IN VITRO ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF THREE KENYAN MEDICINAL PLANTS AND ANALYSIS OF ACTIVE CHEMICAL PRINCIPLES.

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DECLARATION

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

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This thesis has been submittted for examination with our approval as University Supervisors.

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DEDICATION

To my Grandmothers Leonida Odwori and the late Victorina Makale, for you I will undertake all that is desirable.

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ABBREVIATIONS

ACD Labs	Advanced Chemistry Developments Laboratory
AIDS	Acquired Immuno-Deficiency Syndrome
ANOVA	Analysis of Variances
EAH-NMK	East African Herbarium – National Museums of Kenya
HIV	Human Immunodeficiency Virus
ICIPE	International Centre for Insect Physiology and Ecology
JKUAT	Jomo Kenyatta University of Agriculture & Technology
KEMRI	Kenya Medical Research Institute
M.I.C.	Minimum Inhibitory Concentration
NCCLS	National Committee for Clinical Laboratory Standards
PMR	Proton Magnetic Resonance
R_f	Retardation factor
UoNH	University of Nairobi Herbarium
VICRES	Lake Victoria Research Initiative

ABSTRACT

Medicinal plants have been used in centuries as a remedy for human diseases because they contain components of therapeutic value. The development of drug resistance to available antibiotics has also led to investigation of antimicrobial alternatives from medicinal plants. The Acquired Immunodeficiency Syndrome (AIDS) caused by the Human Immunodeficiency Virus (HIV) is an important public health problem in sub – Sahara Africa. Currently the treatment of the disease is mainly directed at secondary microbial infections mainly bacterial and fungal that account for up to 70% of HIV/AIDS defining illness.

From literature search by online sources, databases and search engines, a number of plants used in traditional health systems in Kenya were selected for study, of which basing on ethno botanical application and accessibility to their natural habitats three; *Aspilia mossambicensis* (Oliv.) Wild, *Ocimum gratissimum* (L.) Labiatae. and *Toddalia asiatica* (L.) Lam. (Rutaceae) were investigated with an overall aim of identifying and evaluating the bioactive antimicrobial agents.

Sequential extraction with organic solvents (n-Hexane, ethyl acetate and methanol) realized percentage yields in the range 0.5% (*Ocimum gratissimum* stem bark ethyl acetate extract) to 2.7% (*Toddalia asiatica* root bark methanol extract). Steam distillation enabled extraction of volatile oils with highest yields being 0.45% (*Ocimum gratissimum* leaves).

Antibacterial and antifungal assays were done on the crude extracts and volatile oils derived from these plants by disc diffusion susceptibility testing method. The highest activity was with *Toddalia asiatica* stem bark methanol extract (15mm diameter

zone of inhibition) against Methicillin Resistant *Staphylococcus aureus* for antibacterial assays and the ethyl acetate extract (22mm diameter zone of inhibition) against *Microsporum gypseum* for antifungal assays. *Ocimum gratissimum* leaves' volatile oils elicited activity across all the fungal strains tested against in the range of 12mm to 22mm inhibition zone diameter, this was a confirmation of explosive studies carried out previously hence no basis for further analysis. Statistical data analysis of the bioassays data using the Statistics Package for Social Scientists (SPSS) illustrated significance in the variation of bioactivity; from plant to plant, among different plant parts and for different extracts as expressed by F – statistics.

Bioactivity-guided fractionation of the *Toddalia asiatica* (stem bark methanol extract) yielded Methyl (6-methoxy-2-oxo-*2H*- chromen- 7 –yl) acetate (**F4-C**) **(6)**, which was characterized using Ultra Violet, Infra Red, Nuclear Magnetic Resonance and Mass Spectroscopy and comparison with spectra of authentic samples. By bioautographic selection (**F4-C**) **(6)**, showed antimicrobial activity against Methicillin Resistant *Staphylococcus aureus (10mg/ml)* and *Cryptococcus neoformans (5mg/ml).* **F'9-I'** (structure elucidation to be summed up) elicited antibacterial activity against *Pseudomonas aeruginosa (10mg/ml).*

These results validate the ethno botanical use of *Toddalia asiatica* a Kenyan medicinal plants for conditions that may be of bacterial and fungal etiology.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Microbiology in Health Management

Microbiology which is the study of micro-organisms is a multi-disciplinary subject distributed not only among biologists but also among chemists, biochemists, geneticists, pathologists, immunologists and public health physicians. (Microsoft Encarta Reference Library, 2002). In health management, it addresses diagnosis and treatment as two major components whereby specimen identification by biochemical and molecular / immunological techniques are applied for diagnostic purposes while drug susceptibility aspects being given attention in regard to treatment.

Medicinal plants have been used in centuries as remedy for human disease because they contain components of therapeutic value. Recently, the acceptance of traditional medicine as an alternative form of health care is gaining popularity. Development of drug resistance to the available antibiotics has led to investigation of antimicrobial alternatives from medicinal plants (Bisignano *et al.,* 1996; Lis-Balchin and Deans1996; Maoz and Neeman, 1998). Moreover, the increasing use of plant extracts in the food, cosmetics and pharmaceutical industries suggests that, in order to find bioactive compounds, a systematic study of medicinal plants is very important. Therefore, we

aimed at investigating antibacterial and antifungal activities of extracts from medicinal plant used traditionally in Kenya for infectious diseases.

1.1.1 Microbial Opportunistic Infections

The Acquired Immunodeficiency Syndrome (AIDS) caused by the Human Immunodeficiency Virus (HIV) is the most important public health problem in sub - Sahara Africa. The infection is alarming due to the unique pathogenesis of the virus that decreases the CD4 cells, signaling the emergence of opportunistic infections in the host. Among the various opportunistic infections, bacterial and fungal infections account for up to 70% of HIV/AIDS defining illness (Shailaja *et al.*, 2004).

The opportunistic infections associated with HIV/AIDS include diseases like bacterial pneumonia which is higher in the HIV infected patients than in the general population. Similarly, serious fungal infections in immunocompromized patient are the leading causes of mortality and morbidity especially in HIV/AIDS (Rosen, 1994). Fungal pulmonary infections often precede the appearance of other serious opportunistic infections, but frequently in co-existence with other pathogens (Rosen, 1994). Antibacterial and antifungal agents utilized in the management of opportunistic infections are either bacteriostatic or bactericidal. Therapeutic agents widely used here include the penicillins, glycopeptide antibiotics for example vancomycin, chloroamphenical as antibacterial agents and fluconazole and nystatin for fungi.

Nevertheless the draw back in their application is the emerging resistant strains and associated toxicity (Jabra-Risk, 2006).

1.1.2 Medicinal Plants

Plants have been an integral part of the human society since the start of civilization. Plant biodiversity has always provided a source of new drug candidates for almost all types of disease. Plants with medicinal properties continue to receive attention as scientists survey plants of ethno botanical significance for biological activities ranging from antibiotics to antitumour properties (Bruneton, 1999). The bioactivity-guided fractionation of plant extracts has resulted in the discovery of highly potent and efficacious compounds. The search for antimicrobial agents has taken a definite direction; in developed countries and plants are now providing western medicines with raw materials for drugs used for the treatment of variety of health problems (Bruneton, 1999).

Natural products therefore have been a rich source of biologically active compounds while several plants are documented to be used as antimicrobial agents in folkloric practices (Kokwaro, 1976). Based on their traditional uses and ethno botanical information (Table 1), the plants from the families Rutaceae, Compositae, and Lamiaceae collected from Lake Victoria basin and Central Kenya were studied;

Table 1: Plants selected for the study

Plant (Botanical name)	Family	Region where collected	Name (Community)	Parts used	Ethno botanical Uses
Toddalia asiatica (L.) Lam.	Rutaceae	Lake Victoria basin	Mwikunya (kikuyu), Nyalwet kwach (Luo)	Leaves Fruits Root bark	Cough suppressant
Aspilia mossambicensis (Oliv.) wild	Compositae	Central Kenya	Muti (Kamba), Raywetigo (Luo)	Leaves Root bark	Fever, gonorrhea
Ocimum gratissimum (L) Linn.	Lamiaceae	Central Kenya	Bwari (Luo), Chivumbani (Digo)	Leaves Root bark	Mosquito repellant, Stomach pains

1.1.3 Phytochemical exploration for antimicrobial agents

Major groups of antimicrobial compounds can be isolated from plants due to the fact that plants have an almost limitless ability to synthesize aromatic substances, most of which being phenols or their oxygen-substituted derivatives (Cowan, 1999). In many cases, these substances serve as plants' defence mechanisms against infestation by microorganisms, insects and herbivores. Some of the compounds include; volatile oils which give plants their odours while quinines and tannins are responsible for plant pigment. Many of these compounds are also responsible for the plant flavour (for example the terpenoid capsaicin from chili peppers), and some of the same herbs and species used by humans to season foods yield useful medicinal compounds (Cowan, 1999).

Drug discovery using higher plants can be approached in a number of ways: random selection followed by chemical screening; random selection followed by one or more biological assays; biological activity reports and ethno medical use of plants. The later approach includes plants used in traditional medical systems; herbalism, folklore and shamanism; and the use of databases (Ahmad *et al.*, 2006). The target of which is to isolate new bioactive phytocompounds. When an active extract has been identified, the first task is the identification of the phytocompounds, and this can mean either a full identification of a bioactive phytocompound after purification or partial identification to the level of a family of known compounds. The complex chemical composition of plant

extracts is generally a limiting obstacle to the isolation of antimicrobial compounds. Nevertheless, the use of bioautography agar overlay bioassays allows the detection of active components in a crude plant extract. This method permits the localization of antimicrobial active components that have been separated by Thin Layer Chromatography (Ellof *et al*., 2005).

1.2 Justification of the Study

1.2.1 Problem statement

While the search for antiviral drugs to combat HIV is high on priority, mycobacterium and gram-negative bacterial infections are still among the serious most common pathogenic diseases worldwide. Resistant, mutant bacterial strains have developed as a consequence of over prescription and inappropriate use of antibiotics in the treatment of various diseases. Similarly opportunistic fungal pathogens such as *Candida albicans*, *Candida tropicalis, Cryptococcus neoformans* and dermatophytes have emerged as significant pathogens, the challenge is resistance to the already limited and expensive antifungal agents on the market. Notwithstanding the fact that the cost of drugs is a sizable proportion of the total health expenditure in most developing countries, drug related expenses account for upto 30-50% of the total cost of healthcare (WHO, 2007). The problem is compounded by many people living in rural areas several kilometers from health centres hence not accessible to even primary health care.

Toxicity of these drugs of choice is also an issue yet to be addressed conclusively.

These factors necessitate the search for antimicrobial agents. The plants; *T. asiatica, A. mossambicensis,* and *O. gratissimum* are widely used in management of microbial infections in traditional medicine but there is limited information on the scientific efficacy of these plants against microbial pathogens and especially those of fungal nature. Therefore, there is need to investigate these plants and establish their reputed antimicrobial activity.

1.2.2 Hypothesis

Extracts of *Toddalia asiatica, Aspilia mossambicensis* and *Ocimum gratissimum* possesses identifiable antimicrobial properties.

1.2.3 Objectives

1.2.3.1 General Objective

To establish the in vitro antibacterial and antifungal activity of extracts from three Kenyan medicinal plants used traditionally in management of bacterial and fungal infections and analyze the most active chemical principles.

1.2.3.2 Specific Objectives

- To evaluate antibacterial activity of extracts from the plants.
- To evaluate antifungal activity of extracts from the plants.
- To carry out activity guided fractionation.
- To carry out isolation and characterization of the pure active fractions

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Microbial Opportunistic Infections

Microbial infections are disease-causing microscopic organisms or pathogens that infect human; they include a variety of bacteria, viruses, fungi, protozoan and parasitic worms. In addition, it has been theorized that some proteins called prions may cause infectious diseases (Microsoft Encarta Reference Library, 2002). HIV/AIDS has led to emergency of opportunistic infections mainly mycotic and bacterial in nature which has resulted in significant morbidity and mortality. The primary immune defect upon HIV infection resulting in the decline of CD4 cell counts results in susceptibility to opportunistic pathogens requiring antimicrobial intervention. (Jamjoom *et al.,* 1995). When the HIV infection reduces the number of CD4 cells to below 200 cells per microlitre of blood, the infected individual becomes susceptible to opportunistic fungal and bacterial pathogens characterized by fever, general weight loss and respiratory complications (Shailaja et al., 2004).

2.1.1 Bacterial Infections

2.1.1.1 Bacterial pneumonia

Bacterial pneumonia and less severe respiratory tract infections caused by one or several bacteria such as *Streptococcus pneumoniae, Haemophilus influenza, Escherichia coli,* Salmonella *spp*, Shigella *spp*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* are

the most common pathogens associated with infectious diseases. The role of *Legionella pneumoniae*, *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* is still not clear in HIV/AIDS associated infections (Blasi, 2004).

2.1.1.2 Salmonellosis

This is a diseases caused by Salmonella infection and HIV- positive individual are at risk for salmonellosis with between 20 to 100 times more than HIV – negative people (Sharifi-Mood *et al.*, 2006). Salmonella *spp* can enter the body by eating or drinking contaminated food or water or by contact with infected people or animals.

2.1.1.3 Shigellosis

It is caused by a bacteria called Shigella with species such as; *Shigella sonnei* and *Shigella flexneri*. The infections are more common among HIV – positive people and can lead to either mild or severe cases of shigellosis (Kristjánsson *et al.*, 1994)

2.1.1.4 Pseudomonas aeruginosa

Pseudomonas aeruginosa is a significant opportunistic human pathogen, and the most common gram-negative bacterium associated with nosocomial infections responsible for nosocomially acquired hospital pneumonia, hospital acquired urinary tract infections, surgical wound infections and blood stream infections. Immunocompromized patients such as neutropenic cancer, HIV/AIDS and borne marrow transplant patients are particularly susceptible to such opportunistic pathogens (Cowan, 1999).

