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UCL SCHOOL OF PHARMACY

EVALUATION OF THE *IN VITRO* BIOLOGICAL ACTIVITIES AND PHYTOCHEMICAL PROFILING OF EIGHT *FICUS* SPECIES COLLECTED IN ZAMBIA

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DECLARATION

I, Angela Gono Bwalya declare that this dissertation is my own work. It is being submitted for the degree of Doctor of Philosophy (Ph. D) in the School of Pharmacy, University College London, United Kingdom. It has not been submitted before for any degree or examination in any other University.

Signed:.....this day.....2014

To my sons

Nkandu, Kudakwashe and Luse Bwalya

ABSTRACT

Infectious diseases are responsible for an overwhelming number of deaths and morbidity worldwide. In tropical regions of the world, in particular, developing countries like Zambia, poor health is prevalent and diseases such as malaria, meningitis, pneumonia, tuberculosis and gastrointestinal infections strongly persist. Folkloric medicines are still actively used against some of these infections as primary care before seeking conventional treatment at hospitals. Members of the genus *Ficus* (Moraceae) are traditionally used in Zambia against many diseases caused by bacterial, fungal and protozoal infections. Thus according to the plant parts used traditionally for herbal preparations, aerial and root parts of eight *Ficus* species namely; *F. ingens*, *F. lutea*, *F. natalensis*, *F. ovata*, *F. sansibarica* subsp. *macrosperma*, *F. sycomorus* subsp. *gnaphalocarpa*, *F. sycomorus* subsp. *sycomorus* and *F. wakefieldii* were collected from different parts of Zambia.

The main aim of this thesis was to evaluate the medicinal potential of members of the genus *Ficus*. This was achieved by three objectives, which involved the phytochemical profiling of the crude extracts and subextracts of the *Ficus* for their constituents using chromatographic methods such as thin layer chromatography (TLC), proton Nuclear Magnetic Resonance (^{1}H NMR) and high performance liquid chromatography (HPLC). Secondly, the extracts were screened for various biological activities after which they were evaluated against recombinant FAS-II elongation enzymes, FabG, FabI and FabZ as potential targets in liver stage malaria parasites.

In this case, finely ground dried plant material was extracted with methanol (MeOH) to yield the crude methanol extracts (CR-MeOH) which, were further partitioned to provide a coarse separation of the crude extracts according to polarity. The three subextracts obtained included n-hexane, chloroform ($CHCl_3$) and aqueous methanol (aq-MeOH). The obtained extracts and subextracts were screened for biological activities such as: antifungal and antibacterial activities using the broth dilution and agar disc diffusion assays, antitubercular activity using the MTT assay, antischistosomal activity using the microscopic *in vitro* assay. In addition, antiprotozoal activitites which included antileshmanial activity using an assay against amastigotes of *L. donovani* strain

MHOM/ET/67/L82, trypanocidal activity against *T. cruzi* and *T. brucei rhodesiense* STIB 900 strain, and antiplasmodial activity by modified [^{3}H]-hypoxanthine incorporation assay, using the chloroquine/pyrimethamine resistant K1 strain were performed. Cytotoxity activity was also performed using rat skeletal myoblasts L6-cells. The chemical profiling was done by TLC, NMR and RP-HPLC. Meanwhile the chemical compound isolation for *F. sansibarica* was attempted by different chromatographic techniques and characterization by spectroscopic methods.

The phytochemical profiling revealed the presence of closely related polyphenolic compounds to which some of the biological activities were attributed to. For instance, the antibacterial and the FAS-II enzyme inhibition activities were mostly retained in the aq-MeOH subextracts, which were composed of very polar metabolites including flavonoids. Antiplasmodial activity was observed mostly in the less polar metabolites which were retained in the hexane and $CHCl_3$ subextracts of the stem barks. This pattern was similar with antitrypanosomal and antileishmanial activities, though with lesser sensitivity. The same subextracts including those of the root barks showed the most activity against *M. tuberculosis* with MIC values of 256 and 128 μg/ml, and against *Schistosoma*, for both larval and adult worms. The extracts did not exert any antifungal activity by the agar disc diffusion method we used.

