 3.5 and 3.6).

Plate 3.5. Loaded TB Bactec System

Key: Bactec TB System with Vials Loaded into Racks. The vials contain the broth media into which the extracts and isolated *Mycobacterium* strains are introduced.

into which the extracts and isolated *Mycobacterium* strains are introduced.

Plate 3.6 TB BACTEC system with Broth tubes arranged in racks



Key: Vials with broth medium for culturing *Mycobacterium tuberculosis*

3.5.4 Principles of the Procedure

3.5.5 BACTEC TB Medium (12B) – An Enriched Middle Brook 7H9 Broth Base

Mycobacteria utilize a 14C labeled substrate (fatty acid) present in the medium and release $14Co_2$ into the atmosphere above the medium. When the 7H12 medium vials with growth are tested on the BACTEC 460 instrument, the $14Co_2$ is aspirated from the vial and its radioactivity is determined quantitatively in terms of numbers on a scale from 0 – 999. The numbers are designated as the Growth Index (G.I). The G.I numbers are displayed by the BACTEC 460 instruments and are also printed out along with the identifying rack and bottle numbers.

The daily increase in the G.I output is directly proportional to the rate and amount of growth in the medium. If an inhibitory agent is introduced into the medium, inhibition of metabolism is indicated by reduced production of $14Co_2$ when compared to a control having no inhibitory agent. This basic principle is applied for drug susceptibility testing and in differentiating TB from other *Mycobacteria.*

3.6 Phytochemical Screening

A screening procedure described by Chhabra *et al.,* (1984) was used. 15 g of the powdered plant material were repeatedly extracted with ether (3 x 50 ml) at room temperature, with shaking at intervals of 1h. The resulting solution was filtered using Whatman filter paper No. 1. The solvent was distilled off and the residue dissolved in ethanol (30 – 40 ml) and then divided into two portions. A portion of the extract was tested for the presence of alkaloidal bases and volatile oils while the other portion of the extract was saponified with alcoholic potassium hydroxide (10 ml, 0.5 N) by refluxing in a water bath for 1 – 2 h. The alcohol was distilled off and the residue redissolved in hot distilled water (10 – 15 ml). The non-saponifiables were extracted with ethuer (3 x 10 ml) and tested for the presence of carotenoids, and steroids/triterpenoids. The alkaline aqueous solution was acidified with concentrated hydrochloric acid (pH 3 - 4) and extracted with ether (3 x 15 ml). This ethereal solution was tested for the presence of coumarins, emodins, fatty acids and flavonoids.

The plant material, after repeated extraction with ether was repeatedly extracted with hot methanol (3 x 50 ml). The combined extracts was concentrated under reduced pressure to one-third their original volume and divided into two portions. To one portion of the extract chemical reactions were performed the detection of alkaloidal salts, reducing compounds and tannins, while the other portion was hydrolyzed with hydrochloric acid (10 ml, 10%) by refluxing on a water bath for 30 min. The contents were cooled. Distilled water was added (15 ml) and extracted with ether (3 x 10 ml). The ether extract was tested for the presence of anthracenes, coumarins, flavonoids and steroids/triterpenoids while the acidic aqueous solution was tested for the presence of anthocyanins.

The plant material after extraction with ether and methanol was repeatedly extracted with hot water (3 x 50 ml). The combined extracts was concentrated under reduced pressure to one-third their original volume and divided into two portions. To one of the portions chemical reactions were performed for the presence of alkaloidal salts, polyuronoids, reducing /compounds, starch and tannins. The ether portion was hydrolyzed with hydrochloric acid and screened as hydrolyzed methanol extract.

3.7 Phytochemical screening individual performance tests

Tests were performed on each of the classes of compounds present in the extracts used for bioassay. The specific tests used were as those described by Chhabbra *et al.* (1984).

i) Anthocyanins

Methanol and water extracts were tested for anthocyanins. The appearance of a red colour at $_pH$ 3-4 and the change of colour with $_pH$ 8-9 aqueous solution indicated presence of anthocyanins.

ii) Carotenoids

Methanol extract was tested for carotenoids. 10 ml of the methanol extract was evaporated to dryness and a saturated solution of antimony trichloride in chloroform (3-4 drops) added what is termed as Carr price's reaction. On addition of concentrated sulphuric acid, carotenoids pigments first turn blue and later become red.

iii) Anthracene glycosides

The water and methanol extracts were tested for anthracene glycosides. The appearance of a red colour by addition of ammonium hydroxide (25%) to a portion of water and methanol extracts presence of anthracene glycosides.

iv) Flavonoids

The water and methanol extracts were evaporated to dryness and the residue dissolved in methanol (50%, 1-2 ml) by heating, then magnesium metal and concentrated hydrochloric acid (1-2 ml) were added. A red or orange colour indicated the presence of flavonoids.

v) Polyoses

The water extract was tested for polyoses. The appearance of a red colour upon addition of 2-3 drops of concentrated sulphuric acid and thymol alcohol to apportion of evaporated extract indicated the presence of polyoses.

vi) Starch

Addition of lugol iodine to the water extract caused appearance of a blue colour indicating presence of starch.

vii) Steroids/triterpenoids

The water and methyanol extracts were tested for the presence of steroids. The methanol extract and water extracts were tested by Libbermann-Burchard's reaction. 10ml of each extract were evaporated to dryness and the residue dissolved in 0.5ml of acetic anhydride followed by the addition of 0.5ml of chloroform. The solution was transferred to a dry test tube and 1.2ml of concentrated sulphuric acid added from the side of the tube. A brownish-red or violet ring at the contact zone with a green or violet supernatant denoted the presence of steroids/triterpenes.

viii) Coumarins

The water and methaqnol extracts were tested for coumarins. The extracts were evaporated to dryness and dissolved in a small amount of hot distilled water. UV fluorescence at (=254nm) of these aqueous solutions and the increase in intensity after the addition of ammonium hydroxide (10%) indicated the presence of coumarins.

ix) Fatty acids

The ethanol extract was tested for presence of fatty acids. A portion of the ethanol extract was evaporated on a piece of filter paper and the transparency noted indicating oresence of fatty acids.

x) Tannins

The water and methanol extracts were tested for presence of Tannins by the gelatin-salt block test. The extracts were divided to three parts and sodium chloride solution added to each first portion, 1% gelatin solution to each second portion and the gelatin-salt reagent to each third portion.