2.1.2 Fungal Infections

2.1.2.1 Candidiasis

Candidiasis is a disease caused by a fungus *Candida albicans.* The fungus is a normal flora found on the skin, stomach, colon and rectum, the vagina, and in the mouth and throat (Jawhara and Poulain, 2007). In normal circumstances, *Candida albicans* is harmless and actually helps keep bacteria levels in check. Due to underlying health conditions, sometimes there is an overgrowth of the fungus leading to severe clinical conditions. Both HIV – positive and HIV – negative individual can develop candidiasis (Patel *et al.,* 2006). Candidiasis is a type of mycosis characteristic of pseudo membranes on mucosal surface, aczematoid skin lesions and rarely granulomata of various tissues. Common clinical manifestations are oral thrush, vaginitis, bronchopulmonary disease and meningitis (Rippon, 1988) *Candida tropicalis* is a major cause of septicemia and disseminated candidiasis, especially in patients with lymphoma, leukemia and diabetes. It is the second most frequent encountered medical pathogen next to *C. albicans* and is also found as a normal mucocutaneous flora. Environmental isolation has been made from feces, shrimp and soil (Rippon, 1988)

2.1.2.2 Cryptococcal meningitis

This is a serious infection of the brain and spinal column that occur in people living with HIV. It is caused by a fungus *Cryptococcus neoformans.* The fungus is very common in the environment and can be found in soil and in bird droppings. *Cryptococcus*

neoformans is acquired from air by inhalation to the lungs. The fungus can disseminate to the blood, the spinal column and brain where it can cause severe disease conditions (Eisenman *et al.,* 2007). Cryptococcus is characterized by chronic fetal meningitis (torula meningitis) with other clinical manifestation such as acne-like skin lesions, subcutaneous tumor, pneumonitis (Capoor, *et al.,* 2007)

2.1.2.3 Dermatophytoses

These comprise of dermatological conditions caused by fungi from the genera Microsporum, epidermophyton and Trichophytons. The infection is characterized by *Tinea capitis* (ringworm of the scalp) and *Tinea corporis* (ringworm of the body) which begin as a small papule and spreads peripherally leaving scaly patches of alopecia (baldness). Infected hairs become brittle and break off easily; supportive lesions (kerions) may develop and may be secondary infected with bacteria. The infectious agent can be acquired from contact with infected individual, animals or contaminated soils. (Van Rooij *et al.,* 2006).

2.2 Management of Microbial Infections

Antimicrobial agents used in the treatment of infectious diseases can be classified into two groups; antibiotics, which are natural substances produced by certain groups of microorganisms, and chemotherapeutic agents, which are chemically synthesized (Todar, 2002). The most important property of an antimicrobial agent, is its selective toxicity, that is, it kills bacterial pathogens but has little or no toxic effect on the host.

2.2.1 Treating bacterial Infections

The selection of an antibiotic to treat an infection depends on sensitivity of the causative agent of the infection. (Campo *et al.*, 2007). Drugs used to treat bacterial infections include:

2.2.1.1 Cephalosporins

These destroy the outer wall of the bacteria cell, thereby killing the bacteria but not harming the body cells. Cephalosporins can be administered orally or intravenously. Drugs in the class include Cefaclor, Cefradroxil, Cefazolin (Salzberg and Helmann, 2007)

2.2.1.2 Macrolides

Macrolides alter the chemical activity inside the bacteria, preventing the production of proteins needed for the bacteria to survive. Drugs in the class include azithromycin and erythromycin (Labro and el Benna, 1990).

2.2.1.3 Penicillins

They kill bacteria by destroying the outer wall of the bacteria. Examples include amoxicillin, dicloxacillin, penicillin V (Labro and el Benna 1990).

2.2.1.4 Quinolones

Work by interfering with the enzyme that allows bacteria to reproduce, thereby halting the growth of bacteria and eventually leading to their death. These include Ciproflaxacin, norfloxacin, (Anderson *et al.*, 1998)

2.2.1.5 Sulfonamides and Related Compounds

Bacterial cells produce folic acid, a chemical necessary for their growth. Sulfonamides interfere with the enzymes that help form folic acid, subsequently killing the bacteria. Drugs in the class include sulfadiazine, sulfamethoxazole (Todar, 2002).

2.2.1.6 Tetracyclines

Tetracyclines alter the chemical activity inside the bacteria, preventing production of proteins needed for the bacteria to survive; examples include demeclocycline, doxycline and tetracycline (Rempe *et al.*, 2007).

2.2.2 Treating Fungal Infections

The fungal agents currently available for the treatment of fungal infections are classified by their mode of action. They include:

2.2.2.1 Polyenes

These are antifungal agents (nystatin, amphotericin B) which are fungicidal and have broad spectrum of antifungal activity. The polyenes kill the fungal cell by intercalating into the ergosterol-containing membranes to form channels and destroy the proton gradient causing leakage of cytoplasmic content (Jabra-Rizk, 2006).

2.2.2.2 Azoles

Comprise the second class of antifungal agents and include the imidazoles (clotrimazole, miconazole and ketoconazole) and the triazoles (fluconazole and itraconazole). The azoles inhibit ergosterol biosynthesis through their interaction with the enzyme

lanosterol demethylase, which is responsible for the conversion of lanosterol to ergosterl, leading to the depletion of the erygosterol in the membrane. Fluconazole is used for the treatment of oropharyngeal and vaginal candidiasis, as well as a prophylaxis for fungal infections in neutropenic patients and in HIV – infected individuals (Jabra – Rizk, 2006).

2.2.2.3 Five – flucytosines (5 – FC)

After uptake into the fungal cell, 5-FC ultimately leads to the disruption of DNA and protein synthesis in the fungal cell (Jabra-Rizk, 2006).

2.2.2.4 Echinocandins

Echinocandins inhibits the synthesis of 1,3-β-D-glucan, a fundamental component of the fungal cell wall by inhibition of 1,3-β-D-glucan synthetase, an enzyme complex that forms glucan polymers in the cell wall and is absent in the mammalian cells. The inhibition is effective and specific and a brief exposure leads to cell death (Jabra-Rizk, 2006).

2.3 Antimicrobial Resistance

The ability of a microbial organism to withstand an antibiotic's attack is what is referred to as resistance. When a person takes an antibiotic, the drug kills the defenseless bacteria, leaving behind or "selecting," those that can resist it. These renegade bacteria then multiply, increasing their numbers a millionfold in a day, becoming the

predominant microorganism. The antibiotic does not technically cause the resistance, but allows it to happen by creating a situation where an already existing variant can flourish. (Ricki, 1995)

2.3.1 Antibacterial Resistance

Disease-causing microbes interfere with antibiotics' mechanism of action. For example, penicillin kills bacteria by attaching to their cell walls, then destroying a key part of the wall. The wall falls apart, and the bacterium dies. Resistant microbes, however, either alter their cell walls so penicillin can't bind or produce enzymes that dismantle the antibiotic (Ricki, 1995). In another scenario, erythromycin attacks ribosomes, structures within a cell that enable it to make proteins. Resistant bacteria have slightly altered ribosomes to which the drug cannot bind. The ribosomal route is also how bacteria become resistant to the antibiotics tetracycline, streptomycin and gentamicin (Ricki, 1995).

In this case bacteria acquire genes conferring resistance by either spontaneous DNA mutation, where by bacterial DNA mutate spontaneously for example drug-resistant tuberculosis. Bacterial resistance may also occur by a form of microbial sex called transformation; one bacterium takes up DNA from another bacterium. Penicillin-resistant gonorrhea results from transformation. Most frightening, however, is resistance acquired from a small circle of DNA called a plasmid that can flit from one type of

bacterium to another. A single plasmid then provides a slew of different resistances (Ricki, 1995).

2.3.2 Antifungal Resistance

Antifungal drug resistance is quickly becoming a major problem due to the increasing emergence of resistant strains which arises by varied mechanisms;

The mechanisms of azole resistance for *Candida* is mostly linked to the upregulation of the genes that code for membrane-localized, multi-drug efflux pumps that actively pump the drug from the fungal cells. The over-expression of these proteins results in a multi-drug resistant phenotype (Jabra-Rizk, 2006).

Another major mechanism of drug resistance is the over-expression of the ERG11 gene, which codes for lanosterol demethylase, a key enzyme in the synthesis of ergosterol, which is the main sterol in the fungal cell membrane and the target for fluconazole therapy. The inhibition of ergosterol synthesis by fluconazole compromises the cell membrane integrity, leading to cell growth arrest. In addition, point mutations and the loss of heterozygosity of the ERG11 gene may also contribute to azole resistance (Jabra-Rizk, 2006).

A patient can develop a drug-resistant infection either by contracting a resistant strain to begin with, or by having a resistant microbe emerge in the body once antibiotic treatment begins. Drug-resistant infections increase risk of death, and are often

associated with prolonged hospital stays, and sometimes complications. Irrational use of antibiotics, poor quality generic drugs, sub-optimal dosage are the major sources of resistance currently (Ricki, 1995), a re-address of these can not be over emphasized.

2.4 Medicinal Plants

Plants play an important role in the life of human, as the major source of food, as well as for the maintenance and improvement of health. The World Health Organization (WHO) estimated that about 80% of the world's population still relies on plant-based medicines for their primary health care (WHO, 2002). This is a clear indication of the role of medicinal plants in the maintenance of health and treatment of diseases and as therapeutic alternatives throughout the world (Khalil *et al.*, 2007).

2.4.1 Plants as a Source of antimicrobial Agents

Historically, plants have been a source of novel drug compounds. Plant derived medicines have made a huge contributions to human health and well-being. There are numerous plant derived drugs; the isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha*, and related species. It has been used for many years as an amoebicidal drug and treatment of abscesses due to the spread of *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine, an alkaloid occurring naturally in the bark of *Cinchona* tree. Apart from its continued usefulness in the treatment of malaria, it has been used to relieve nocturnal leg cramps (Iwu *et al.*, 1999).

Plants have an almost limitless ability to synthesize aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Cowan, 1999). Useful antimicrobial plant phytochemicals can be divided into several categories,

2.4.1.1 Phenols and phenolic acids

These are the simplest bioactive phytochemicals consisting of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds which are in the highest oxidation state. The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner *et al.*, 1996), and fungi (Critchfield *et al.*, 1996). Catechol and pyrogallol both are hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two −OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased hydroxylation results in increased toxicity (Cowan, 1999)

The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfuryl groups or through more nonspecific interactions with the proteins (Mason *et al.*, 1987).

2.4.1.2 Quinones.

These are aromatic rings with two ketone substitutions. They are ubiquitous in nature and are highly reactive. The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone-hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems (Cowan, 1999)

In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al.,* 1996), often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism (Cowan, 1999).

2.4.1.3 Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. They are known to be synthesized by plants in response to microbial infection (Dixon *et al.,* 1983). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al.,* 1996). Flavonoid compounds exhibit inhibitory effects against multiple viruses for instance the

effectiveness of flavonoids such as swertifrancheside against HIV (Pengsuparp *et al.*, 1995).

Delineation of the possible mechanism of action of flavones and flavonoids is hampered by conflicting findings. Flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than are those with the −OH groups; this finding supports the idea that their microbial target is the membrane (Chabot *et al.*, 1992). Lipophilic compounds would be more disruptive of this structure. However, other authors have also found the opposite effect; that's the more hydroxylation, the greater the antimicrobial activity (Sato *et al.*, 1996). It is safe to say that there is no clear predictability for the degree of hydroxylation and toxicity to microorganisms.

2.4.1.4 Tannins.

"Tannin" is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000. They are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumor activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996). One of their molecular actions is to complex with proteins through nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Stern *et al.*, 1996).

2.4.1.5 Coumarins

These are phenolic substances made of fused benzene and α-pyrone rings. As a group, coumarins have been found to stimulate macrophages (Casley-Smith, 1997), which could have an indirect negative effect on infections. More specifically, coumarins have been used to prevent recurrences of cold sores caused by Herpes Simplex Virus -1 in humans (Cowan, 1999). Hydroxycinnamic acids which is related to coumarins seem to be inhibitory to gram-positive bacteria (Fernandez *et al.,* 1996). Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity

2.4.1.6 Terpenoids and Essential Oils

The fragrance of plants is carried in essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes, and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}) (Baker and Engel, 1992).

Terpenoids are synthesized from acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol, camphor (monoterpenes) and farnesol, artemisin (sesquiterpenoids). Terpenenes or terpenoids are active against bacteria (Barre *et al.,* 1997), fungi (Ayafor *et al.,* 1994), viruses (Fujioka and Kashiwada, 1994), and protozoa (Ghoshal *et al.,* 1996). The mechanism of action of

terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Mendoza *et al.,* 1997).

2.4.1.7 Alkaloids

Heterocyclic nitrogen compounds, diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family (Atta-ur-Rahman and Choudhary, 1995), are commonly found to have antimicrobial properties (Omulokoli *et al.,* 1997). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Phillipson and O'Neill, 1987). Ethno pharmacology thus deserves attention, so that initial screening of potentially pharmacologically active plants described is made more useful.

2.4.2 Plants studied

In spite of the great advances observed in modern medicine, plants still make an important contribution to health care. This is due in part to the recognition of the value of traditional medical systems and the identification of medicinal plants from indigenous pharmacopoeias, which have significant healing power (Lewis, 2001). Medicinal plants are distributed worldwide, but they are most abundant in tropical countries and a wide range of plants in several families of the plant kingdom have been used for centuries in folk medicine (Calixto, 2000).

2.4.2.1 Toddalia asiatica (L.) Lam. (Rutaceae)

Belongs to the family Rutaceae, commonly it is referred to as the wild orange and is known in vernacular as; *Nyalwet-kwach* (Luo), *Katemwe* (Tugen), *Mkuro / Chikombe za Chui* (Digo), *Mdakakomba* (Swahili), *Mwikunya* (Kikuyu), *Olebarmonyo* (Masai).