Detailed phytochemical investigation of the leaves of *F. sansibarica* was performed, and led to the isolation of two compounds; epicatechin and apigenin-6-*C*-glucoside from the chloroform and aq. MeOH subextracts respectively. The predominant constituent of the CR-MeOH extract of *F. sansibarica* was identified as having a molecular weight of 432 g/mol by LC-MS analysis which could be set as an identification chemical marker for *F. sansibarica*.

The results highlight the potential that *Ficus* species could have as a valuable source for potent compounds which can be identified as scaffolds for the development of novel liver stage antimalarial drugs. Our results support previous research on the antimicrobial activity of *Ficus* species and they also provide an *in vitro* scientific basis supporting the use of *Ficus* species in traditional herbal preparations against some bacterial and parasitic infections.

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ABBREVIATIONS

°C	Degree celsius
δ	Delta, for chemical shift
µg	Microgram
µl	Microlitre
µM	Micromolar
A	Absorption
ACP	Acyl carrier protein
ACTs	Artemisinin combination therapies
AIDS	Acquired immunodeficiency syndrome
Alt.	Altitude
APS	Ammonium persulfate
BSAC	British Society for Antimicrobial Chemotherapy
CFU	Colony forming unit
CO_2	Carbon dioxide
CoA	Coenzyme A
cm	Centimetre
^{13}CNMR	Carbon nuclear magnetic resonance
cpm	Count per minute
kDa	Kilodalton
DAD	Diode array detector
DHA	Dihydroartemisinin
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
e.g	*lat. exempli gratia* (for example)

EGCG	Epigallocatechin gallate
et al.	*lat. et aliter* (and others)
FAB	Fatty acid biosynthesis
FabA	β-hydroxydecanoyl-ACP dehydrase
FabB	β-ketoacyl-ACP synthase
FabG	β-ketoacyl-ACP-reductase
FabI	Enoyl-ACP-reductase
FabZ	β-hydroxyacyl-ACP-dehydratase
FAS-I	Type I fatty acid synthase
FAS-II	Type II fatty acid synthase
Fig.	Figure
FBS	Fetal bovine serum
g	Gram
GPS	Global positioning system
HCl	Hydrochloric acid
hr	Hour
HEPES	4-(2-Hydroxyethyl) piperazine-1-ethanesulfonic acid
His	Histidine
HIV	Human immunodeficiency virus
^{1}HNMR	Proton nuclear magnetic resonance
HPLC	High performance liquid chromatography
Ib	Pound
IC_{50}	50% inhibitory concentration
i.e.	*lat. id est* (that is)
in	Inche
IPTG	Isopropyl-β-D-thio-galactoside
IR	Infrared spectroscopy

IRS	Indoor residual spraying
ITNs	Insecticide-treated nets
Lat.	Latitude
LC-MS	Liquid chromatography coupled with mass spectrometer
L	Litre
Log.	Longitude
LLINs	long-lasting insecticidal nets
LSHTM	London School of Hygiene and Tropical Medicine
M	mole per litre
mA	Milli ampere
max.	Maximum
MDR-TB	Multi-drug resistant tuberculosis
MEM	Minimum essential medium
mg	Milligram
MHB	Mueller-Hinton broth
MHz	Mega hertz
min	Minute
MIC	Minimum inhibitory concentration
ml	Millilitre
mM	Millimolar
MRSA	Methicillin resistant *Staphylococcus aureus*
MS	Mass spectrometry
MTT	3-4,5-dimethylthiazol-2-yl-2, 5 diphenyl tetrazolium bromide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCCLS	National Committee for Clinical Laboratory Standards
Ni-NTA	Nickel-nitrilotriacetic acid

nm	Nanometre
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
OCC	Open column chromatography
PEG	polyethylene glycol
pH	Potentium hydrogenii
PMSF	Phenylmethanesulfonylfluride
ppm	Parts per million
RBC	Red blood cells (erythrocytes)
rpm	Revolutions per minute
Sec	Second
SDS	Sodium dodecyl sulphate
SPE	Solid-Phase extraction
TB	Tuberculosis
TEME	Tetramethylethylenediamine
TLC	Thin layer chromatography
TRIS	Tris hydroxymethylaminomethane
U	Unit
VLC	Vacuum liquid chromatography
UV	Ultraviolet
UZL	University of Zambia herbarium
V	Volt
VS	Vanillin sulphuric acid reagent spray
W	Watt
W/V	Weight by volume
WHO	World Health Organization
XDR-TB	Extensively drug-resistant tuberculosis