Precipitation with the gelatin-salt reagents was indicative of the presence of tannins. Precipitation with sodium chloride solution (control) indicated a false-positive test. Positive tests were further confirmed by the action of a few drops of dilute ferric chloride to the test extracts, which gave a blue black/green black coloration.

xi) Reducing compounds

Methanol and water extracts were tested for reducing compounds. To a portion of the extracts a few drops of Fehling's solution were added. The appearance of a red precipiatate on heating the mixture indicated the presence of reducing compounds.

xii) Saponins

The water extract was tested for the presence of saponins. The formation of the honey comb froth in a portion of the water extract which persists after shaking for 10 secs, and a positive Libberman'a Burchard' reaction in the hydrolysed solution indicated the presence of saponins.

xiii) Volatile oils

Volatile oils were tested in the ethanol extract. An aromatic smell in the evaporated ethanol extract indicated the presence of volatile oils. The results were recorded in the table given above in slight aromatic, moderate aromatic and strong aromatic smell.

xiv) Alkaloids

The water, ethanol/alcohol and methanol extracts were used to test the presence of alkaloids in the screened extracts. In the ethanol extract, ethanol was evaporated and the residue macerated with hydrochloric acid (2%, 1.5 ml), filtered, basified with ammonium hydroxide (10%) and extracted with ether. The ether soluble portion was evaporated, dissolved in hydrochloric acid (2%, 1.5 ml), divided into three parts. To one part was added Mayer's reagent and to another Wagner's reagent while the third acted as a blank. A white yellowish white precipitate was formed indicating presence of alkaloids.

In the water extract, aqueous solution was basified with ammonium hydroxide (10%) and extracted with ether (3 x 10 ml). the ether solution was dissolved with hydrochloric acid (10%) and the acidic aqueous solution was divided into three parts and tested as follows; to one of the portion Mayer's reagent and to another Wagner's reagent while the third acted as a blank. A faint turbidity production indicated presence of alkaloids in the extracts.

xv) Emodins (polypeptide)

A portion of the ether solution was .evaporated and the residue dissolved in benzene. If on addition of ammonium hydroxide (25%), a red colour appeared, the presence of emodin was concluded.

3.8 Data analysis

Analysis of the results was done by use of Multi-linear Regression Logistics. A test for statistical significance was set at 1% .and 5% levels of confidence. The classes of compounds in the extracts were tested using colour reaction. Dependent variable [Growth Index] was numerical / independent variables [Concentration of Extracts] were categorical hence multiple logistic regression analysis was used. The χ^2 was used to establish the differences in GI among different concentrations of independent variables. The p value was used to indicate significance, $p < 0.05$ was considered statistically significant.

CHAPTER FOUR

RESULTS

The survey carried out in Kisii Central on the Medical Herbal Plants used by herbalists to treat TB revealed a number of species used (Table 4.1).

Table 4.1. Plant Species used to treat TB in Kisii in order of their frequency as mentioned by herbalists interviewed.

Botanical Name	Family	Vernacular Name	Part used
1. *Spermacoce princeae* *	Rubiaceae	Omotakiebo	Leaves + roots
2. *Synadenium glaucescens* *	Eurphorbiaceae	Egesongoirua	Leaves
3. *Warburgia ugandensis* *	Canellaceae	Esoko	Leaves + bark
4. *Carissa edulis* *	Apocynaceae	Omonyangateti	Leaves
5. *Toddalia asiatica* *	Rutaceae	Ekenagwa ekiegarori	Leaves
6. *Solanum aculeastrum* *	Solanaceae	Entobo entominto	Leaves
7. *Vernonia auriculifera*	Compositae	Omosabakwa	Leaves
8. *Parinari curaterifolia*	Rosaceae	Omoraa	Leaves
9. *Clerodendrum myricoides*	Verbenaceae	Omonyasese	Leaves
10. *Leonotis mollisima*	Labiatae	Risibi	Leaves
11. *Senna didymobotrya*	Caesalpinaceae	Omobeno	Leaves
12. *Vernonia lasiopus*	Compositae	Omosocho	Leaves + bark

(*) Plants chosen for further study because they were the most frequently mentioned by the herbalists interviewed.

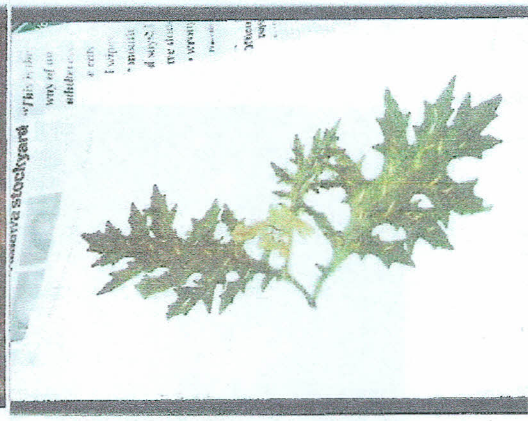
The concoctions were prepared by boiling the different plant parts in a mixture form and then given to patients to drink three times a day. Most herbalists interviewed used *Spermacoce princeae* (75%), *Synadenium glaucescens* (60%), *Warburgia ugandensis* (55%), *Carissa edulis* and *Toddalia asiatica* (both at 54%) to treat Tuberculosis.

Among the common plants which featured greatly were *Carrisa edulis, Spermacoce princeae, Warburgia ugandensis, Solanum aculeatistrum, Toddalia asiatica* and *Synadenium glaucescens* which some of their plates are given below.

Plate 4.1 Carissa edulis in family Apocynaceae

Plate 4.2 Spermacoce princeae in fam
Rubiaceae

Plate 4.3 Warburgia ugandensis in family Canellaceae



4.4 Solanum aculeastrum in family
Solanaceae

Plate 4.5 Toddalia asiatica in family Rutacea leaves with flowers

Plate 4.6 *Toddalia asiatica* in family Rutacea leaves with fruits

Table 4.2. Yields of extracts in mg per 25 Grams of Plant Material

Plant Species	Hexane	DCM	Methanol	Water	Total
1.Spermacoce princeae	2.40	1.18	2.14	2.96	8.68
2. Synadenium glaucescens	1.16	3.40	4.14	4.00	12.70
3. Warburgia ugandensis	2.10	6.21	4.67	6.71	19.69
4. Carissa edulis	1.90	4.15	3.15	5.16	14.36
5. Toddalia asiatica	2.17	3.67	3.67	4.31	13.82
6. Solanum aculeastrum	0.32	1.16	1.16	2.17	4.81

Water yielded the highest crude extract on average suggesting that most plant compounds are very polar. Hexane recorded the lowest yield which indicates that it might be less effective in extracting compounds from plant parts/poor solvent or most compounds are highly polar. *Warburgia ugandensis* gave the highest yields on average suggesting a higher presence of compounds in relation to other plant extracts used. *Solanum acculeastrum* registered the lowest amount of the extracts which indicates little amout of compounds in the extracts.