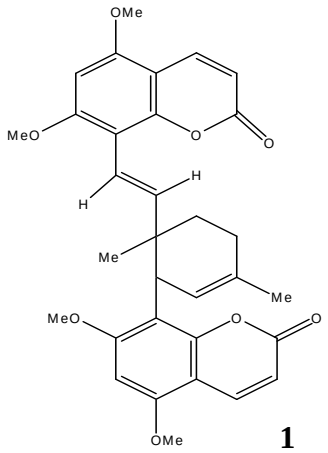
Figure 1: *Toddalia asiatica* (Tsai *et al.*, 1998)

Toddalia asiatica root extracts has been used medicinally as a traditional remedy for cough, treatment of indigestion nasal and bronchial pains (Kokwaro, 1976). In the continuous search for phytochemicals active against pathogenic infections, *T. asiatica* has received considerable attention. For instance 30 compounds classified as coumarins, alkaloids, a benzoquinone and an amine which showed strong anti-platelet aggregation activity *in vitro* have been isolated (Tsai *et al.,* 1998). Some fractions rich in quaternary alkaloids have shown anti-HIV activity (Rashid *et al.,* 1995). *T. asiatica* volatile oil have been shown to have anti-inflammatory activity (Kavimani *et al.,* 1996), extracts isolated from the roots have been shown to posses antiplasmodial activity (Oketch-

Rabah *et al.,* 2000). However no antimicrobial studies have been reported for *T. asiatica.*

Toddalia asiatica has been claimed severally in indigenous medicine to have a range of applications; antipyretic properties, diuretic activity, a cardiotonic stimulant (Sharma *et al.,* 1980); pain reliever and for haemostatics (Ian-Lih *et al.,* 1997) and anti-platelet aggregation activity (Ian-Lih *et al.,* 1998). Basing on these, chemical investigations have been carried out leading to isolation and characterization of a number of compounds. Examples of coumarins isolated include:

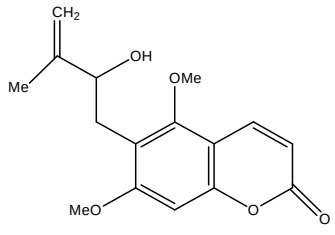
Toddasin (1)



1

(Sharma *et al.,* 1981)

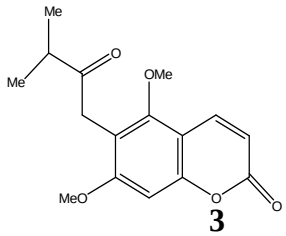
Toddanol (2)



2

(Sharma *et al.*, 1981)

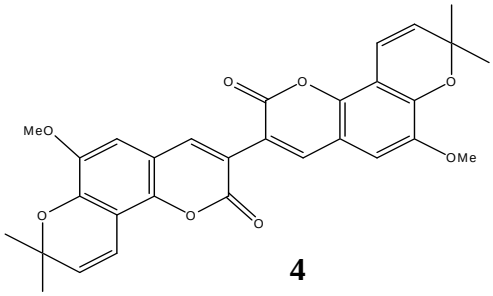
Toddanone (3)



3

(Sharma *et al.*, 1981)

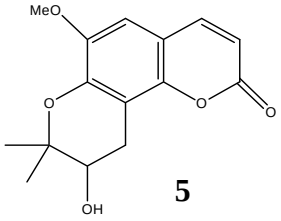
Toddasiatin (4)



4

(Ian-Lih *et al.*, 1997)

Toddanin (5)



5

(Ian-Lih *et al.*, 1998)

2.4.2.2 Aspilia mossambicensis (Oliv.) Wild.

Aspilia mossambicensis is a shrub belonging to the family Compositae. In vernacularly it is refereed to as follows; *Muti* (Kamba), *Raywetigo* (Luo), *Eraji* (Nyankore), *Ihwala*

(Sukuma), *Lilelie* (Lunyore), *Mubiawawala* (Busoga), *Mutanzi / Maruwa / Muhepe* (Digo), *Nyanganyanga* (Shambaa) (Kokwaro, 1976).

Figure 2: *Aspilia mossambicensis* (Ofulla *et al.*, 1995)

Across Africa, the plant is used in herbal medicine for various infections of bacterial origin such as gonorrhea, stomach trouble, cystitis and corneal opacity (Adeniyi and Odufuwora, 2000). Other traditional uses include treatment for lumbago, malaria, tuberculosis, lung and breast tumors (Kokwaro, 1976). Reports suggest that *Aspilia* leaves produce red oil known as thiarubrine-A, which kills viruses, fungi, and parasitic worms (Rodriguez *et al.*, 1985). Total leave extracts of *A. mossambicensis* have been shown to have antiplasmodial activity (Ofulla *et al.*, 1995). However antimicrobial information is limited.

2.4.2.3 Ocimum gratissimum (L) (Labiatae)

Commonly known as the African basil or wild basil, it is refereed to as *Bwari* (Luo), *Chivumbani* (Digo), *Jrumba yaza* (Nyamwezi) (Kokwaro, 1976).

Figure 3: *Ocimum gratissimum* (Wild, 1994)

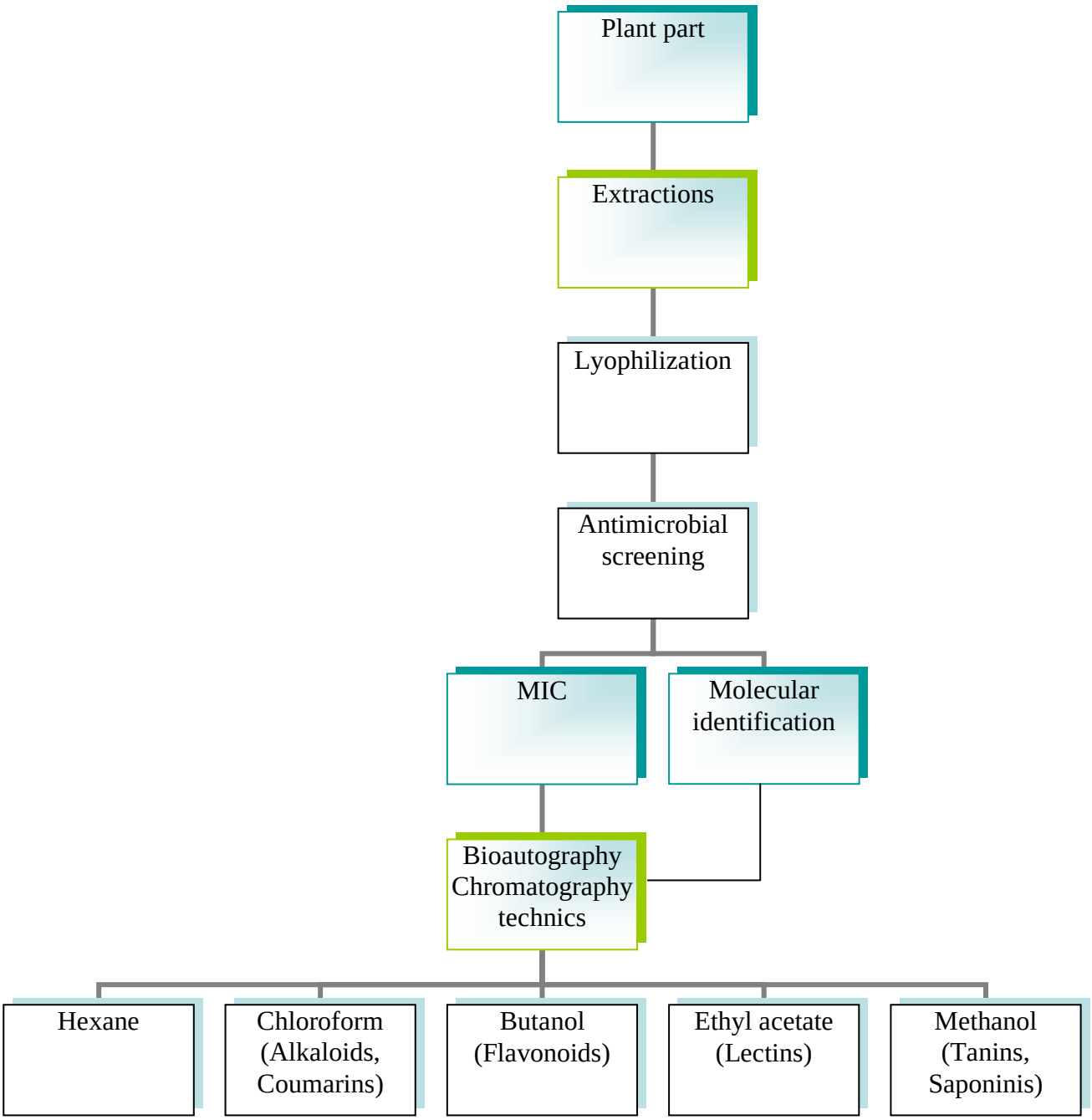
A perennial herb, woody at base, belonging to the family lamiaceae, *O. gratissimum* is used by the Luo community to drive away mosquitoes by laying branches in the house, a decoction of the roots is drunk for stomach pains and vapour from boiling leaves is inhaled for the treatment of nasal and bronchial catarrh (Kokwaro, 1976). Reports on the biologically activity of *O. gratissimum* are numerous; *in vitro* activity of the ethanolic crude extract, ethyl acetate, hexane, and chloroformic fractions, essential oil, and eugenol towards 25 isolates of *Cryptococcus neoformans* (Lemos *et al.,* 2005), the essential oil (EO) of *Ocimum gratissimum* inhibition of *Staphylococcus aureus* at a concentration of 0.75 mg/ml (Nakamura *et al.,* 1999), steam distillation extracts of *O.*

gratissimum leaves showed inhibitory effects on selected bacteria with a minimum inhibitory concentration (MIC) range from 0.1% for *S. aureus* to 0.01% for *E. coli* and *S. typhimurium*, and 0.001% for *S. typhi*. (Adebolu and Oladimeji, 2005). These and several other studies imply more studies should be carried out on the plant also its literature on antimicrobial activity is a good consideration for substantive research to isolate the bioactive compounds.

2.5 Bioactive phytocompound identification

Different approaches to drug discovery using higher plants can be distinguished: random selection followed by chemical screening; random selection followed by one or more biological assays; biological activity reports and ethno medical use of plants (Eloff *et al.,* 2005). When an active extract has been identified, the task to be taken is the identification of the bioactive phytocompounds, and this can mean either a full identification of a bioactive phytocompound after purification or partial identification to the level of a family of known compounds. Scheme 1 below illustrates a plausible extraction – to – identification of bioactive phytocompounds.

Scheme 1: Phytocompound extraction – to – identification



(Mendonça-Filho, 2006)

Plants are chosen randomly or either based on literature, followed by botanical identification and voucher specimen deposition in local herbariums. Collected plant material can be used fresh or dried in whichever case they are ground to optimize solvent contact. Extractions proceed in regard to polarity of solvents from the apolar hexane to polar methanol which is more effective in isolating bioactive compounds (Wild, 1994).

After extraction the volume must be concentrated by lyophilization before screening. The high concentration of the extract guarantees the identification of the bioactivity, if present. Using low concentrations in drop tests may lead to false negative results (Wild, 1994).

The antimicrobial screening by disk diffusion agar assay is an efficient and inexpensive assay to identify antimicrobial activity. The microbes inoculums is expressed as McFarland scale; this permits the antimicrobial activity to be compared within antibiotic controls and between different microorganism groups. (Bajorath, 2002) When antimicrobial activity is detected the minimum inhibitory concentration (MIC) must be determined to continue other antimicrobial assays of interest.

A bio-guided chromatography technique such as bioautography preceded by solvent separation is essential to initiate the bioactive phytocompound identification process; fraction collection with chromatographic assays, preparative TLC are also valid

techniques. In the TLC assays, Rf values can be determined and polarity of chemical groups elucidated. Spectroscopic techniques like NMR, HPLC/MS, and GC/MS are finally used to identify a bioactive phytocompound.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Preliminary experimental design

Experimental design for bioassays involved varying treatments as follows;

- Test group: Consisted of the organisms (selected ATCC strains and clinical isolates) plus the extracts at different concentrations. This determined whether the extracts are effective as antibacterial, antifungal agents.
- Positive control: This consisted of organisms plus known antibiotics (gentamycin and fluconazole). This ensured that the utilized organisms were susceptible to common chemotherapeutics and were not resistant strains.
- Negative controls: Organisms plus the pure solvents, this was necessary to confirm that the solvents used for extraction and dissolution had no inhibitory action on their own.

All determinations involving quantitative data were carried out in triplicate.

3.1.1 Collection of the Plants from the Field

Three Kenyan medicinal plants; *Aspilia mossambicensis, Ocimum gratissimum* and *Toddalia asiatica* were chosen from literature information on their traditional use and considering accessibility to their natural habitat The plant species were collected from their natural habitats; *Aspilia mossambicensis* (1^{O} 06'S 36^{O} 26'E) & *Ocimum gratissimum* (1^{O} 06'S 36^{O} 27'E) in the Mai Mahiu forest while *Toddalia asiatica* (0^{O}

03'S 34^{O} 49'E) Kajulu hills, Kisumu district. Mr. G. M. Mungai from the East African Herbarium Nairobi Kenya confirmed their identities and voucher specimens were deposited in the East African Herbarium – National Museums of Kenya Nairobi (EAH-NMK) and the University of Nairobi Herbarium (UoNH).

Upon successfully authentication by plant taxonomist at the respective herbariums where specimens were deposited. The voucher specimen accession numbers were assigned as shown in table 2;

Table 2: Voucher Specimen Numbers

Plant	Herbarium	Voucher Specimen Number
Toddalia asiatica	UoNH	MWITARI 007
Aspilia mossambicensis	EAH-NMK	CM 145
Ocimum gratissimum	EAH-NMK	CM 144

The samples for extraction were collected in paper bags to the Medicinal Chemistry Laboratory, Centre for Traditional Medicine & Drugs Research – Kenya Medical Research Institute for processing and extraction.

3.1.2 Reagents

Organic solvents; *n*-hexane, dichloromethane, ethyl acetate, chloroform (GPR), methanol, acetone, ammonia were sourced from Kobian, Nairobi, Kenya. chloroform (Analar), methanol (Analar), vanillin, sulphuric acid, potassium hydroxide, anisaldehyde, acetic acid, acetic anhydride, antimony chloride, ninihydrine, bismuth

subnitrite, potassium iodide, sodium nitrate all were obtained from Sigma chemical company, St. Louis, USA. Silica gel (70 – 230 mesh) (Macherey-Nagel GmbH & Co.) for column chromatography and silica gel DGF_{254} (Riedel-de Haen GmbH & Co.) for thin layer chromatography (TLC) and preparative TLC (PTLC) were from the respective companies.

All the GPR grade solvents were distilled to remove possible impurities prior to use. Re-usable glassware (test tubes, conical flasks, measuring cylinders, beakers, volumetric and round bottomed flasks) were soaked in hot water with liquid detergent before washing thoroughly and rinsing with distilled water. They were then dried in an electric oven at $105^{O}C$ for 1 hour and allowed to cool slowly to room temperature before use.

3.1.3 Initial Processing of the Samples

Toddalia asiatica root bark organic solvents extracts were kindly provided by Prof. J. I. Jondiko and T. Nyahanga of Maseno University through the VICRES collaborative project. Briefly the dry root bark powder was sequentially extracted with hexane, ethyl acetate and methanol under room temperature to obtain extracts.

For the other samples upon collection, each of the plant was separated into parts as the root bark, stem bark and leaves. Approximately 1500grams of each part was refrigerated awaiting volatile oils extraction while the rest were dried at room temperature by spreading evenly in the drying rooms and ground into fine powder using a Willy mill. 200g plant powder of each part was extracted sequentially with Hexane then ethyl

acetate and finally methanol to obtain the organic solvents extracts. The extracts were filtered through Whatman No. 1 filter paper and solvent removed in vacuo using a rotary evaporator. The extracts were then stored in sterile air-tight containers at $4^{O}C$ in readiness for biological activity evaluation. (Harborne, 1998; Olila *et al.*, 2001)

Refrigerated plant parts were steam distilled using the clavenger apparatus (Hickman head) to extract volatile oils, each part separately. Volatile oils obtained was dried with Sodium nitrate crystals and stored under refrigeration in air tight containers ready for bioassay (Adebolu and Oladimeji, 2005).