EVALUATION OF THE *IN VITRO* BIOLOGICAL ACTIVITIES AND PHYTOCHEMICAL PROFILING OF EIGHT *FICUS* SPECIES COLLECTED IN ZAMBIA

1. INTRODUCTION

Infectious diseases account for an overwhelming number of deaths and morbidity worldwide. Infectious diseases that thrive in hot and humid conditions are referred to as tropical diseases and these include malaria, leishmaniasis, schistosomiasis, onchocerciasis, lymphatic filariasis, chagas disease, African trypanosomiasis and dengue (WHO, 2013). These illnesses are caused by pathogenic microorganisms such as viruses, bacteria, fungi, protozoa and other multicellular parasites. Of the main parasites, schistosoma and protozoa cause some chronic disabling conditions termed as neglected tropical diseases (NTDs). These diseases thrive and spread easily in communities with inadequate sanitation, mainly in poor regions of the world. Thus, over one billion people worldwide, mainly in Africa are affected. The most vulnerable groups of people affected by the diseases are young children and pregnant mothers. Over the years, delivery of healthcare has greatly improved, so have the pathogenic microorganisms also evolved to adapt to their ever disturbed environments. This has greatly compromised the advancements that have been made in the diseases management due to efficacious drug interventions. Suffice it to say, the challenge to manage diseases is more pronounced in poor regions of the world where most infectious diseases are prevalent. According to the World Health Organisation (WHO, 2010), these infections contribute to more than 40% of health problems worldwide. As a consequence, rigorous and efficient methods to control the diseases should be established, as the discovery and development of new drugs is key factor to achieving this goal.

Infectious diseases are more widespread and persistent in tropical regions of the world which are endowed with high levels of biodiversity. These are regions that also accommodate most of the developing and poor countries which have inadequate health facilities and services. Zambia falling in this category, poor health is prevalent and diseases such as malaria, meningitis, pneumonia, tuberculosis and gastrointestinal infections have remained a challenge and hence continued to debilitate individuals in most communities. The human immunodeficiency virus (HIV) and Acquired immunodeficiency syndrome (AIDS) epidemics have also exacerbated the situation.

Another factor that contributes to poor health is that many communities especially in rural areas have to travel very long distances to access the most basic healthcare, meaning that complicated and severe infections are mostly never remedied. Hence the use of traditional medicine for primary healthcare is practiced.

The use of plants to alleviate the mentioned diseases has always been practiced worldwide (Heinrich *et al*., 2004). The work presented in this thesis thus involves the screening of crude extracts and subextracts of the aerial plant parts and root barks of eight selected *Ficus* species for antibacterial, antifungal, antimycobacterial and antiparasitic activities. In addition, the extracts were screened for the inhibition of three recombinant FAS-II enzymes and the malaria prophylactic activities. Furthermore, the chemical compound isolation and characterization of the extracts are also presented. This introduction will thus give a brief overview of the pathogenic organisms which were screened for. These include dermatophytes, yeasts, moulds, gram-positive and gram-negative bacteria, mycobacteria, and parasites like protozoa. The brief description of the selected eight *Ficus* species will be outlined.