4.2 DRUG SUSCEPTIBILITY TEST

The laboratory *Mycobacterium tuberculosis* isolate was also tested for primary drugs susceptibility and the results recorded in Table 4.3.

Table 4.3. Drug susceptibility Test Results

Drug	Day / G.I					
	1	2	3	4	Average (G.I)	Results
Control	5	12	21	49	+24	
Streptomycin	41	92	269	567	+267	R
Rifampin	27	71	161	462	+291	R
Ethambutol	74	81	69	71	+4	S
Isoniazid (INH)	41	92	215	581	+266	R

Key : Where R indicates resistance of the strain to the drug and S susceptibility to the drug. The G.I readings for negative vials (susceptibility) generally remain within avalue of 1-9. Any time avail reads G.I 10 or more is considered positive/growth occur (resistance).

The isolate registered a high resistance rate to the common primary drugs used for treatment of tuberculosis. The same laboratory isolate was cultured in the four extracts in which the result indicated susceptibility in a few extracts.

4.2.1 Bioassay

Preliminary screening of the six crude plant extracts was done against a laboratory isolate resistant to rifampin, isoniazid and strepyomycin.

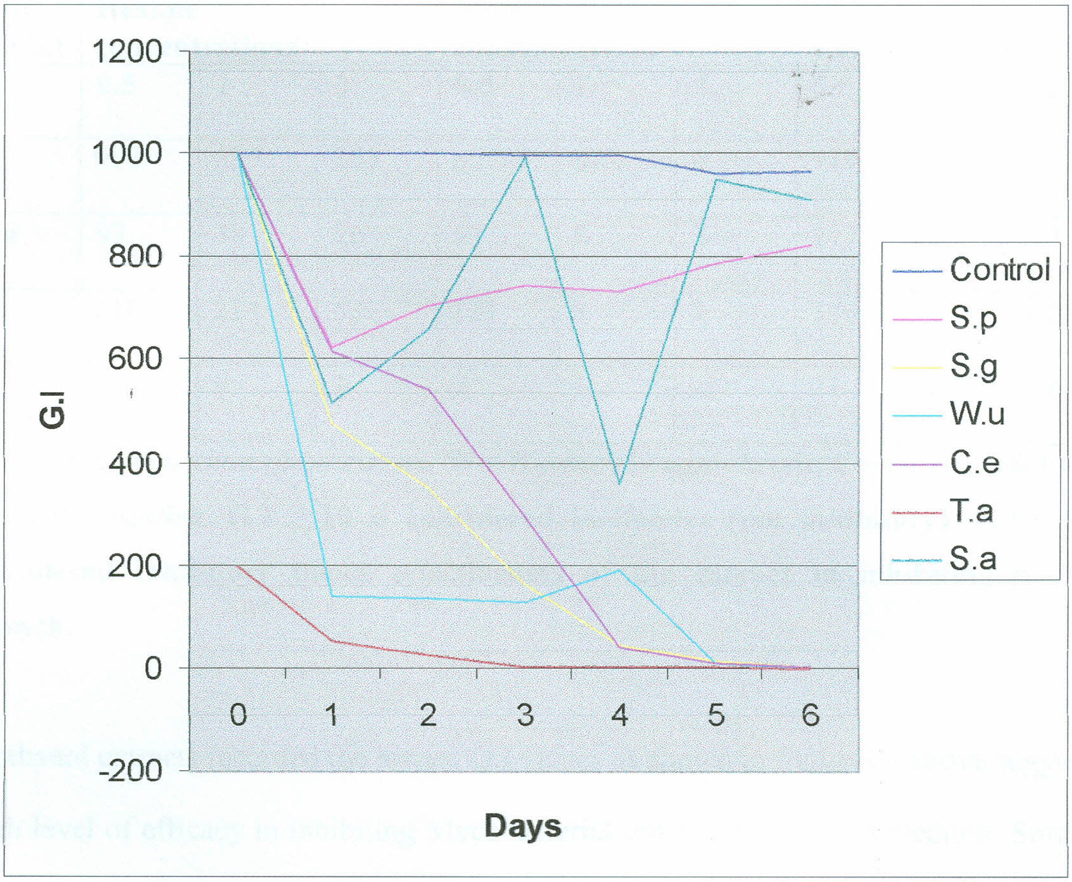
Table 4.4. Methanol extracts of 2 mg/ml Activity on the Laboratory *Mycobacterium* *Isolate*

Day	Control	*S.p*	*S.g*	*W.u*	*C.e*	*T.a*	*S.a*
0	999	999	999	999	999	199	999
1	999	624	478	139	616	56	517
2	999	709	352	137	543	25	659
3	997	745	162	129	297	5	991
4	998	735	45	193	43	2	359
5	961	790	17	13	10	2	949
6	967	825	4	1	2	-1	912

Key : the extracts which showed G.I of less than 10 were assayed further for their activity on the *Mycobacterium* isolates in different concentrations of 0.5, 1and 2mg/ml.

The extracts S.g, W.u, C.e, and T.a from *Synadenium glaucescens, warburguia ugandensis, Garissa edulis,and Toddalia asiatica respectively* were active at 2 mg/ml and effectively inhibited the growth of Mycobacterium tuberculosis. They were therefore selected for further analysis of their activity in various concentrations at 1 and 0.5 mg/ml.

Figure 4.5. Activity of Crude extracts *in vitro* inhibition of *Mycobacterium* strain



The control vials had no crude extract and indicated high G.I while *Toddalia asiatica* extracts registered the least G.I indicating effective inhibitory activity against *Mycobacterium tuberculosis. Spermacoce princea, Synadenium glaucescens, Solanum aculeastrum* extracts recorded higher G.I values hence less effective in inhibiting growth of *Mycobacterium tuberculosis. Warburgia ugandensis* and *Carissa edulis* had activity and effectively inhibited the growth of *Mycobacterium tuberculosis* but after 4-5 days of inoculation.

Table 4:6 G.I in Different Extracts Concentrations

Plant extract	Hexane concentrations			Methanol concentrations			Water concentrations		
	0.5	1	2	0.5	1	2	0.5	1	2
Sg	999	889	253	657	87	6	919	817	615
Wu	92	38	26	53	8	2	217	75	3
Ce	516	214	62	121	9	2	379	221	25
Ta	18	9	1	7	1	-5	21	8	6

Key: Sg-*Synadenium glaucescens*, Wu-*Warburgia ugandensis*, Ce-*Carissa edulis*, Ta-*Toddalia asiatica*. G.I $\geq$ 10 is considered ineffective (not inhibitory), G.I $\leq$ 10 is considered inhibitory hence effectiveness of the extract in inhibiting bacterial growth.