3.2 Biological Activity Evaluation

Following extraction of the plants' parts with organic solvents of differing polarities and volatile oils distillate, susceptibility testing of select pathogenic strains that cause respiratory, gastrointestinal infections and other opportunistic pathogens as cryptococcal meningitis and dermatological conditions was carried out.

3.2.1 Microbial Test Organisms

Selection of microbial test organisms was based on the recommendation of the National committee for clinical laboratory standards (NCCLS, 1992) as the important causative agents for the respective ailments. Standard reference microbial organisms (bacteria and fungi) were obtained from the Centre for Microbiology Research, KEMRI (courtesy of Dr. C. Bii) while clinical isolates were sourced from stocked strains in bacteriology and mycology laboratories at the centre, all preserved at $-20^{O}C$.

3.2.1.1 Bacterial Strains

Bacterial test strains were chosen in terms of their gram-staining properties as gram positive / gram negative and in consideration to the ethno botanical exploitation of the plants as follows;

Gram positive;

- Methicillin Resistant *Staphylococcus aureus* (clinical isolate)

Gram negative;

- *Pseudomonas aeruginosa* (ATCC 27853),
- *Escherichia coli* (ATCC2592)
- *Salmonella typhi* (clinical isolate)

3.2.1.2 Fungal Strains

Similarly the fungal test organisms were chosen as per the following categories:

Yeasts as;

- *Candida albicans (*ATCC 90028*)*
- *Candida tropicalis(*ATCC 750*),*
- *Cryptococcus neoformans(*ATCC 66031*)*

Moulds (dermatophyte)

- *Microsporum gypseum (*Clinical isolate*)*

3.2.2 Preparation of Test Organisms

From stocked isolates, bacterial strains were sub-cultured on Müller Hinton agar No. CM0337 (Oxoid Ltd, Basingstoke, Hampshire, England), and incubated at 37^{o}C for 24 hours to attain freshly growing strains. The fungal; strains were sub-cultured onto Sabouraud Dextrose Agar No. CM 0041 (Oxoid LTD, Basingstoke, Hampshire, England) and incubated for 72 hours at 30^{o}C to obtain freshly growing strains (Rajakaruna *et al.*, 2002).

3.2.3 Antimicrobial Assays

In the assays, negative controls were the solvents used in extraction (n-hexane, ethyl acetate and methanol) and for dissolution (Dimethyl sulfoxide). The standard drugs used were gentamycin (10µg mg disc) for bacteria and fluconazole (25µg mg disc) for fungi all sourced from Sigma-Aldrich Canada Ltd, Ontario Canada.

3.2.3.1 Bioactivity Testing

From a 24hr culture of bacterial strain, 0.5 McFarland turbidity standard (10^{8} colony forming units [CFU]/ml.) were prepared in sterile distilled water and inoculated uniformly onto Mueller Hinton agar. A disc 6mm diameter was impregnated with 10µl of the test extract (100mg/ml) and aseptically placed on the inoculated plates and incubated at 37^{o}C for 24hrs, clear zones of inhibition were measured and compared with that produced by the standard drug (Gentamycin). Antifungal assays were done similarly

except that Sabouraud Dextrose Agar was used and the plates inoculated at 30^{o}C for 72hrs with fluconazole as the standard drug. All tests were performed in triplicate and the antibacterial / antifungal activity was expressed as the mean of inhibition diameters (mm) produced by the plant extracts. This was represented with an antimicrobial index (Rajakaruna *et al.*, 2002) ranging from 0 to 5+.

3.2.3.2 Minimum Inhibitory Concentrations (MIC)

The Minimum Inhibitory Concentrations (MIC) of the bioactive extract was then determined by impregnating paper discs with 10µl of the reconstituted samples at concentrations ranging from 0.0015 to 1mg/ml. The discs were then transferred aseptically into Mueller Hinton Agar and Sabouraud Dextrose agar plates inoculated with the test bacteria/fungi. The MIC was regarded as the lowest concentration that produced a visible zone of inhibition (Sindambiwe *et al.,* 1999).

3.2.4 Bioautographical selection

To confirm the antibacterial, antifungal activity of the column fractions purified, bioautography was carried out.

3.2.4.1 Bioautography of Column fractions

Aliquots (10 µL) of the purified column isolates **F4-C, F12-D, F3-A, F'2-C', F'1-F', F'8-I'** and **F'9-I'** were spotted on silica gel Kieselgel DGF_{254} TLC plates and eluted with Chloroform: Methanol (98:2) with five drops of glacial acetic acid. After evaporation of the organic solvents the TLC plates were placed into sterile Petri dishes (10 x 30 cm) and flooded with 100 ml of Müller Hinton (No. CM0337 OXOID LTD)

(38 g/L) agar seeded in 1% with an aqueous microorganism suspension (10^8 cells/ml) (Wilson, 2000). A TLC plate was prepared and flooded as above for each of the following organisms; Methicillin Resistant *Staphylococcus aureus* (clinical isolate), gram positive bacteria, *Pseudomonas aeruginosa (*ATCC 27853*),* gram negative bacteria and *Cryptococcus neoformans (*ATCC 66031*)* fungi.

The plates were incubated at 30^0C for 24 hrs (bacteria) and 48 hrs (fungi), and then flooded with 50 ml of microbiological agar (10 g/l) containing 0.05% of MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) (M-2128 lot. 40H5021 Sigma). Cell growth inhibition indicating antimicrobial active compounds were detected as yellowish TLC spots on a purple background, allowing the chromatographic retention to be observed. Measurement was done for the spots and R_f values recorded against a solvent front of 13 cm (Vanden and Vlietinck, 1991).

3.2.4.2 MIC of Bioautography Selected Spots

The bioautography selected antibacterial, antifungal pure compounds **F4-C** and **F'9-I'** were subjected to Preparative TLC, localized based on chromatographic R_f, scraped out of the plates and extracted with Dimethyl Sulphoxide (DMSO). Solvents were evaporated by standing the vials for 48 hours. The residues were then suspended (10 mg/ml) in 1000 µl H_2O: DMSO (2: 8) prior to successive dilutions on a 96-well microtiter plate. Finally an aliquot (100 µl) of culture medium (Müeller Hinton, 8 g/l) containing 0.5 McFarland of an aqueous microorganism suspension (Methicillin

Resistant *Staphylococcus aureus* (clinical isolate), (gram positive bacteria), *Pseudomonas aeruginosa* (ATCC 27853), (gram negative bacteria) and *Cryptococcus neoformans* (ATCC 66031) fungi.) was added to a set of wells for each, affording bioactive compound fractions to final concentrations of 10, 5, 2.5, 1.25, 0.625, 0.312 and 0.156 mg/ml. Controls were prepared by substituting test fraction with aqueous-DMSO plus inoculum. The plates were incubated at 30^{o}C, for 24/48 hours. Aliquots (50 µl) of aqueous MMT (0.05%) were added to the wells, and reduction of the tetrazolium salt (yellow) to formazan (purple) by living cells observed within 30 min. MIC was regarded as the lowest well concentration at which reduction of the tetrazolium salt did not occur (Vanden and Vlietinck, 1991).

3.2.5 Statistical data analysis

The results were then subjected to statistical analysis for qualification of the variability. Statistical Package for Social Scientist (SPSS version 12.0) was utilized, which enabled analysis of variance by one way - ANOVA to establish the significance of variability between and within different groups (plant parts, solvents, concentrations, or organisms) with bioactivity as the dependent variable to establish the significance at 0.05 level of confidence.

Frequencies in regard to plant part assayed, organism tested against and case processing summary was also carried out to check on any bias in the assays setting.

3.3 Phytochemical Evaluation

Plants being endowed with vast groups of compounds due to their limitless ability to synthesize, phytochemical screening was thus based on bioactivity guide to isolate and identify the most active chemical principles.

3.3.1 Screening of phytocompounds

From initial bioassays, the plant extracts exhibiting biological activity were screened for groups of chemical constituents. TLC plates were developed with Chloroform: Methanol (98:2) with five drops of glacial acetic acid, before spraying with TLC visualization reagents giving specific reactions (table 3). The tests were carried out according to the methods of Harborne (1998).

Table 3: TLC visualization reagents

Group of Compounds	Visualizing reagent
Organic compounds	Vanillin sulphuric acid
Phenolics	Ethanolic potassium hydroxide
Terpenoids	Anisaldehyde
Anthraquinones	Methanolic potassium hydroxide (Kedde reagent)
Flavonoids	Ammonia fumes
Steroids	Liebermann-Burchard reagent
Cardiac glycosides	Carr-Price reagent
Amines	Ninihydrine
Alkaloids	Dragendorff reagent

3.3.2 Isolation of Active Chemical Principles

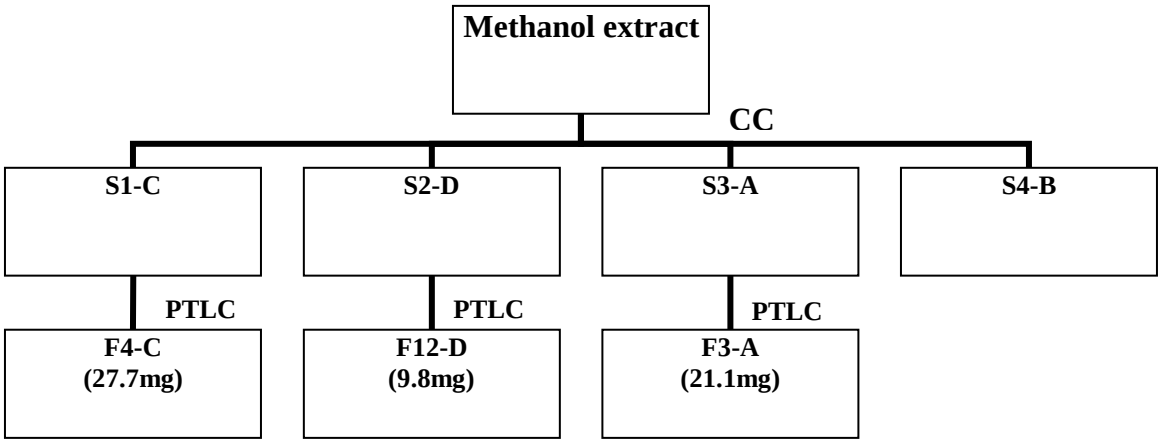
Bioassay guided fractionation was adopted based on Minimum Inhibitory Concentrations (MIC). *Toddalia asiatica* stem bark methanol and ethyl acetate extracts which exhibited the highest activity from preliminary bioassays were selected for isolation and screening of their chemical principles by chromatographic techniques and spectroscopic analysis.

Gradient elution column chromatography (CC) was performed with solvents of increasing polarity. Thin layer chromatography (TLC) and preparative TLC (PTLC) were performed on glass plates coated with silica gel containing fluorescent indicator and eluted with appropriate solvent.

3.3.2.1 Isolation of compounds from *T. asiatica* stem bark methanol extract

5 g of crude methanol extract was loaded onto the packed column and gradient elution then carried out. The eluants were collected manually attaining 41 fractions, and analyzed by TLC before being pooled according to content. Four fractions; **S1-C, S2-D, S3-A** and **S4-B** were in substantial amounts for further purification with PTLC and crystallization to give **F4-C, F12-D** and **F3-A** as pure compounds. These were washed with pure methanol and allowed to re-crystallize. The procedure is summarized in scheme 2.

Scheme 2: Isolation of compounds from the *T. asiatica* stem bark methanol extract



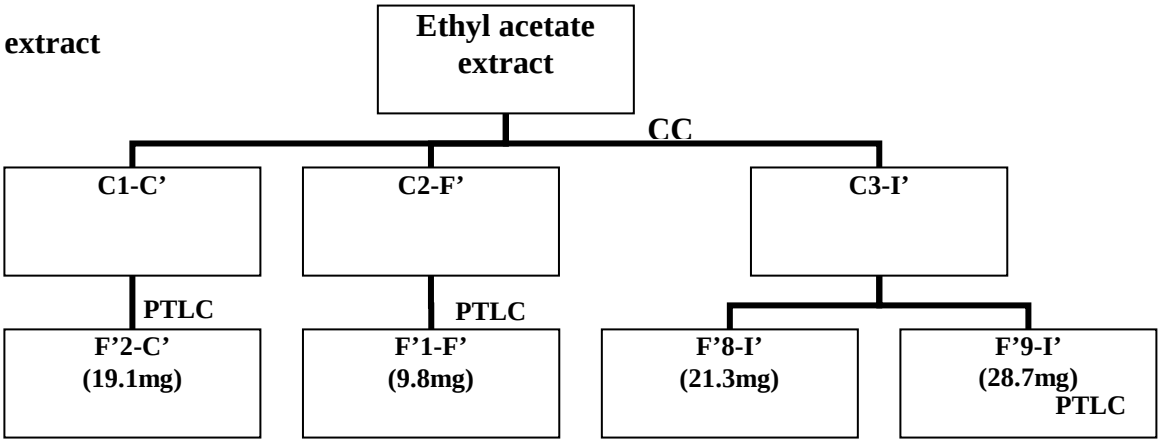
(Kigondu, 2007)

3.3.2.2 Isolation of compounds from *T. asiatica* stem bark ethyl acetate extract

3 g of the crude extract was loaded onto the packed column and gradient elution carried out as described above to afford 35 fractions which were pooled according to content as per TLC analysis. Fractions **C1-C', C2-F'** and **C3-I'** that were in substantial amounts were purified with PTLC and left to crystallize. Four pure compounds; **F'2-C' F'1-F' F'8-I'** and **F'9-I'** were washed with pure methanol and allowed to re-crystallize. The isolation is represented in

scheme 3.

Scheme 3: Isolation of compounds from the *T. asiatica* stem bark ethyl acetate extract



(Kigondu, 2007)

The pure compounds were screened for biological activity to pick out the active ones; **F4-C** from the methanol extract and **F'9-I'** from the ethyl acetate which were taken up for spectroscopic analysis

3.3.3 Spectroscopic Analysis

To ascertain the active chemical principles spectroscopic assays was performed for the two pure compounds exhibiting antibacterial, antifungal activity; **F4–C** and **F'9–I'.** **U**ltraviolet spectra were recorded on a Beckman DU-6 spectrophotometer (USA) at a scan speed of 300 nm/min with a slit of 1 cm path length at the Medicinal Chemistry laboratory– Centre for Traditional Medicine and Drug Research, KEMRI.