1.1 FUNGAL INFECTIONS

Mycotic infections have increased over the years because of the increase and widespread use of therapies that depress the immune system such as organ transplants and also the frequent and often indiscriminate use of broad-spectrum antibacterial agents (Johann *et al.*, 2007; Maschmeyer and Haas, 2006; Ghannoum and Rice, 1999). Antibacterial agents generally destroy the body's natural microflora which, inhibit the thriving of certain fungi. In most cases, fungal infections are opportunist and are usually classified by the nature of tissues they colonize. Tissue colonization can be categorized at five levels, which include:

i) superficial colonization which is restricted to the outer layers of the skin and hair
ii) cutaneous colonization which develops a little deeper into the epidermis, hair and nails

iii) subcutaneous colonization which affects the dermis, subcutaneous tissues and muscles
iv) systemic colonization which generally originates from the lungs
v) opportunistic infection which develops because there is already an underlying compromising site where the immune system has been weakened

In this thesis, focus is mostly on communicable fungi from human to human of which the anthropophylic fungal taxa of interest include dermatophytes in particular, members of the genera *Trichophyton* such as *Trichophyton interdigitale* and *Trichophyton tonsurans*, which cause *Tinea capitis* (scalp ringworm). Yeasts from the genus *Candida* and also a mould, *Aspergillus* which is acquired from the environment will be screened.

1.1.1 Dermatophytes

Dermatophytes occur worldwide and are responsible for many fungal infections affecting the skin, nails and hair (Pakshir *et al.*, 2009; Ferńandez-Torres *et al*., 2003). They are a homogenous group of fungi with numerous species belonging to three main genera; *Microsporum*, *Epidermophyton* and *Trichophyton*. Dermatophytes feed on keratinized material for their proliferation, to cause a wide range of clinical symptoms of which dermatophytosis (tinea or ringworm) is one of the most common fungal infections, especially in children (Pakshir *et al.*, 2009).

1.1.1.1 Treatment

There are several types of drugs used to treat dermatophytosis. They include amines, azoles and carbamates. Examples of these drugs are shown in Figure 1. Many infections respond to topical antifungal therapy and the most common azoles used include miconazole, clotrimazole and ketoconazole. Other types of antifungal moieties that have been added to treatment regimens include tolnaftate and terbinafine hydrochloride which are synthetic carbamates and allylamines respectively. However, serious and chronic infections of *Tinea capitis* (scalp ringworm) and *Tinea unguium* (nail) may require systemic treatment with oral medications even though sometimes treatment may fail due to resistance (Pakshir *et al*., 2009).