Methanol extracts recorded the lowest G.I values as shown in figure 4.6 above suggesting high level of efficacy in inhibiting Mycobacterial growth in the broth medium. Since G.I was used to determine the efficacy of the extracts it is therefore important to note that methanol effectively removed the anti-mycobacterial agents from the crude extracts used. Hexane and water did not effectively extract anti-mycobacterial agents as they recorded high levels of growth indices (G.I).

4.2.2 Analysis of data using Multi-linear Regression Logistics

Table 4.7. The Growth Index of *Mycobacterium tuberculosis* in various plant extracts

Extract	**Growth index (effectiveness)**	**P value**
Synadenium glaucescens	582 (0^{ns})	0.277
Carissa edulis	172 (0)	0.601
Toddalia asiatica	7.3 (1)	0.049
Warburgia ugandensis	57 (0)	0.602

Key : ns The value of zero indicates no activity while one indicates activity. P < 0.05 was considered to be significant. There was no significant relationship between the growth of *Mycobacterium tuberculosis* with the type of extracts used. Analysis of the effectiveness of the various plant extracts on Mycobactreial growth using Linear Regression Logistics indicates *Toddalia asiatica* extracts with significant performance in inhibiting Mycobactreial growth at 0.05 levels of significance.

Table 4.8. The Growth of *Mycobacterium tuberculosis* in various Solvents

Solvent	**Growth index (effectiveness)**	**P value**
Hexane	260	0.617
Methanol	79	0.048 *
Water	276	0.616

Key : * The value of zero indicates no activity while one indicates activity p 0.05 was considered to be significant there was a significant relationship between the solvents used and growth of mycobacterium. Methanol with $p< 0.048$ was more effective in extracting anti-mycobactrial compound.

There was a significant relationship between the solvents used and growth of mycobacterium. Methanol with $p< 0.048$ was more effective in extracting ant-mycobacterial compounds.

Table 4.9 The growth index of mycobacterium tuberculosis in various plant extract concentrations.

Concentrations (Mg/ml)	**Growth index (effectiveness)**	**P value**
0.5	333	0.022 *
1	198	0.416
2	83	0.415

Key : The value of zero indicates no activity while one indicates activity. $P< 0.05$ was considered to be significant. There was a significant difference in relations to the concentration of the extract used. .Concentrations 1 and 2 had the highest inhibitory activity while .0.5 had the lowest inhibitory activity.

4.3 Summary of Phytochemical Screening Results

The results are summarized in the Table 4.10 below. The screening covered mainly nitrogenous compounds, acetogenins, polyketides, isoprenoids and carbohydrates.

Table 4.10. Phytochemical Screening Results

Extracts	Compounds Screened	Results	Comments
Ethanol extracts	i) Alkaloidal bases ii) Volatile Essential oils	A heavy yellowish white ppt formed aromatic smell	High concentration o alkaloids Moderate concentratio of volatile oils present
Water extracts	i) Carotenoids *ii) Steroids* iii) Alkaloidal salts iv) Reducing compounds v) Starch vi) Tannins vii) Flavonoids	Blue colour *Green precipitation formed* Yellow whitish precipitation Red precipitation is formed Blue colour appears White precipitation Orange colour formed	Slight amount of *carotenoids* Steroids present in lo amount Alkaloids present High amount o reducing compound present Starch present Tannins present Flavonoids present
Methanol	i) Alkaloidal bases ii) Reducing compounds	Yellow white precipitation	Presence of alkaloids Presence of reducin

	iii) Tannins iv) Anthocyanins v) Flavonoids	Red precipitation Heavy black precipitation A red colour A red colour	cpds Tannins present Presence of flavonoids
Ether	i) Coumarins *ii) Emodins* iii) Fatty acids iv) Flavonoids	A strong fluorescence *A red colour appear* Transliscence spot on filter paper A red precipitate appears	Presence of coumarins *Presence of emodins* Fatty acids present Presence of flavonoids

Screening for nitrogenous compounds was mainly concerned with alkaloids whereas that for acetogenins and polyketides covered mainly tannins, flavonoids, coumarins, emodin, anthocyanins, enthracene glycosides and fatty acids. Three plant sample (50%) tested positive for tannins. Five plants (83%) tested positive for flavonoids. Four plants (67%). Gave a positive test for coumarins. Two plants (33%) showed the presence of anthocyanins. Three plants(50%) tested positive for anthracene glycosides, three plants (50%) showed the presence of fatty acids and two plants (33%) tested positive for emodins.

Screening for isoprenoids was confined to steroids/triterpenoids; saponins, volatile oils and carbohydrates included polyuronoids, polyoses, reducing compounds and starch. Five plants (83%) tested positive for steroids/triterpenoids, six plants (100%) showed the presence of saponins, five plants (83%) tested positive for volatile oils while 3 plants (500%) showed the presence of carotenoids. Three plant samples (50%) showed the presence of polyuronoids. Four samples (677%) tested positive for polyoses, six plants (100%) positively tested for reducing compounds while five plant samples (83%) showed a positive reaction for starch.

CHAPTER FIVE

DISCUSSIONS AND CONCLUSIONS

5.1 Mycobacterial activity of Selected Plant Extracts

Out of the 6 plant species studied only 4 showed activity against *Mycobacterium* isolate, at 2 mg/ml (Table 4.5). *Spermacoce princeaei*(omontakiebo) crude extracts which was found to be commonly used by herbalists had high G.I indicating resistance of *Mycobacterium* to this extract. *Solanum aculeastrum* preferred by a number of herbalists to treat respiratory infection showed resistant to *Mycobacterium* in the extracts. Crude extracts for *Synadenium glaucescens* (egesongorua) *Warbugia ugandensis* (Esoka), *Carrisa eduklis* (omonyangateti) and *Toddalia asiatica* (ekenagwa ekiegarori) registered impressive activity at 2 mg/ml. for crude extract to be considered active the growth index of *Mycobacterium* in broth medium should be < 10. Values above 10 are considered inactive (Basco et al., 1995).