Infra-Red spectra were recorded on a Shimadzu Fourier – Transform Infra-Red (FTIR-8400) spectrophotometer using liquid cell at the Chemistry Department Jomo Kenyatta University of Agriculture & Technology.

Nuclear Magnetic Resonance (NMR); ^{1}HNMR ^{13}CNMR and COSY spectra were determined at the University of Nairobi Department of Chemistry using a varian HY 200 NMR spectrometer, Year Hold Helium (OXFORD) 200MHz NMR system in deuterated acetone-d6 and mass spectroscopy at the International Centre for Insect Physiology and Ecology - Research & Development Department laboratory using a VG 12 - 250 Upgrade (VG ANALYTICAL) Quadruple Organic Mass Spectrometer, (Biotech England) as solid probe.

Melting temperature was carried out at the Centre for Traditional Medicine and Drug Research, KEMRI Medicinal Chemistry laboratory by aid of a Gallenkamp melting point apparatus (variable heater).

CHAPTER FOUR

4.0 RESULTS

4.1 Initial Processing of Samples

For each extraction of 200g powdered plant material, percentage yields were determined; *T. asiatica* root bark and leaves had the highest yield of 2.7% and 2.3% respectively while for volatile essential oils *O. gratissimum* leaves had the highest yield (0.45%) (Tables 4 and 5).

Comparison of the average percentage yields for the different solvents extractions of the plant parts, it was revealed; Methanolic leave extracts produced the highest yield of 2.17% followed by hexane extract with 1.75%. Lowest yields were encountered with ethyl acetate stem bark where the average percentage yield was as low as 1.08%.

$$\% \text{ yield} = \frac{\text{Total weight of extract obtained}}{\text{Total weight of plant material extracted}} \text{ X } 100$$

(Kigondu, 2007)

Table 4: Percentage yields for sequential extractions (%yield = extracted weight / 200 X 100)

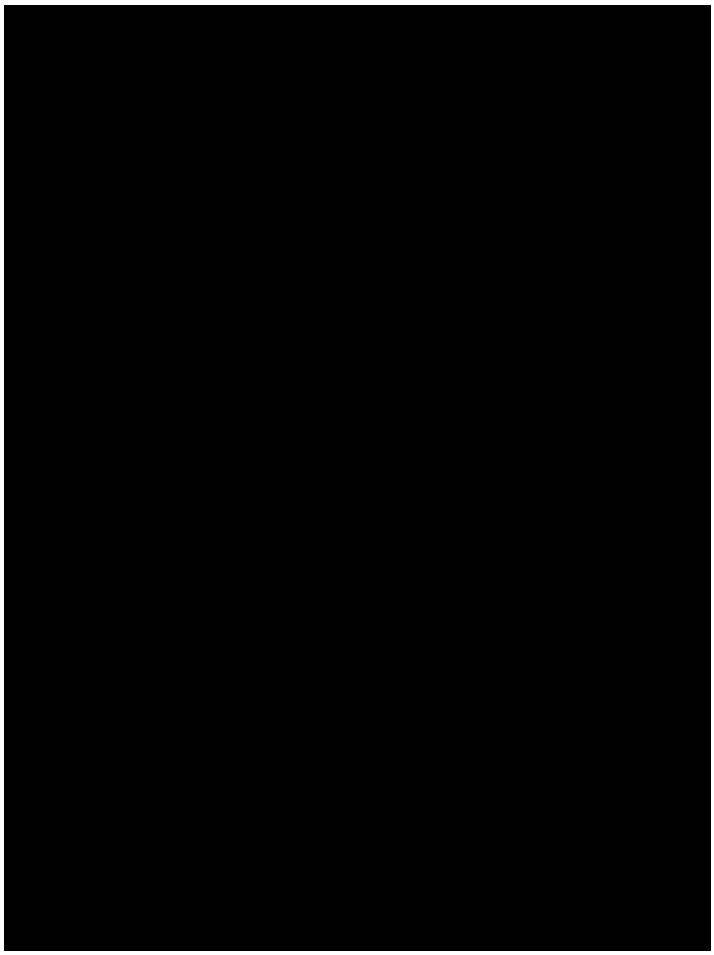
Solvent	Plant	Part extracted	Extract Weight (grams)	% yield
Hexane	*Toddalia asiatica*	Leaves	4.6	2.30
		Stem bark	3.7	1.85
		Root bark	5.6	2.70
	Ocimum gratissimum	Leaves	2.3	1.15
		Stem bark	0.9	0.45
		Root bark	1.2	0.60
	Aspilia mossambicensis	Leaves	3.6	1.80
		Stem bark	3.2	1.60
		Root bark	2.4	1.20
Ethyl acetate	*Toddalia asiatica*	Leaves	1.2	0.60
		Stem bark	2.7	1.35
		Root bark	2.8	1.40
	Ocimum gratissimum	Leaves	2.9	1.45
		Stem bark	1.0	0.50
		Root bark	1.5	0.75
	Aspilia mossambicensis	Leaves	3.8	1.90
		Stem bark	2.8	1.40
		Root bark	2.5	1.25
Methanol	*Toddalia asiatica*	Leaves	3.5	1.75
		Stem bark	3.6	1.80
		Root bark	4.1	2.05
	Ocimum gratissimum	Leaves	5.5	2.75
		Stem bark	1.3	0.65
		Root bark	2.1	1.05
	Aspilia mossambicensis	Leaves	4.0	2.00
		Stem bark	3.5	1.50
		Root bark	3.5	1.75

Table 5: Percentage yields for volatile oils steam distillates (%yield = extracted weight / 1000 X 100)

Solvent	Plant	Part extracted	V. oil Weight (grams)	% yield
Volatile oil distillates from water vapour	*Toddalia asiatica*	Leaves	3.1	0.31
		Stem bark	0.1	0.01
		Root bark	1.4	0.14
	Ocimum gratissimum	Leaves	4.5	0.45
		Stem bark	0.5	0.05
		Root bark	0.1	0.01
	Aspilia mossambicensis	Leaves	0.2	0.02
		Stem bark	0.1	0.01
		Root bark	4.1	0.41

Comparison of the solvents in terms of average percentage yields was gotten to take the format illustrated by figure 4

Figure 4**: Comparison of average % yields for the extraction solvents**



4.2 Biological Activity Evaluation

4.2.1 Bioactivity Testing

The diameters of the zones of inhibition for triplicate tests were measured and tabulated (appendix 1a) then averages appropriately determined. The largest zone, indicating highest activity was with *T. asiatica* stem bark methanol extract (15mm) against M.R.*S.aureus* for antibacterial assays (table 6) and the ethyl acetate extract (22mm) against *M. gypseum* for antifungal assays (table 7). On the other hand the least activity or no activity was evidenced in several extracts with no zone of inhibition being realized (6mm).

Table 6: Antibacterial activity of plants' crude extracts

Solvent	Plant	Part extracted	Conc^{n} mg/ml	*BIOACTIVITY INDEX*			
				E. coli	***S. typhi***	***P. aeruginosa***	***M.R.S. aureus***
DMSO	*Control*			0	0	0	0
Gentamicin	*Standard drug*		50	5+	4+	4+	4+
Hexane	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	0
	Ocimum gratissimum	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	2+
		Root bark	100	0	0	2+	1+
	Aspilia mossambicensis	Leaves	100	0	0	0	0
		Stem bark	100	0	0	2+	2+
		Root bark	100	0	0	2+	0
Ethyl acetate	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	0
	Ocimum gratissimum	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	2+
		Root bark	100	0	0	0	0
	Aspilia mossambicensis	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	1+
		Root bark	100	0	0	0	0
Methanol	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	2+	0	2+	3+
		Root bark	100	0	0	0	2+
	Ocimum gratissimum	Leaves	100	0	0	0	0
		Stem bark	100	0	0	1+	2+
		Root bark	100	0	0	2+	2+
	Aspilia mossambicensis	Leaves	100	1+	0	2+	2+
		Stem bark	100	0	0	1+	2+
		Root bark	100	0	0	0	1+
Volatile oils	*Toddalia asiatica*	Leaves	100	0	0	0	0
	Ocimum gratissimum	Leaves	100	0	0	0	0
	Aspilia mossambicensis	Root bark	100	0	0	0	2+

In terms of antimicrobial activity index (Rajakaruna *et al.,* 2002), most of the extracts are seen to display antibacterial activity amounting to antimicrobial index 2+; this can be considered moderate activity in consideration that the highest is at 5+ and lowest 1+. *A. mossambicensis* root bark volatile oil is realized to also exhibit activity in the same range (2+) as the organic solvents extracts.

Table 7: Antifungal activity of plants' crude extracts

Solvent	Plant	Part extracted	Conc^{n} mg/ml	BIOACTIVITY INDEX			
				C. neoformans	***C. albicans***	***C. tropicalis***	***M. gypseum***
DMSO	*Control*			0	0	0	0
Fluconazole	*Standard drug*		50	4+	5+	5+	5+
Hexane	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	2+	2+	0	2+
		Root bark	100	2+	0	0	2+
	Ocimum gratissimum	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	1+
	Aspilia mossambicensis	Leaves	100	0	0	0	0
		Stem bark	100	0	1+	0	1+
		Root bark	100	0	1+	0	0
Ethyl acetate	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	3+	0	0	4+
		Root bark	100	2+	0	0	2+
	Ocimum gratissimum	Leaves	100	1+	0	0	1+
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	1+
	Aspilia mossambicensis	Leaves	100	0	1+	0	0
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	0
Methanol	*Toddalia asiatica*	Leaves	100	0	0	0	0
		Stem bark	100	4+	2+	2+	5+
		Root bark	100	1+	0	0	2+
	Ocimum gratissimum	Leaves	100	0	0	0	0
		Stem bark	100	0	0	0	0
		Root bark	100	0	0	0	0
	Aspilia mossambicensis	Leaves	100	0	0	0	0
		Stem bark	100	0	1+	0	0
		Root bark	100	0	0	0	0
Volatile oils	*Toddalia asiatica*	Leaves	100	0	0	0	0
	Ocimum gratissimum	Leaves	100	4+	2+	1+	2+
	Aspilia mossambicensis	Root bark	100	0	0	0	0

Antimicrobial Activity Index: **0** = zone of inhibition <8mm (no inhibition)
1+ = 8 – 9 mm
2+ = 10 – 14 mm
3+ = 15 – 19 mm

4+ = 20 – 24 mm
5+ = 25 mm and above

The controls (solvents used; hexane, ethyl acetate, methanol and Dimethyl sulfoxide) showed no inhibition effect as evident from their bioactivity index of 0. The standards; for antibacterial assays the drug gentamicin had an index of 4+ and 5+ which was not quite far from that given by the extracts. The plant extracts showed antifungal activity that compared well to the standard (fluconazole) *T. asiatica* stem methanol extract registered highest bioactivity index in the range of 2+ to 5+ for all the fungal strains tested. The lowest bioactivity index was that of *O. gratissimum* stem bark.

4.2.2 Minimum Inhibitory Concentrations (MIC)

Plant extracts that showed significant activity were further subjected to bioassay to determine the minimum inhibitory concentration which was regarded as the lowest concentration that produced a visible zone of inhibition (Appendix 2a & 2b). The MICs were thus registered for antibacterial (table 8) and antifungal (table 9) appropriately;

Table 8: MICs of plants' crude extracts for antibacterial assays

Test organism	Solvent	Plant/part extract	M.I.C. (mg/ml)
	***Gentamicin**	**Standard**	**0.5**
Escherichia coli	Methanol	*T. asiatica* stem bark	25
Pseudomonas aeruginosa	Hexane	*O. gratissimum* root bark	6.25
		A. mossambicensis root bark	50
	Methanol	*T. asiatica* stem bark	12.5
		A. mossambicensis leaves	50
Methicillin Resistant Staphylococcus aureus	Hexane	*A. mossambicensis* stem bark	6.25
		O. gratissimum stem bark	50
	Ethyl acetate	*O. gratissimum* stem bark	3.1
	Methanol	*T. asiatica* root bark	25
		T. asiatica stem bark	12.5
	Volatile oils	*A. mossambicensis* root	50

*MIC for the standard drugs gentamicin was adapted from NCCL's Interpretative standards for dilution & disc diffusion susceptibility testing tables (Murray *et al.*,. 1999).

Table 9: MICs of plants' crude extracts for antifungal assays

Test organism	Solvent	Plant/part extract	M.I.C. (mg/ml)
	***Fluconazole**	**Standard**	**0.025**
Cryptococcus	Hexane	*T. asiatica* root bark	50
neoformans	Ethyl acetate	*T. asiatica* stem bark	6.25
	Methanol	*T. asiatica* stem bark	6.25
	Volatile oils	*O. gratissimum* leaves	12.5
Candida albicans	Methanol	*T. asiatica* stem bark	50
	Volatile oils	*O. gratissimum* leaves	25
Candida tropicalis	Methanol	*T. asiatica* stem bark	50
Microsporum	Hexane	*T. asiatica* stem bark	25
gypseum		*T. asiatica* root bark	3.1
	Ethyl acetate	*T. asiatica* stem bark	6.25
	Methanol	*T. asiatica* stem bark	6.25

*MIC for the standard drugs were determined and interpreted according to NCCL's procedures and quality control (Murray *et al.,*. 1999).

The lowest concentrations that produced visible zones of inhibition for all extracts tested ranged between 3.1 and 50 mg/ml. *Toddalia asiatica* stem methanol extract was the most active with an MIC of 3.1 mg/ml against the dermatophyte *Microsporum gypseum.*

For volatile oils, substantial activity is realized with *O. gratissimum* leaves' oil with MIC values in the range of 12.5 mg/ml to 25 mg/ml (table 12). This is in agreement with several studies (Jedlickova *et al.,* 1992; Nakaruma *et al.,* 1999; Orafidiya *et al.,* 2000

and Pessoa *et al.,* 2002) previously carried out. Antibacterial activity was also exhibited by volatile oils from *A. mossambicensis* against M. R. *S. aureus* (MIC, 50 mg/ml).

4.2.3 Bioautographical Selection

4.2.3.1 Bioautography of pure compounds

Detection of the chromatograms with MTT indicated biological activity from only two pure compounds **F4-C** and **F'9-I'** whose Retardation factor (R_f) values were 0.77 and 0.57 respectfully (table 10). The others elicited no activity against the organisms.