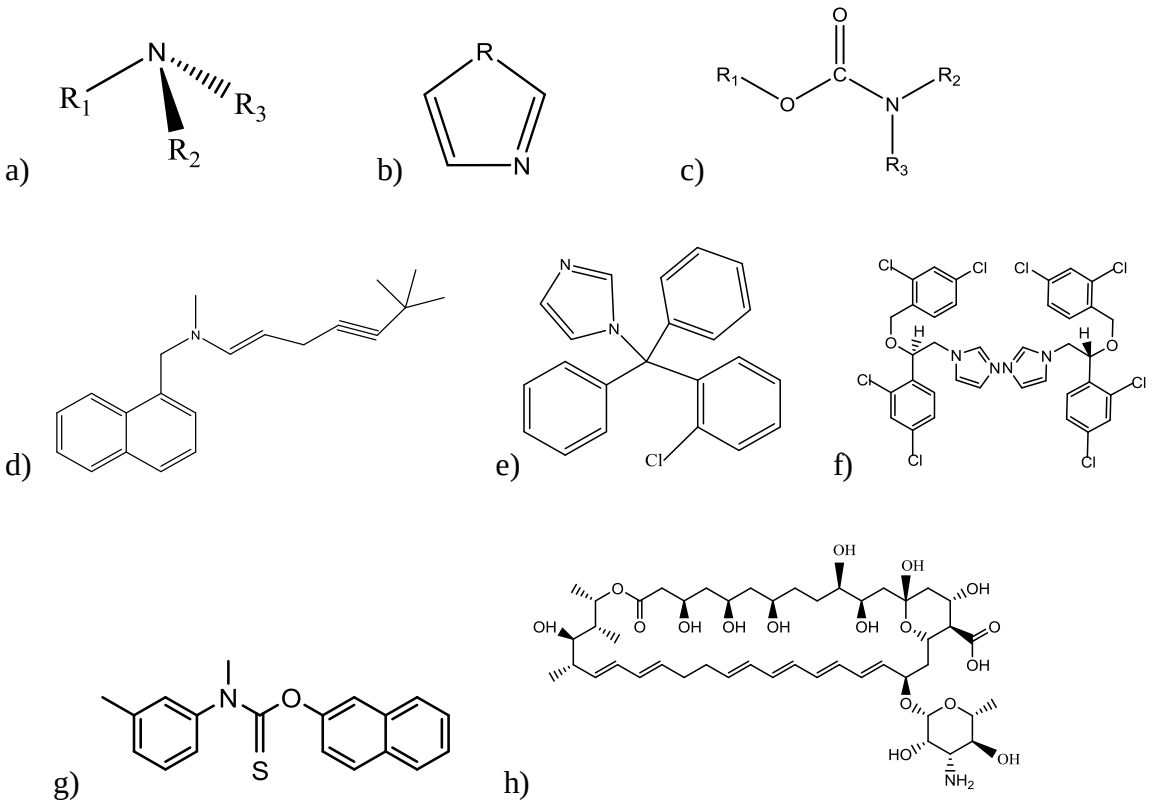


Figure 1: Common antifungal drugs. (a) Amine. (b) Azole. (C) Carbamate. (d) Terbinafine ([(2*E*)-6, 6-dimethylhept-2-en-4-yn-1-yl](methyl)(naphthalen-1-ylmethyl) amine). (e) Clotrimoxazole (1-[(2 chlorophenyl) (diphenyl) methyl]-1*H*-imidazole). (f) Miconazole (*RS*)-1-(2-(2, 4-Dichlorobenzyloxy)-2-(2, 4 dichlorophenyl) ethyl)-1*H*-imidazole). (g) Tolnaftate (synthetic thiocarbamate) (*O*-2-naphthyl methyl-(3-methylphenyl) thiocarbamate). (h) Nystatin

1.1.2 Yeasts (Candidiasis)

Candidiasis is a fungal condition caused by yeasts of the genus *Candida*. The most common species that cause adverse health problems is *Candida albicans*, a diploid fungus (a yeast) and a causal agent for opportunistic oral and genital infections in humans. Systemic fungal infections are one of the main causes of morbidity and mortality in immunocompromised patients infected with HIV and suffering from AIDS, those with cancer and are on chemotherapy, those having to undergo organ or bone marrow transplantation, as well as the elderly (Samie *et al*., 2010; Johann *et al.*, 2007). The extensive use of broad-spectrum antibiotics, corticosteroids and anticancer agents has also added to the increase in yeast infections (Gisela *et al*., 1994), since these agents tend to destroy the natural microflora in the body.

1.1.2.1 Treatment

Candidiasis is mostly treated with antifungal agents that possess an azole moiety (Figure 1b). These include topical agents like clotrimazole and ketoconazole, fluconazole and topical nystatin whose images are shown in Figure 2. There have been several suggestions on the mode of action of azoles. One such established thought is how azoles initially target the heme protein which, together with cytochrome P-450 inhibits 14α-demethylase enzyme to cause the disruption of ergosterol biosynthesis. This leads to the accumulation of toxic sterol precursors, which in turn alter the integrity of the structure and function of the plasma membrane (Ghannoum and Rice, 1999).