5.2 Anti-mycobacterial activity of the Plant Extracts at various concentrations

Out of 36 extracts essayed for Mycobacterial sensitivity, only 13 (36%) were active (G.I<10). Hexane extracts (10) were not active (17%). Water extracts 9 were active while 3 were inactive. Methanol extracts recorded highest activity suggesting that most active compounds are polar hence methanol extracts of *Synadenium glaucescens* were active at 2 mg/ml. *Warbugia ugandensis* extracts were active at 1mg/ml, 2 mg/ml for methanol and 2 mg/ml for water. *Carissa edulis* extracts were active at 1 mg/ml and 2 mg/ml for methanol while *Toddalia asiatica* extracts were active at 1 mg/ml, 2 mg/ml for hexane, 0.5 mg/ml, 2 mg/ml methanol and 1 mg/ml water (Table 4.6). *Toddalia asiatica* extracts activity was high with total inhibition of growth at 2 mg/ml. Most extracts didn't show any activity but lack of any activity in *vitro* not have in *vivo* extrapolations since compounds may act as pro-drugs which must undergo metabolic changes for them to be active. In addition the inactive plants may not have direct effect to the *Mycobacterium* but may deal with tuberculosis symptoms.

Other plant extract showed moderate activity of G.I. less than 50. *Synadenium glaucescens* at 1 mg/ml water, *Warburgia ugandensis* at 1 and 2 mg/ml hexane, *Carissa edulis* at 2 mg/ml water, also *Toddalia asiatica* at 0.5 mg/ml hexane and 0.5 mg/ml water for *Toddalia asiatica. A detailed* study to evaluate its efficiency at lower concentrations is necessary because it seems to be a plant with potential anti-mycobacterium agents.

5.3 Summary on Bioassays

Out of the six plants species used, four (67%) were found to have at least one extract with growth index of less than 10 hence active. Only two plants (33%) were active at 1 mg/ml and 2 mg/ml *Toddalia asiatica* extracts. Methanol extracts were active at all concentrations except at 0.5 mg/ml for *Synadenium glaucescens* and 0.5, 1 mg/ml for *Warburgia ugandensis.* Methanol extracts accounted for 67% of the proportions of the extracts used suggesting that most compounds active against *Mycobacterium* are highly polar and soluble in organic solvents.

For water extracts only three had G.I of less than 10 (25%) at 2 mg/ml *Warburgia ugandensis,* 1 and 2 mg/ml of *Toddalia asiatica* extracts had activity. The rest of the extracts were inactive suggesting that compounds active against *Mycobactetium* in most plants are not soluble in water.

Although many extracts (23) out of the 36 extracts did not show any in *vitro* activity (61%) it should be noted that lack of in *vitro* activities does not always indicate lack of activities. In some instances extracts with no in *vitro* activities have shown good in *vivo* activity in animal models (Gessler *et al.,* 1995). This could be direct low absorption or the parent molecule may need to undergo structural changes during metabolism for it to be active. Herbal preparations may also act by more than one mechanism, such as having an indirect effect on the immune system or other pathways that are not yet clear. It is therefore important to investigate even the extract with G.I more than 10 for in *vivo* activity.

5.4 CONCLUSION

From the results four of the six herbal plants selected showed anti-mycobacterial activity and this supports the reason why they are commonly used by herbalist for treatment of tuberculosis. The other two plants may be playing a role in treatment of clinical manifestations. Methanol extracts were found to be more active than hexane and water extracts which had less activity. This suggests that most active principles are extracted in polar, organic solvents.

With emergence of multi-drug resistance tuberculosis the active compounds can be characterized, isolated and used to standardize a popular crude drug based on traditional use of these plants as anti-mycobacterial agents. Some extracts showed growth index values above 10 this could not be interpreted to mean resistance of *Mycobacteria* in the extracts since in *vivo* experiments can show activity.

a) The following plants collected and identified had antimycobacterial properties:-

- *Synadenium glaucescens*
- *Warburgia ugandensis*
- *Carissa edulis*
- *Toddalia asiatica*

b) *Toddalia asiatica* had the highest antimycobacterial activity

c) Methanol registered the best solvent activity

d) Antibacterial compounds were present in plant extracts

The presence of several phytochemical compounds known to be medicinal importance points out as source for ant-mycobacterial agents to address the problem of MDR-TB.

ater extracts	Methanol extracts
kaloid	Alkaloids
eroids	Reducing compounds
rotenoids	Tannins
ducing compounds	Antocyanins
irch	Flavonoids
nnins	
vonoids	

5.5 Recommendation for Future Work

(i) The extracts that exhibited good in *vivo* anti-mycobacterial activity could be subjected to in *vivo* studies in animal models so as to extrapolate in *vitro*/in *vivo* activities of the extracts.

(ii) The characterized active principles should be isolated and tested individually for anti-mycobacterial activity.

(iii) The cases where only leaves/bark or roots were used, studies should be carried out to study different parts of the same plant and compare their activities.

(iv) It is important also to carry out studies on cytotoxicity of the extracts.

(v) Development of plant extracts as capsules of tablets can be undertake to avail crude drugs that are cheaper and affordable to the local population.

(vi) It could also be interesting to study the activity of the combined drugs such as ethambutol and crude extracts.

REFERENCES

Baker, J., Borris, R.P., Carter, B., Cragg, G.M., Gupta, M.P., Iwu, M., Madullid, D.R. and Tyler, V.E., (1995). Natural products drug discovery and development: New perspective on international collaboration, *Journal of Natural Products,* 58 (9): (1325-1327).

Basco, L.K., Mitaku, S., Skaltsounins, A.L., Ravelomanaintsoa, N., Tillequin, F., Koch, M. and Le Bras J. (1995). In *vitro* activities of Acridone Alkaloids against *Plasmodium falciparum.* Antimicrobial and chemotherapy, 5:1169-1171.

Bloom, B.R. and Murray C.J.K. (1992). Tuberculosis: commentary on are-emergent Killer. *Science,* 257: 1055-1064.

Burdon, K.L. and Williams, R.P. (2001). Microbiology (5th ed.). mackmillan Publishers, New York, pp102-04, 398, 470, 584.

Chaisson, R.e. (2004). *Mycobacterium tuberculosis:* file://A:/virulent factors. htm. pp5.

Chhabra, S.C. Uiso, F.C. and Mshiu, e.N. (1984). Phytochemical screening of Tanzania medicinal Plants, *Journal of ethnopharmacology* 11:157-179.

Countenary, S. (1996). Technical report on Tuberculosis epidemic. WHO, Geneva, pp 67-78.

Dormandy, T. (2004). The white death: *A history of tuberculosis.* (http://www-micr.msb.le.ac.uk).(p1-5).

Dubos, R. and Dubos, J. (1987). The white plague. Rutgers University Press, New Brunswick, NJ, USA, pp 1-5.

Frieden, T., (2004). Toman's Tuberculosis case Detection, Treatment and Monitoring. *WHO technical report,* Geneva pp45-107.

Gay, L., (1992). Educational Research: Competencies for analysis and Application., New York: Macmillan.