Table 10: Bioactivity of Column Isolates

Test organism	**Column Isolate**	**Distance moved (cm)**	**Bioactive spot R_f value**	**Inference**
Methicillin Resistant *Staphylococcus aureus*	F4-C	10.0	0.77	Active
	F'9 – I'	7.4	0.00	Not active
Pseudomonas aeruginosa	F4-C	10.0	0.00	Not active
	F'9 – I'	7.4	0.57	Active
Cryptococcus neoformans	F4-C	10.0	0.77	Active
	F'9 – I'	7.4	0.00	Not active

4.2.3.2 MIC of Bioautography Selected Spots

The MIC of bioautography selected spots R_f 0.77 and 0.57 of **F4-C** and **F'9-I'** respectively as determined by the reduction of the tetrazolium salt to formazan by living cells was 10mg/ml against both M.R.*S.aures* and *P. aeruginosa* and 5mg/ml against *C. neoformans.* (Table 11).

Table 11: MIC of Purified Compounds

Test organism	**Column Isolate**	**Bioactive spot R_f value**	**MIC (mg/ml)**
Methicillin Resistant *Staphylococcus aureus*	F4-C	0.77	10
Pseudomonas aeruginosa	F'9 – I'	0.57	10
Cryptococcus neoformans	F4-C	0.77	5

4.2.4 Biological activity statistical data management

Statistical analysis was employed in order to make conclusions with certainty in regard to the biological activity of the plant extracts. Considering frequencies in regard to plant part assayed, a valid percent of 33.3 is listed across all the parts assayed, 12.5 for all organisms tested against and a 0.0% cases excluded in case processing summary (appendix 7a). These confirmed there was no bias in sample preparation of plant parts and organisms tested against in relation to ethnobotanical application.

Analysis of variance using One Way – ANOVA (Appendix 7b) to establish whether variation in biological activity was significant illustrated quite significant variation in bioactivity; from plant to plant, among different plant parts, for extracts with different concentrations and amongst different test organisms was encountered with a calculated value of 0.00 and F values of 19.128, 19.566, 17.552 and 9.210. Nevertheless there was minimal significance in the variation of bioactivity of extracts of different solvents with a calculated value of 0.190 and an F value of 1.662.

4.3 Phytochemical evaluation

4.3.1 Screening of phytocompounds

TLC spray reagents used for preliminary screening of phytocompounds showed that alkaloids and flavonoids were present in all the studied plants, alkaloids occurred in all parts but for flavonoids there was no detection in the *A. mossambicensis* stem bark. Terpenoids, anthraquinones, phenolics, steroids, amines and cardiac glycosides were shown to be present in atleast one part of each plant (table 12).

Table 12: Phytochemical profile of the plants

Botanical Name	Plant part	Phytocompounds							
		Alkaloids	**Flavonoids**	**Terpenoids**	**Anthraquinones**	**Phenolics**	**Steroids**	**Amines**	**Cardiac glycosides**
Aspilia mossambicensis	Leaves	+ +	+	-	+	+	-	+	-
	Stem	+	-	-	-	+	-	-	+
	Root	+	+ +	-	-	-	+	-	+
Toddalia asiatica	Leaves	+ +	+	+	+	-	+	+	+
	Stem	+ +	+	+	+	-	+	+	-
	Root	+ +	+	-	+	+	+ +	+ +	+
Ocimum gratissimum	Leaves	+ +	+ +	+	+	+ +	+ +	+	+
	Stem	+	+ +	-	+	+	+	+	-
	Root	+ +	+ +	+ +	+	+	+ +	+ +	+

Key

+ + Abundant

+ Trace

- Not detected

4.3.2 Analysis of active chemical principles

Active pure compounds isolated were analyzed using molecular identification assays by means of spectroscopic techniques for structural elucidation.

4.3.2.1 Methyl (6-methoxy-2-oxo-2H-chromen-7-yl) acetate (F4-C) (6)

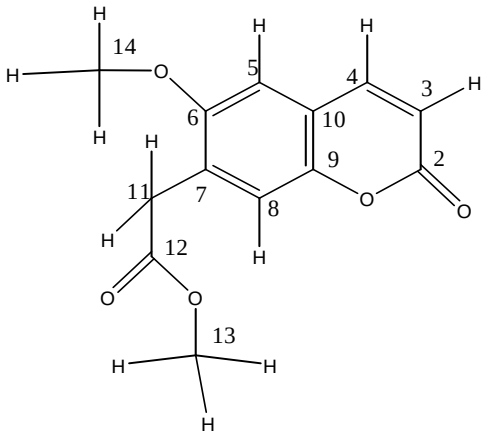
F4-C was isolated as cream needles [27.7mg, R_f 0.77 (SiO_2, 49:1 $CHCl_3$: MeOH)]. The melting temperature was 139 – 141^{O}C. UV absorption was observed at λ_{max} 240nm.

The IR spectrum (appendix 4a) showed characteristic bands at ν_{max} 2945.1 and 2831.3 (C-H stretch), 1716.5 (COOH) confirmed by 1203.5 (C-O stretch); characteristic of esteric carbonyl, 1683.7 (overtones due to C-H out of plane bending indicative of aromaticity). EIMS revealed *m/z* 248 [M^+] (24%) $C_{13}H_{12}O_5$; 233 [M^+ - CH_3] (9%); 217 [M^+ - OCH_3] (1%); 189 [M^+ - $COOCH_3$] (2%); 175 [M^+ - CH_2COOCH_3] (15%) (appendix 6a).

^{1}H, ^{13}C and COSY data (appendix 5a, 5b and 5c) is summarized in table 13, calculated ^{1}H and ^{13}C chemical shifts using ACD labs HNMR/CNMR predictor version 5.12 are also tabulated and as they are found to compare well with the observed values as well as the value reported in previous studies (Sharma *et al*., 1981, Ishii *et al*., 1992) of similar compounds;

Table 13: NMR (field, Acetone-d6) data for F4-C

Position	δ_H(200Hz)			δ_C(50Hz)			COSY
	Observed	Calculated	Reported	Observed	Calculated	Reported	
1	-	-	-	-	-	-	
2	-	-	-	150.3	161.3	-	
3	6.12 *d*	6.26	6.24	112.8	117.1	-	H3
4	8.04 *d*	7.65	7.94	139.5	143.7	-	H4
5	7.16 *d*	7.01	-	105.6	106.7	106.6	H5
6	-	-	-	146.0	151.9	-	
7	-	-	-	128.1	124.1	-	
8	7.78 *d*	7.13	-	117.6	115.0	-	H8
9	-	-	-	144.9	151.0	-	
10	-	-	-	115.0	117.5	-	
11	3.96 *s*	3.78	3.80	30.3	36.7	-	
12	-	-	-	159.6	169.6	171.4	
13	4.11 *s*	3.98	-	60.7	52.0	-	
14	3.97 *s*	3.80	3.96	61.1	55.7	61.4	



As observed in the ^{1}H and ^{13}CNMR; 13 carbons were present, 2 methoxy group carbons are revealed at chemical shifts δ 60.7 and δ 61.1 for C-13 and C-14 respectively, C-14

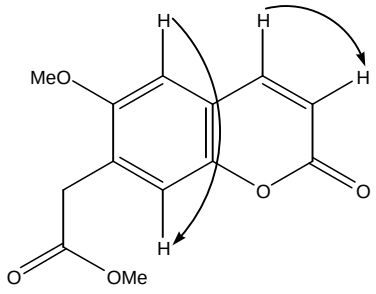
compares well to the literature value at δ 61.4. An esteric carbonyl carbon is suggested by the shift observed at δ 159.6 for C-12 and a benzylic carbon observed at δ 30.3 (C-11). Several chemical shifts observed above δ 100 to δ 160 suggest aromatic carbons.

The usual resonances in the ^{1}HNMR spectrum at δ 3.97 (*s*, 3H for OMe), at δ 6.12 and δ 8.04 (*d*, 1H each, *J*=10Hz) for the pyran-ring protons is a clear indication of a coumarin nucleus with a methoxy substitution (Sharma *et al.,* 1981). The chemical shifts at δ 7.16 and δ 7.78 (*d*, 1H each, *J*=2.2) presenting distance correlation was attributed to C-5 and C-8 para to each other hence suggesting that a side chain (CH_2COOCH_3) was linked to C-7 while methoxy group to C-6 of the coumarin nucleus. This assigning was in line with a related compound (toddanol) by Sharma (1981). The side chain contained methoxy protons δ 4.11 *s* (C-13) and a singlet at δ 3.96 for benzylic protons of C-11 (obscured under OMe signal) as described in the elucidation of dihydrotoddanol (Sharma *et al.,* 1981).

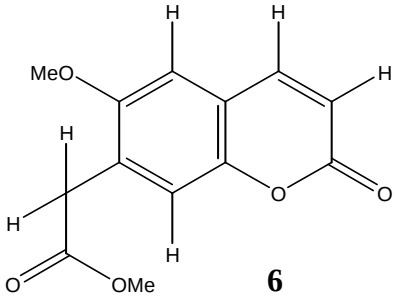
Toddanol and dihydrotoddanol, similar compounds with the coumarin nucleus elucidated by Sharma *et al.,* (1981) are reported with chemical shifts that are in agreement to the observed; proton shifts for C-3, C-4, C-11 and C-14 observed at δ 6.12, δ 8.04, δ3.96 and δ 3.97 respectively compare well to the reported values of δ 6.24, δ 7.94, δ3.80 and δ 3.96 respectively as indicated in the table above. Characteristic methoxy (C-13 & 14) ^{13}C chemical shifts are observed δ 60.7 and δ 61.1; this is in

agreement with the reported values at δ 61.4. C-5 observed shift at δ 105.6 is in line with the reported value of δ 106.6.

Assignment of proton positions was facilitated by the COSY data (appendix 5c) that illustrated proton correlation as shown below



H-3 and H-4 were determined to correlate as evidenced from the HNMR spectrum where they couple at a magnitude of 10Hz. Similarly H-5 and H-8 display coupling at equal magnitudes 2.2Hz. From the MS, IR and NMR data and the related compounds reported, **F4-C** was proposed to be;



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4.3.3.2 F'9-I'

The compound was isolated as a yellow fine powder [28.7mg, R_f 0.57 (SiO_2, 49:1 $CHCl_3$: MeOH)]. Melting temperature was 95 – 97^{O}C with UV absorption being observed at λ_{max} 206nm. IR spectrum showed characteristic bands at ν_{max} 3354.0 (OH or NH), 1716.5 (C=O) carbonyl functionality confirmed by C-O at 1207.4 and a C-N band observed at 1253.6 (appendix 4b). The EIMS (appendix 6b) suggested fragmentation pattern involving a tropylium ion at *m/z* 219 caused by either fission of the molecule at a point β – of an olefinic bond or loss of / movement of lone electrons (Sharma *et al.*, 1979). 2D NMR spectroscopy analysis is currently in progress for confirmation of a plausible structure for the compound **F'9 – I'**.

CHAPTER FIVE

5.0 DISCUSSION

5.1 Initial Processing of Samples

Comparing the quantity of sequential extraction using the various solvents it was noted that the highest percentage yield was with the methanol and the lowest ethyl acetate. The plant materials studied therefore could be endowed with components that are mainly polar and non polar other than components of intermediate polarity as evidenced by the decreasing order of extract quantity per solvent (methanol > hexane > ethyl acetate).

Distribution of volatile oils in the parts of the three plants investigated varies as for *T.asiatica and O. gratissimum,* it is the leaves that yields sufficient amounts compared to the stem bark and root bark however for *A. mossambicensis* more volatile oil is realized from the root bark. This difference could be ascribed to the fact that secondary metabolites are distributed differently from plant to plant.

5.2 Biological Activity Evaluation

5.2.1 Bioactivity Testing

The results obtained in this study indicate a considerable difference in antimicrobial activity between extracts obtained with different solvents (n-hexane, ethyl acetate and methanol) with methanol extracts being more active than the other extracts. This activity

was more pronounced against fungal organisms and Gram-positive bacteria than against Gram-negative bacteria. Although, antifungal compounds of the plants investigated are not well known, the presence of flavonoids and coumarins-related compounds and a certain degree of lipophilicity might be responsible for the activity. Lipophilicity toxicity is due to the interactions with the membrane constituents and their arrangement (Tomas-Barberan *et al.,* 1990).

The reason for the different activity between Gram-positive and Gram–negative bacteria could be ascribed to the morphological differences between these micro-organisms. Gram-negative bacteria have an outer phospholipidic membrane carrying the structural lipopolysaccharide components. This makes the cell wall impermeable to lipophilic solutes, while porins constitute a selective barrier to the hydrophilic. The Gram-positive bacteria should be more susceptible having only an outer peptidoglycan layer which is not an effective permeability barrier (Nostro *et al.,* 2000). In spite of this permeability difference, the methanol extracts also show some inhibition on *P. aeruginosa* a gram-negative bacteria hence they may be considered to have a broader spectrum of inhibitory activity than the other extracts.

The oil from the leaves of *O. gratissimum* had antimicrobial activity both antibacterial and antifungal; this is in line with the explosive studies previously carried out. The findings of this study are thus a confirmation of the extensive reports on oil from *O. gratissimum* leaves which goes back to Jedlickova *et al.,* (1992) exploring antibacterial

properties to Lemos *et al.,* (2005) giving an account of the chemical constituents of the oil and pointing out eugenol as a major component (57%) responsible for activity. Regardless of exhibiting higher activity, a basis for further analysis of *O. gratissimum* oil does not therefore arise. The low activity or non susceptibility of the test organism to the oil of other plants may also be due to the higher volatility of the oil, leading to the escape or evaporation of the oil.

Both crude and purified extracts of *T. asiatica* had activity with MIC values that are comparable. However in another case (sample F12 – J) bioactivity was lost upon purification which could be attributed to synergistic effects that are lost on separation and purification. In this test system, *S. typhi* was not susceptible to any of the plant extracts; treatment of diseases such as typhoid that are due to it may not be applied. For the other organisms there was variation in susceptibility ranging from extract to extract.

5.2.2 Minimum Inhibitory Concentrations (MIC)

Antimicrobial activity is detected at higher concentrations of extract upto 50mg/ml of crude extract compared to 0.5 mg/ml for standard drugs, an indication that the active ingredient is present in low quantities requiring use of large amounts of crude extracts. The difference in biological activity is attributed to the fact that plants differ phytochemically and the extraction / purification procedures may alter composition. Absence of in-vitro biological activity does not warranty disapprove of the ethno botanical utilizations as this may suggest extracts acting in an indirect way where active ingredient exist as a precursor requiring activation in-vivo.

From this investigation it is revealed that activity varies greatly from plant to plant in regard to the part where by higher activity may be witnessed in the leaves of one plant while for the other activity is more pronounced in the root bark.

5.2.3 Bioautographical Selection

5.2.3.1 Bioautography of pure compounds

Bioautography results, **F4-C** showed well-defined inhibition bands in assays with the gram positive bacteria Methicillin resistant *Staphylococcus aureus* and the yeast *Cryptococcus neoformans*. This band is in correspondence with Coumarins and flavonoids bands, whose presence is demonstrated by previous studies (Tsai *et al*., 1998, Sharma *et al*., 1981). Inhibition bands shown by **F'9-I'** in assays with gram negative *Pseudomonas aeruginosa* corresponds to Terpenoids and amines bands. Other authors have showed the presence of terpenes and their bacteriostatic and fungistatic properties (Bourrel *et al*., 1993). However it is difficult to compare the data because of some variables like the choice of extraction method and antimicrobial tests.

5.2.3.2 MIC of Bioautography Selected Spots

From broth dilution method, the quantitative data obtained can be considered sufficient for consequent studies aimed at identifying the single active principle(s) as there is close correlation to the positive control used.

5.3 Phytochemical evaluation

5.3.1 Screening of phytocompounds

Phytochemical screening of the test plant extract using TLC visualization reagents showed that the plant extracts were positive for organic compounds such as; flavonoids, phenolics, terpenoids, anthraquinones, steroids and amines while negative for alkaloids and cardiac glycosides. The presence of flavonoids, phenolic or terpenoids in plants has been shown to be responsible for antimicrobial activity in plants (Nostro *et al.,* 2000). Their role is to protect plants against microbial or insect damage (**Cowan, 1999)**. The antibacterial and antifungal activity of some of the plants tested could therefore be attributed to the presence of such compounds.

5.3.2 Analysis of active chemical principles

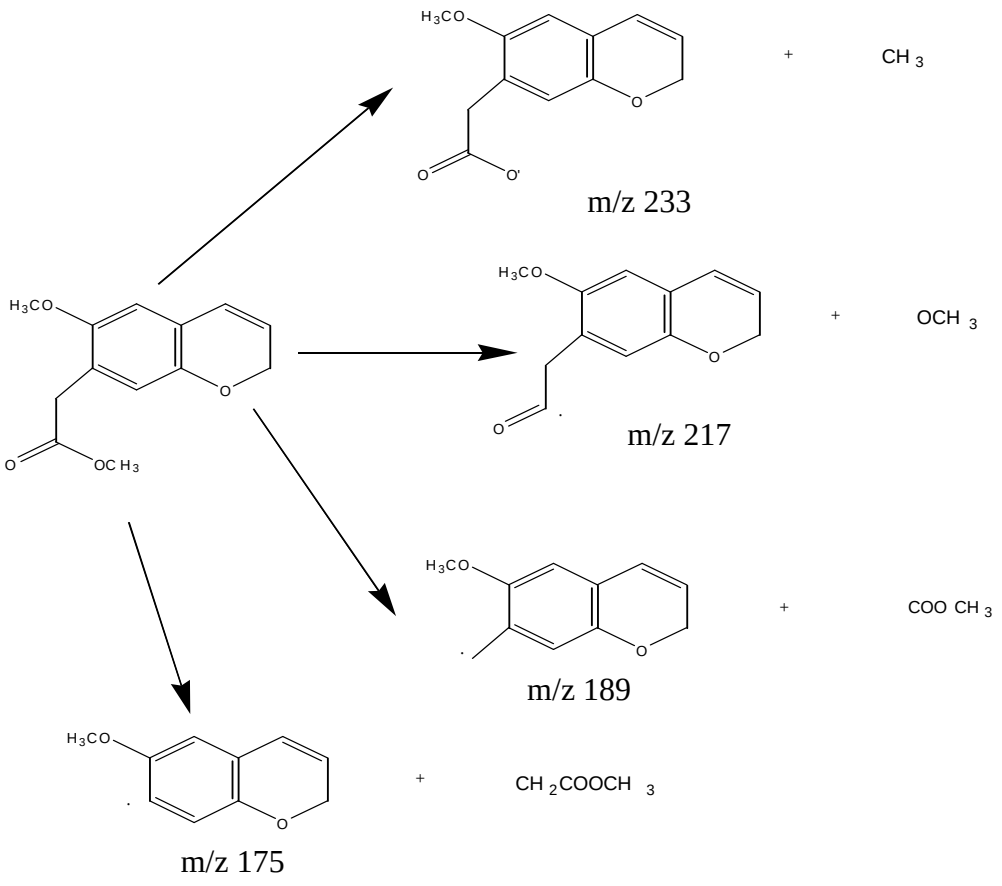
Ultraviolet, infrared, nuclear magnetic resonance and electron impact mass spectroscopic methods yielded sufficient data that was utilized to reveal the active chemical principle(s).

5.3.2.1 Methyl (6-methoxy-2-oxo-2H-chromen-7-yl) acetate (F4-C) (6)

Ultra violet absorption in the near UV region is a clear implication that the compound has electronic energy changes associated with presence of unsaturated groups and atoms with unshared pairs of electrons (Baker and Engel, 1992). Quite a number of infra red absorptions are realized in the finger print region hence adequately bringing out the aromaticity and functionality of the compound.

A comparison between the observed NMR spectra values and calculated values (ACD labs HNMR/CNMR predictor version 5.12) illustrated agreement. This was observed for proton shifts at H-3 and H-4 observed at δ 6.12 and δ 8.04 respectively while calculated values were δ 6.26 and δ 7.65 respectively. H-5 and H-4 observed at δ 7.16 and δ 3.97 while calculated values at δ 7.01 and δ 3.80 respectively. The ^{13}C chemical shifts were also in agreement with the calculated values; C-5 and C-7 observed at δ 105.6 and 128.1 respectively while calculated as δ 106.7 and δ 124.1 respectively (table 14).

Mass spectroscopy data from the EIMS spectra gives the following fragmentation pattern below, which confirmed the proposed structure.



CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 Conclusion

The plant extracts of *T. asiatica, O. gratissimum and A. mossambicensis* contain several antibacterial, antifungal compounds that are identifiable.

Highest antimicrobial activity is encountered in the stem bark of *Toddalia asiatica.*

Activity was more pronounced against fungal organisms and Gram-positive bacteria than against Gram-negative bacteria.

Difference in activity between Gram-positive and Gram–negative bacteria could be ascribed to the morphological differences in terms of outer phospholipidic membrane (gram negative).

Flavonoids & coumarins related compounds and the lipophilicity toxicity arising due to interactions with the membrane constituents and their arrangement (Tomas-Barberan *et al.,* 1990) also account for the activity exhibited.

Regardless of exhibiting higher activity a basis for further analysis of *O. gratissimum* oil does not arise considering previous studies that avail exhaustive information.

The low activity or non susceptibility of the test organism to the oil of other plants may also be due to the higher volatility of the oil, leading to the escape or evaporation of the oil.

Loss of activity upon purification could be attributed to synergistic effects that are lost on separation and purification.

Absence of in-vitro activity does not warranty disapprove of the ethno botanical utilizations as this may suggest extracts acting in an indirect way where active ingredient exist as a precursor requiring activation in-vivo

In this test system, *S. typhi* was not susceptible to any of the plant extracts; treatment of diseases such as typhoid that are due to it may not be applied. For the other organisms there was variation in susceptibility ranging from extract to extract.

Analyzed chemical principles reveals a coumarin, structural with lipophilic end groups that are responsible for the toxicity and hence the bioactivity exhibited. This is in agreement with on-going phytochemical studies of *Toddalia asiatica* results obtained differently by Prof. J. I. Jondiko (Maseno University - Kenya).

6.2 Recommendation

These results validate the ethno botanical uses of *Toddalia asiatica*, its worthwhile recommending that since its potent antibacterial, antifungal activity is being noted here, further work should be directed to Methyl (6-methoxy-2-oxo-2H- chromen- 7 –yl) acetate, 2D NMR to confirm the proposed structure which as far as known the compound is not yet reported.

Substantive fractionation should be carried on the stem bark of *T. asiatica* with the aim of phytochemical profiling of the active principles which may call for explicit methods such as LC-MS and extend the bioassay to other micro-organisms for possible pre-clinical development.

Toxicity studies should be carried out on the crude active fractions to establish their safe levels for use by humans. This will go along way in availing information to assist in calculation of the safety dosage required for complete microbial clearance. This information availed and utilized will be a step forward in traditional medicine practice.

Though exhibiting minimal antibacterial activity, the volatile oil from *A. mossambicensis* root bark should be subjected to explorative analysis involving bioassaying at higher concentrations and hyphenated chromatographic assays such as GC-MS be carried out to reveal the chemical constituents.

The current findings validate the ethnobotanical exploitation of *Toddalia asiatica* a Kenyan medicinal plant for conmditions that are of bacterial and fungal etiology. However issues of conservation should be emphasized and put in place in regard to harvest since in the study it's the stem bark that is highly efficacious.

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APPENDICES

Appendix 1a

DATA SHEET – Antibacterial Bioassay of crude extracts.

S/No.	Solvent / Extract	Conc^{n} (mg/ml)	BIOACTIVITY (Zone size in mm)															
			S. aureus			*E. coli*			*S. typhi*			*P. aeruginosa*			*M R.S. aureus*			Remarks
01	**STD-Gentamicin**	0.1				20	24	22				18	20	22				
02	**Ctrl- solvents**	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
03	A. moss- R - Hex	0.1				-	-	-	-	-	-	10	14	12	-	-	8	
04	A. moss- R- EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
05	A. moss- R- MeOH	0.1				-	-	-	-	-	-	-	-	-	8	10	8	
06	A. moss- S - Hex	0.1				-	-	-	-	-	-	10	8	12	10	12	12	
07	A. moss- S- EtOAc	0.1				-	-	-	-	-	-	-	-	-	10	8	10	
08	A. moss- S- MeOH	0.1				-	-	-	-	-	-	8	10	8	10	10	8	
09	A. moss- L - Hex	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
10	A. moss- L- EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
11	A. moss- L- MeOH	0.1				8	10	10	-	-	-	10	12	12	10	10	8	
12	O. grati-R- Hex	0.1				-	-	-	-	-	-	14	14	12	10	10	8	
13	O. grati-R- EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
14	O. grati-R- MeOH	0.1				-	-	-	-	-	-	12	14	14	10	12	12	
15	O. grati-S- Hex	0.1				-	-	-	-	-	-	-	-	-	10	12	12	
16	O. grati-S- EtOAc	0.1				-	-	-	-	-	-	-	-	-	14	14	14	
17	O. grati-S- MeOH	0.1				-	-	-	-	-	-	10	8	10	10	10	12	

cont.

S/No.	Solvent / Extract	Conc^{n} (mg/ml)	BIOACTIVITY (Zone size in mm)															
			S. aureus			*E. coli*			*S. typhi*			*P. aeruginosa*			*M R.S. aureus*			Remarks
18	O. grati-L- Hex	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
19	O. grati-L- EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
20	O. grati-L- MeOH	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
						-	-	-	-	-	-	-	-	-	-	-	-	
21	T. asia-R - Hex	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
22	T. asia-R - EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
23	T. asia-R - MeOH	0.1				-	-	-	-	-	-	-	-	-	12	10	12	
24	T. asia-S - Hex	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
25	T. asia-S - EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
26	T. asia-S - MeOH	0.1				12	12	14	-	-	-	12	12	12	16	16	14	
27	T. asia-L - Hex	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
28	T. asia-L - EtOAc	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
29	T. asia-L - MeOH	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
30	A. moss – V.oils	0.1				-	-	-	-	-	-	-	-	-	10	10	8	
						-	-	-	-	-	-	-	-	-				
31	O. grati – V. oils	0.1				-	-	-	-	-	-	-	-	-	-	-	-	
32	T. asia – V. oils	0.1				-	-	-	-	-	-	-	-	-	-	-	-	

Appendix 1b

DATA SHEET – Antifungal Bioassay of crude extracts.

S/No.	Solvent / Extract	Conc^{n} (mg/ml)	BIOACTIVITY (Zone size in mm)												
			C. .neoformans			*C. albicans*			*C. tropicalis*			*M. gypseum*			Remarks
01	**STD-Fluconazole**	0.1													
02	**Ctrl- solvents**	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
03	A. moss- R - Hex	0.1	-	-	-	10	8	10	-	-	-	-	-	-	
04	A. moss- R- EtOAc	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
05	A. moss- R- MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
06	A. moss- S - Hex	0.1	-	-	-	8	10	10	-	-	-	8	8	10	
07	A. moss- S- EtOAc	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
08	A. moss- S- MeOH	0.1	-	-	-	10	8	8	-	-	-	-	-	-	
09	A. moss- L - Hex	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
10	A. moss- L- EtOAc	0.1	-	-	-	10	10	8	-	-	-	-	-	-	
11	A. moss- L- MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
12	O. grati-R- Hex	0.1	-	-	-	-	-	-	-	-	-	10	8	10	
13	O. grati-R- EtOAc	0.1	-	-	-	-	-	-	-	-	-	8	10	10	
14	O. grati-R- MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
15	O. grati-S- Hex	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
16	O. grati-S- EtOAc	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
17	O. grati-S- MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
18	O. grati-L- Hex	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
19	O. grati-L- EtOAc	0.1	8	10	10	-	-	-	-	-	-	8	10	8	
20	O. grati-L- MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	

S/No.	Solvent / Extract	Conc^{n} (mg/ml)	BIOACTIVITY (Zone size in mm)												
			C. .neoformans			*C. albicans*			*C. tropicalis*			*M. gypseum*			Remarks
21	T. asia-R - Hex	0.1	12	12	12	-	-	-	-	-	-	18	18	20	
22	T. asia-R - EtOAc	0.1	10	10	10	-	-	-	-	-	-	12	14	12	
23	T. asia-R - MeOH	0.1	10	10	10	-	-	-	-	-	-	12	12	14	
24	T. asia-S - Hex	0.1	10	12	10	12	12	12	-	-	-	14	12	14	
25	T. asia-S - EtOAc	0.1	16	16	14	-	-	-	-	-	-	22	24	22	
26	T. asia-S - MeOH	0.1	22	20	22	12	12	10	14	14	14	26	26	24	
27	T. asia-L - Hex	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
28	T. asia-L - EtOAc	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
29	T. asia-L - MeOH	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
30	A. moss – V.oils	0.1	-	-	-	-	-	-	-	-	-	-	-	-	
31	O. grati – V. oils	0.1	22	20	22	14	14	14	10	8	10	12	12	10	
32	T. asia – V. oils	0.1	-	-	-	-	-	-	-	-	-	-	-	-	

NB

- **– No zone of Inhibition ; zero bioactivity**

A. moss – *Aspilia mossambicensis*
O. grati – *Ocimum gratissimum*
T. asia – *Toddalia asiatica*

R – root bark
S – stem bark
L – leaves

Hex – hexane extract
EtOAc – ethyl acetate extract
MeOH – methanol extract

Appendix 2a

DATA SHEET – MICs for antibacterial active extracts.