Gessler, M.c., Tanner, M.., Chollet, J., Nkunya, M.H.H. and Heinrick, M. (1995). Tanzanian medicinal plants used traditionally for the treatment of malaria: in *vivo* antimalarial and in *vitro* cytotoxic activities. Phytotherapy Research, Nairobi, pp 1-143.

Gisesa, W. (2004). An Ethnopharmacological investigation of plants used by Abagusii traditional medical practitioners. PhD Thesis, Kenyatta University, Nairobi, pp 1-143.

Greenwood, D., slack, C.R. and Petherer, F.J. (2002). Medical Microbiology (16th edn). Churchil Livingstone, Edinburgh, pp 148-9.

Guracha, W.G. (1994). Assessment of knowledge attitude and practice of TB Patients on treatment and control. Project Report submitted to AMREF, pp 4-7.

Hamburger, M., Marston, A. and Hostttman, K. (1991). Advancdes in drug research testa, B.ed, Academic press, London, pp 167-216.

Hocking, G.M. (1969). Plant flavour and aromatic values in medicine and pharmacy, In, current topics in plant Science. J.E. Gunkel, ed. Academic press New York. Pp 273-288.

Huang, P.L., Huang, H.C. and Lee-Huang, S. (1992). Developing drugs from traditional medicinal plants, Chemistry and Industry, 8:290-293.

Kingdom, A.D. (1998). A phytochemical approach to Bio-screening of natural products in: Drug Bioscreening Fundamentals of drug Evaluation Techniques in pharmacology, Graceway publishing company Inc.: 113-131.

Kokwaro, J.O. (1993). Medicinal Plants of East Africa. East Africa Literature Bureau, Nairobi. Pp 42-253.

Kurian, J.C. (1995). Plants that heal. Oriental Watchman Publishing House, Pune, India, pp 2-5.

Lee, B.R., Janice, H. and Tanne, T. (2004). Timebomb: *the global epidemic of multi-Drug Resistant tuberculosis* (http://www-micro.msb.le.ac.uk) pp 4.

Lewis, H.L. (1977). Medical Botany – plants affecting man's health John Willey and Son, Canada, pp 36-9.

Mann, M. (1995). Secondary metabolites. Oxford University Press, London, pp 2.

Matu, E.N. and Staden, J.V. (2003). Antibacterial and anti-inflammatory activity of some plants used for medicinal purposes in Kenya. *J. Ethnopharmacology,* 87:35-41.

Miller of Health, Republic of Kenya (1992). Kenya National plan of Action for Tuberculosis control: pp 16-18.

Ministry of Health, Republic of Kenya (1992). Kenya National plan of Action for Tuberculosis control: 16-18.

Munguti, K. (1997). Indigenous knowledge in the management of malaria and visceral leishmaniasis among Turgen of Kenya. *Indigenous knowledge and development monitor* 5: 10-12.

Nagendran, C.R. and Ochora, J. (1994). Leaf and Nodal Anatomy in two species of *Warbudgia* J. Swamy. *Botanical Journal*, 11:106-07.

Nkunya, M., Weenen, h. and Bray, D. (1990). Proceedings of an International Conference of experts from developing countries on traditional medicinal plants. Arusha, November, 1990. pp 1-19.

Obonyo, B. and Owino, W. (1997). Promoting access to health care through efficiency improvements: priorities and policy options IPAR, Nairobi, pp11-15.

Ochora, J. (2001). Medicinal uses of *Melia volkensii* var. keniae among the Giriama community of Kenya, phD Thesis, Jomo Kenyatta University of Agriculture and Technology, Nairobi.

Okemo, P.O., (1996). Antimicrobial efficacy of selected medicinal plants used by Kenyan herbal doctors. phD thesis, Kenyatta University, Nairobi. Pp 1-192.

Oketch-Rahab, H.A. (1996). Antimalarial and anti-laishmanial activity of medicinal plants. phD Thesis, Royal Danish school of pharmacy Copernhagen, Denmark.

Olawayo, A. (1990). *Proceedings of an international conference of experts from developing countries on traditional medicinal plants,* Arusha, Tanzania.

Porter, J.B.H. and McAdam, K.P.W.J., (1994). Tuberculosis: Back to the future, John Wiley and sons, Chichester. Pp 5-90.

Sahims, C. (2004). The Tuberculosis Threat: http://www.sahim.net/archive/Briefcases/reg/2004/03/reg-review-04-03-31.htm. pp 1.

Sindiga, I. (1994). Indigenous medicinal knowledge of the Masaai, *Indigenous Knowledge and Development monitor* 2:16-18.

Snider, D.E. and Roper W.L. (1992). The new tuberculosis. *New England, Journal of Medicine,* 326: 703 – 705.

Sofowora, A. (1982). Medicinal plants and Traditional medicine in Africa. John Willy and Sons .Limited, Chishester pp 23 -25.

Viskum, K. and KOK-Jensen, A. (1997). Multi-drug resistant tuberculosis in Denmark. 1993 – 1995. *the International Journal of tuberculosis and Lung Disease.* 1:299.

WHO, (1978), WHO report on health, WHO report. Geneva.

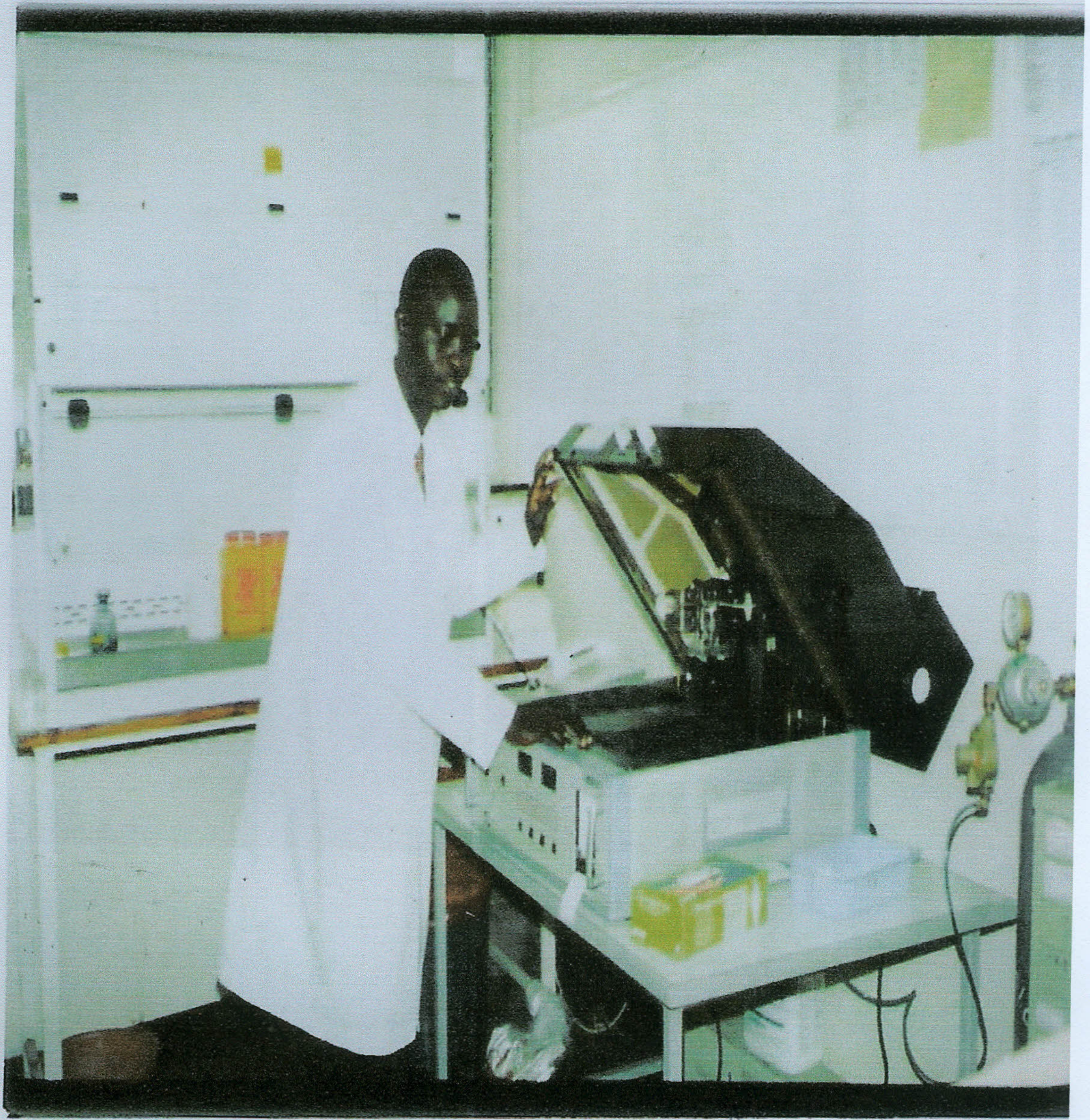
WHO, (1999). Global tuberculosis control report. *WHO Technical Report,* Geneva, pp 1-2.

WHO, (2003). Global tuberculosis control report. *WHO report,* Geneva. Pp 87-96.

WHO, (1004). WHO report on health, *WHO report*. Geneva. Pp 42.

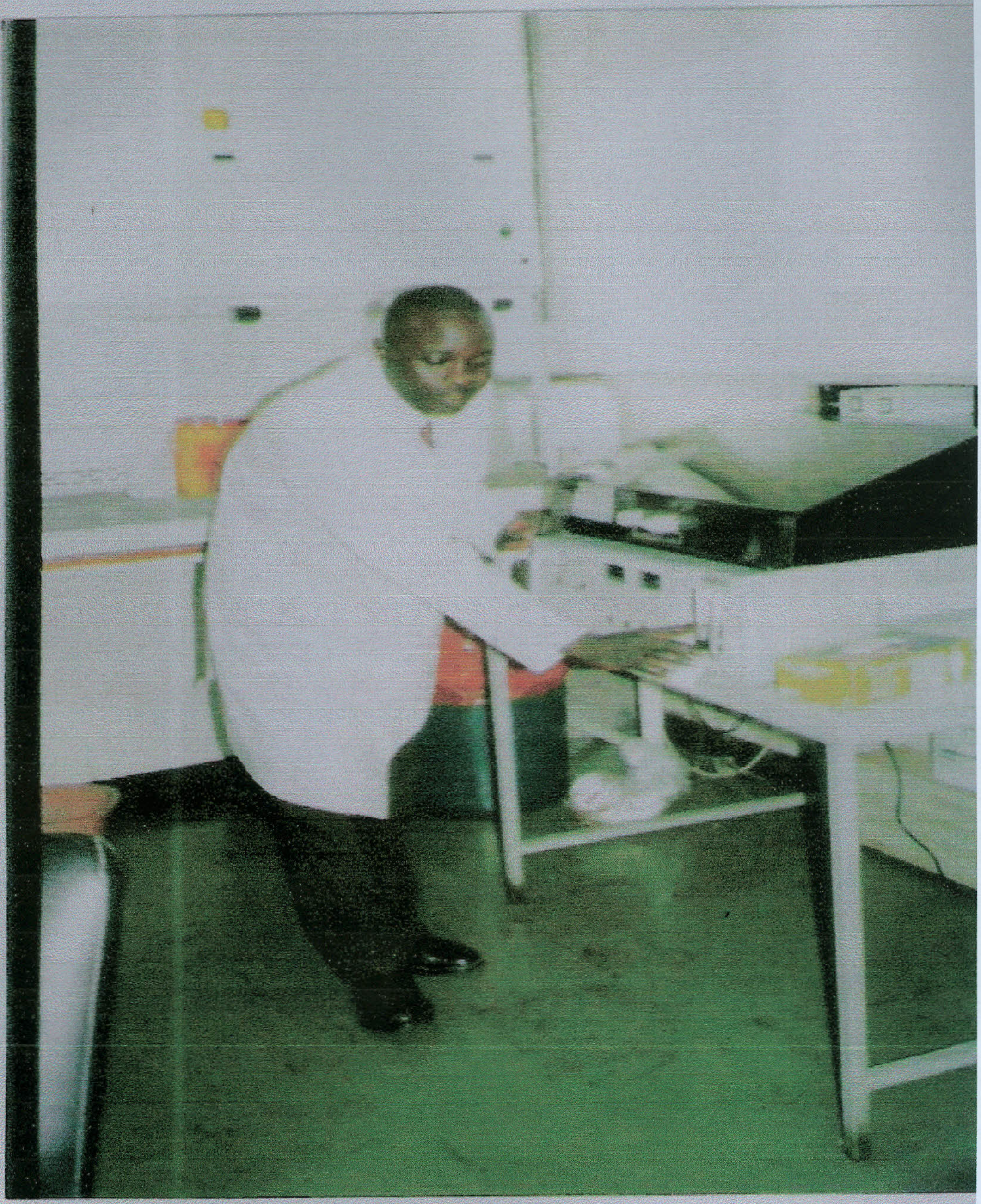
Appendix I

LOADING OF BACTEC TB SYSTEM



Appendix II

READING OF G.I (GROWTH INDEX)