Solvent / Extract	INHIBITORY CONCENTRATIONS (Zone size in mm)																				
	1 mg/ml			0.5 mg/ml			0.25 mg/ml			0.125 mg/ml			0.0625 mg/ml			0.031 mg/ml			0.0015 mg/ml		
ANTIBACTERIAL																					
E. coli																					
STD - Gentamicin	24	26	24	**20**	**19**	**19**															
T. asiatica stem - MeOH	**12**	**14**	**12**	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
M.R.S. aureus																					
T. asiatica root - MeOH	16	16	14	12	14	14	**12**	**12**	**12**	8	-	-	-	-	-	-	-	-	-	-	-
O. gratissimum stem - EtOAc	14	16	14	12	14	14	12	12	14	10	8	10	10	10	12	**8**	**8**	-	-	-	-
A. mossambicensis stem - Hex	12	14	14	12	12	14	12	12	12	10	8	10	**8**	**8**	-	-	-	-	-	-	-
O. gratissimum stem - Hex	12	10	12	**10**	**8**	**10**	8	-	-	-	-	-	-	-							
A. mossambicensis root – v. oils	10	10	8	8	-	8															
P. aeruginosa																					
STD - Gentamicin	20	20	18	**16**	**16**	**18**															
O. gratissimum root - Hex	14	14	12	12	12	14	10	8	10	8	8	10	**8**	**8**	**8**	-	-	-	-	-	-
A. mossambicensis root - Hex	12	14	12	**10**	**8**	**8**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A. mossambicensis lvs - MeOH	14	12	12	**10**	**8**	-	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T. asiatica root - MeOH	12	14	12	12	12	14	10	10	8	**8**	**8**	**8**	-	-	-	-	-	-	-	-	-

Appendix 2b

DATA SHEET – MICs for antifungal active extracts.

Solvent / Extract	INHIBITORY CONCENTRATIONS (Zone size in mm)																				
	1 mg/ml			0.5 mg/ml			0.25 mg/ml			0.125 mg/ml			0.0625 mg/ml			0.031 mg/ml			0.0015 mg/ml		
ANTIFUNGALS																					
C. neoformans																					
STD - Fluconazole	18	16	16	14	112	12	**10**	**8**	**8**												
T. asiatica root - Hex	12	14	12	**10**	**10**	**12**	8	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T. asiatica stem - MeOH	22	24	22	18	20	18	14	16	14	12	10	10	**8**	**10**	**8**	8	-	-	-	-	-
T. asiatica stem - EtOAc	16	14	16	14	14	16	12	10	12	10	10	8	**8**	-	**8**	-	-	-	-	-	-
O. gratissimum – volatile oils	22	24	20	18	18	16	10	12	10	**8**	-	**8**	-	-	-	-	-	-	-	-	-
C. albicans																					
STD - Fluconazole	18	16	16	**14**	**10**	**8**															
T. asiatica stem - MeOH	12	14	12	**8**	**6**	**8**	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O. gratissimum – volatile oils	14	14	12	10	12	10	**8**	-	**8**	-	-	-	-	-	-	-	-	-	-	-	-
C. tropicalis																					
STD - Fluconazole	18	16	16	**14**	**10**	**8**															
T. asiatica stem - MeOH																					
M. gypseum																					
STD - Fluconazole	18	16	16	14	10	8															
T. asiatica root - Hex	18	16	18	16	16	16	14	12	14	14	14	16	14	12	12	**8**	**10**	**8**	-	-	-
T. asiatica stem - Hex	14	16	14	12	12	10	**8**	-	**8**	-	-	-	-	-	-	-	-	-	-	-	-
T. asiatica stem - EtOAc	22	20	22	18	16	16	16	14	14	14	14	12	**12**	**10**	**8**	8	-	-	-	-	-
T. asiatica stem - MeOH	26	24	26	18	16	18	16	16	14	12	10	12	**10**	**8**	**8**	-	-	-	-	-	-

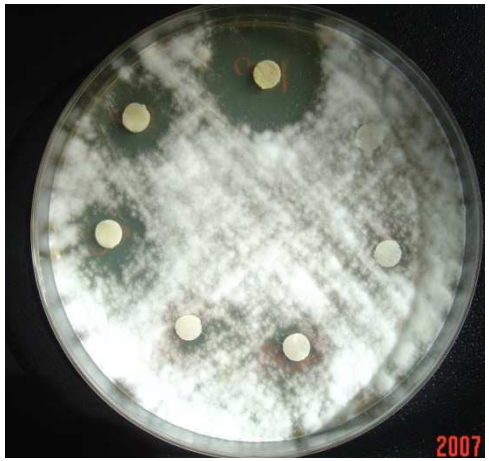
NB

MIC – Highlighted values (zone of inhibition in mm)

Appendix 3

Inhibition zones of antibacterial, antifungal activity.

Zones of inhibition of M.R.*S. aureus*

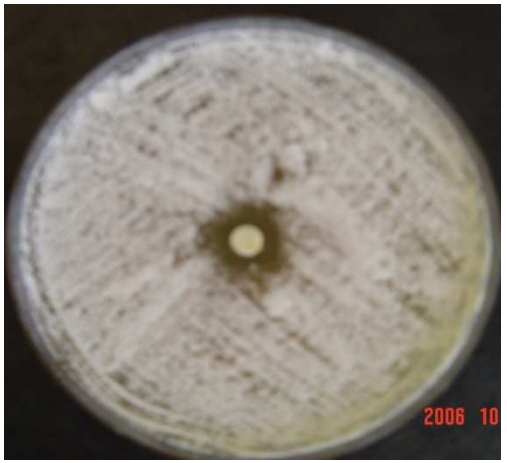


Zones of inhibition of *M. gypseum*

Zones of inhibition of *C. neoformans*


No inhibition of *E.coli* except at the centre

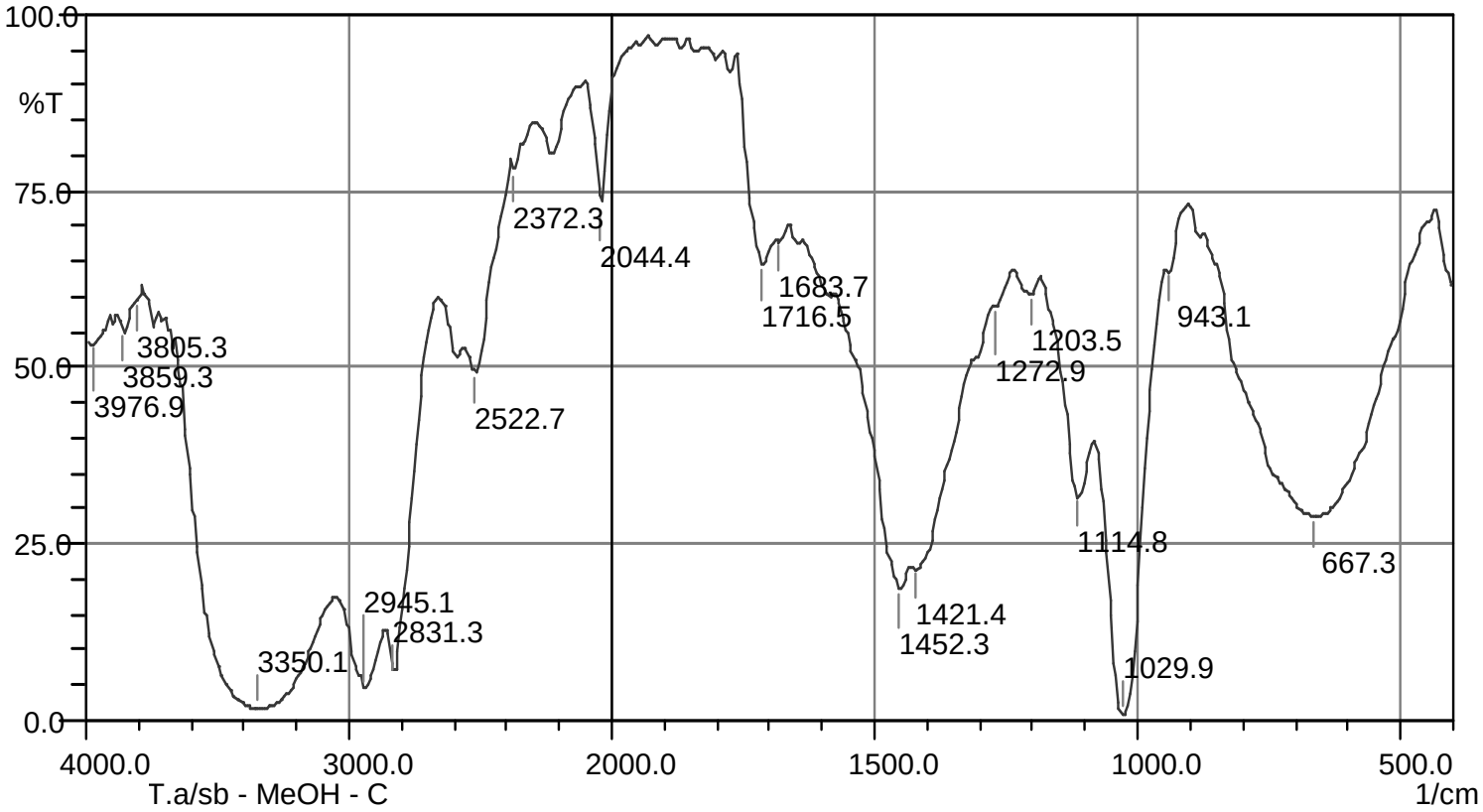
Zone of inhibition of *C. albicans*



Zone of inhibition of *C. tropicalis*

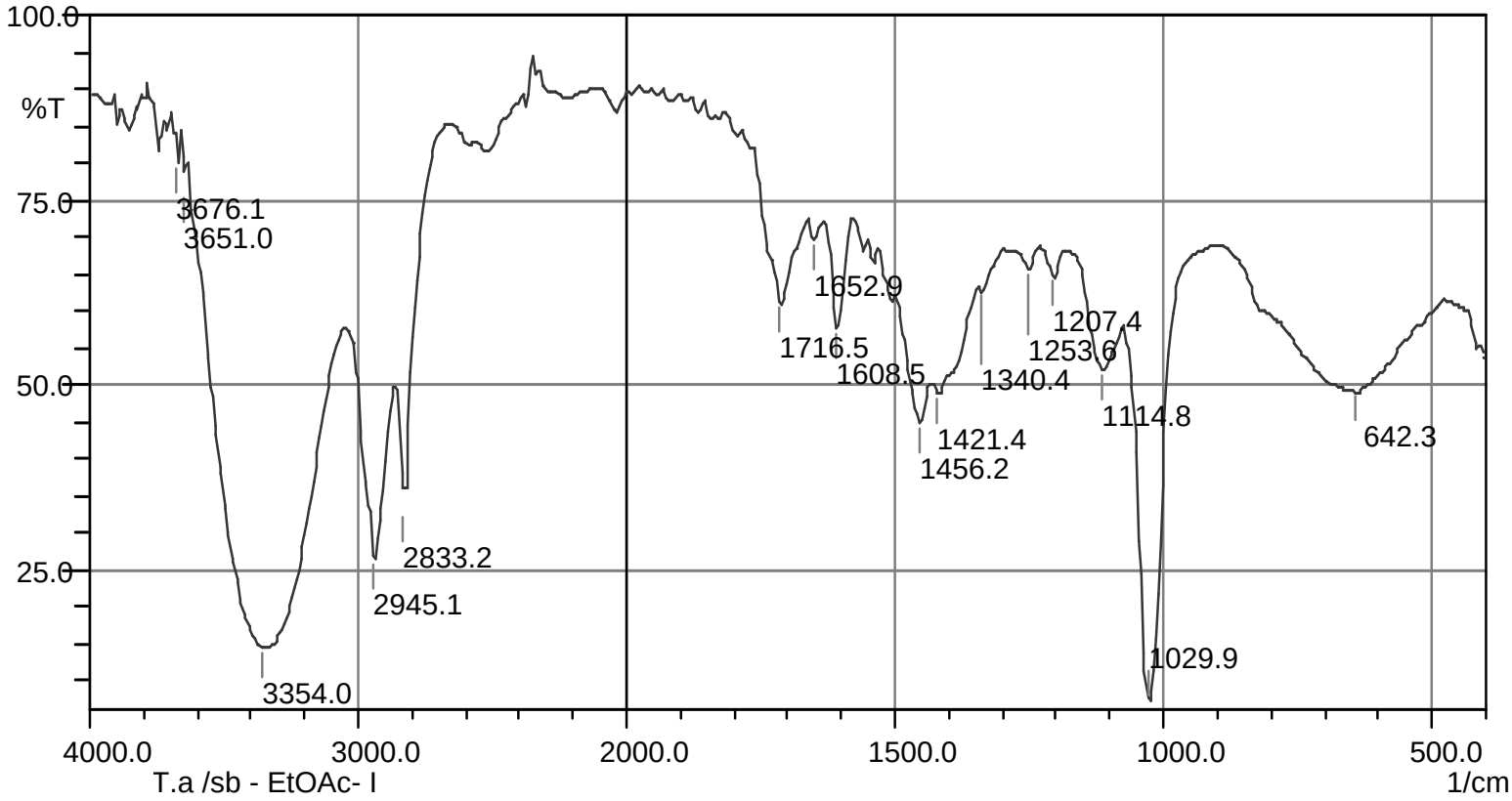
Appendix 4a

Infra red spectroscopy spectrum of F4-C



Appendix 4b

Infra red spectroscopy spectrum of F'9-I'



Appendix 7b

Statistical Analysis – Analysis of Variance (ANOVA)

- Plant

	Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	93.287	2	46.644	19.128	**.000**
Within Groups	3679.726	1509	2.439		
Total	3773.013	1511			

- Plant part

	Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	95.370	2	47.685	19.566	**.000**
Within Groups	3677.643	1509	2.437		
Total	3773.013	1511			

- Extraction solvent

	Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	8.295	2	4.147	1.662	**.190**
Within Groups	3764.718	1509	2.495		
Total	3773.013	1511			

- Concentration of extract

	Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	246.754	6	41.126	17.552	**.000**
Within Groups	3526.259	1505	2.343		
Total	3773.013	1511			

- Test organisms

	Sum of Squares	df	Mean Square	F	**Sig.**
Between Groups	155.087	7	22.155	9.210	**.000**
Within Groups	3617.926	1504	2.406		
Total	3773.013	1511			

In all the above analysis of variance the Dependent variable is biological (antibacterial, antifungal) activity.

Appendix 5b

^{13}C NMR spectrum for F4-C

Appendix 5c

COSY NMR spectrum for F4-C