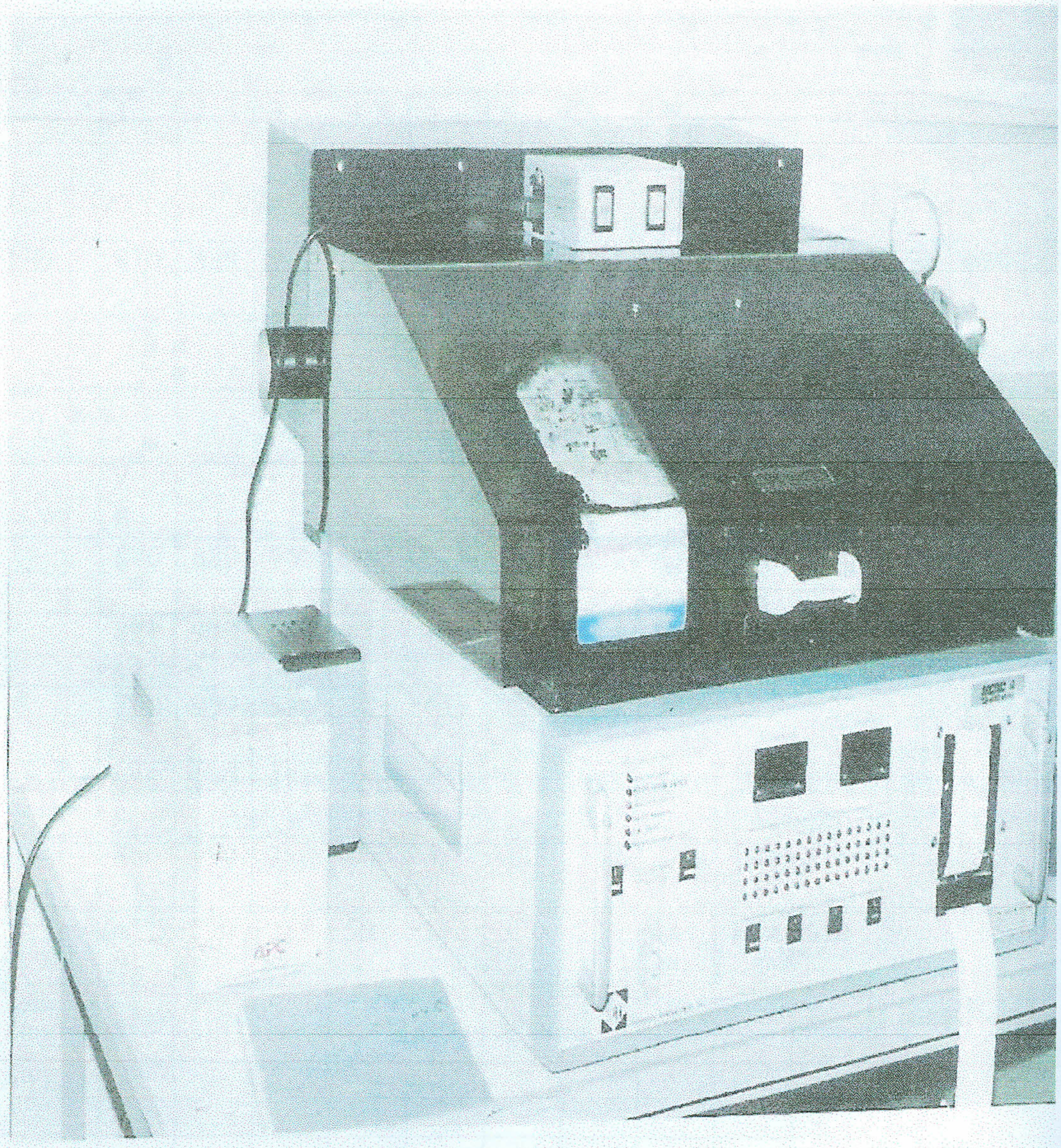


Appendix III

BACTEC TB SYSTEM OPEN SHOWING RACKS WHERE VIALS ARE LOADED

Appendix IV

BACTEC TB SYSTEM RUNNING



Appendix V

TB WORKING SHEET 1

DAY …………………………						DATE ………………………………………							
	1	2	3	4	5	6	7	8	9	10	11	12	13	Dat rele
BACTEC NO.	870	889	999	708	580	778	988	689	617	702	560	820	611	11-
ZN VIAL														
B/A														
48 Hours	870	889	708	580	778	988	689	617	706	560	520	520	611	13-
1 DAY	821	999	620	411	620	666	411	800	458	420	560	800	590	
2 DAYS	700	999	536	385	536	575	385	752	321	320	330	650	459	
3 DAYS	686	999	471	250	471	350	432	555	295	287	190	470	460	
4 DAYS	500	999	350	100	411	452	342	230	199	190	55	320	560	
5 DAYS	451	999	251	50	342	370	23	111	170	121	5	200	550	
ZN/BA DATE														
														Jex

Appendix VII

HERBALIST'S QUESTIONNAIRE

1. Do you treat TB?

Yes [ ] No [ ]

2. Which other respiratory infections do you treat? (*Please tick against what you treat)*

Asthma	
Ordinary cough	
Other	

3. Please list signs by which you recognize TB

4. What are the causes of TB?

5. How did you learn to treat TB? (*Please tick whatever is applicable*)

i) Learnt from parent	
ii) Learnt from relatives other than parents	
iii) Learnt from other healers	
iv) Knowledge came to me in a dream	
v) Other source (s); elaborate	

6. How many people gather plants for you?

7. Are the gatherers the same people in 3 above?

Yes ☐ No ☐

8. What material (s) do you use in treatment? (*Please tick whatever is appropriate*)

Plant parts ☐ Animal parts ☐

Mineral ☐ Other ☐

(Specify) (Specify)

9. Please fill in the table to tell us about your way of treating TB.

Respiratory infection	Plant spp. (Vernacular)	Part used	Time of collection	formula	dose	Route of transmission	Preservation method

10. How do you package herbal medicine? (*Please tick whatever is appropriate*).

Plastic bottle ☐ Plastic paper ☐

Glass bottle ☐ News paper ☐

Other (Specify) ________________

11. How long can you keep your medicinal plants before they loose their healing properties?

Appendix VIII

PAYMENT VOUCHER

NATIONAL MUSEUMS OF KENYA

P.O. Box 40658, Nairobi; Tel: 742131/4; Fax: 742161/4

No. 108340

Date: 06/09/2005

Received from Amburga Bichanga

The sum of Shillings Two thousand one hundred only

being payment of identification of plants & capsule

WITH THANKS

Shs. 2100/=

Cash/Cheque

For: The National Museums of Kenya