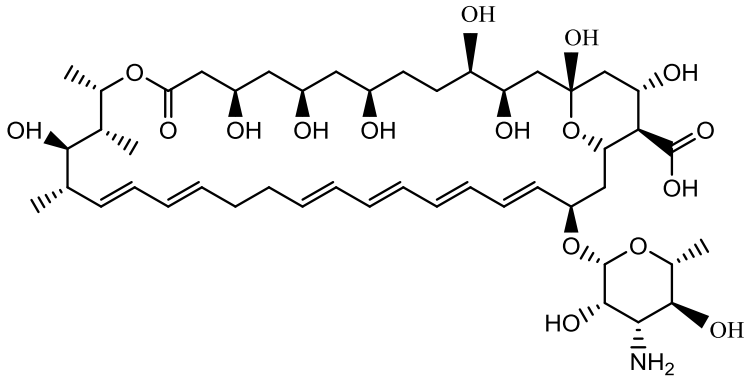


Figure 2: Common drug for candidiasis – Nystatin

1.1.3 Moulds (Aspergillosis)

Aspergillosis is the group of diseases caused by fungi of the genus *Aspergillus*. Aspergillosis infection varies in that it can be due to allegy (e.g asthma or sinusitis) or be invasive due to ones weakened immune system. The most common of these diseases is that caused by the species *Aspergillus fumigatus*, a filamentous fungus which is present in the environment, but does not normally cause illness. Other causes of aspergillosis include *Aspergillus flavus, A. niger* and *A. terreus* (Richardson, 2005). However, diseases surface as a result of immunosuppression in individuals and also in those who already have other lung conditions (Richardson, 2005).

1.1.3.1 Treatment

While the treatment of mycotic infections depends on the tissues affected, many are treated with drugs that have an azole moiety. Of the most widely used are members of the triazole family (Figure 3a), and voriconazole (Figure 3c) is the first-line drug used for invasive aspergillosis (Denning and Hope, 2010). Triazoles have been useful owing to their wide spectrum of activity in that they are effective against invasive aspergillosis, candidiasis, some dermatophyte infections and other endemic mycoses. Also triazoles are said to have potent pharmacokinetic profiles which include rapid absorption and oral bioavailability of more than 90%, and to top it all, it is well tolerated with mild side effects (Maschmeyer and Haas, 2006).

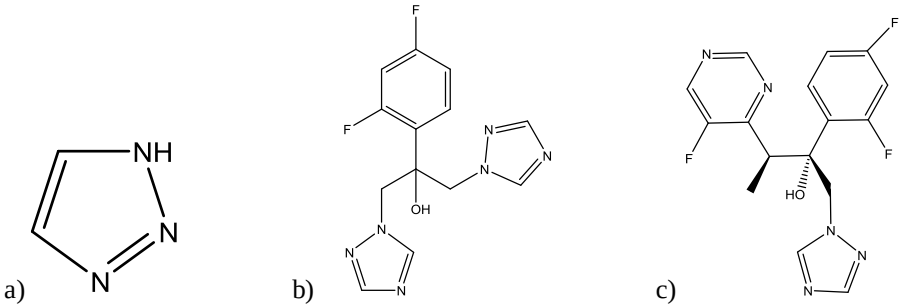


Figure 3: Common drugs for Aspergillosis. a) Triazole moeity. b) Fluconazole. c) Voriconazole

1.2 BACTERIAL INFECTIONS

1.2.1 Bacterial infections

Bacteria are part of the normal human microflora and are beneficial to the body existing on the skin and mucous membranes without causing any harm. However, when allowed entry into the body, or the immune system of the individual fails to fight off the invasion, bacteria becomes pathogenic and thereby causing deseases. Diseases caused by bacteria can include skin infections, chest infections, cerebral and food-borne illnesses.

Bacteria are normally classified according to the structure of their cell walls. In this case, they can be classified as; gram-positive bacteria which have up to about twenty times as much murein or peptidoglycan in their cell walls compared to gram-negative bacteria, the second class. The classification thus relies on the positive or negative results from Gram's staining method, which uses complex purple dye and iodine. Gram-positive bacteria are identified when they retain the dye due to the many layers of complex polymers of amino sugars which are cross-linked to the amino acids alanine, glutamate, lysine or diaminopimelic acid.

1.2.1.1 Treatment

Bacterial infections are treated with a wide range of drugs, and due to the inappropriate and irrational use of some of the drugs, favourable conditions for resistant strains to emerge have been provided. Over the years, bacterial strains have developed new resistance mechanisms which have made them less susceptible to antibacterial drugs, making it difficult and expensive to treat bacterial infections (WHO, 2013). As a result high percentages of hospital-acquired infections have been caused by the resistant bacterial strains (WHO, 2013).

The treatment for MRSA includes glycopeptides such as vancomycin and teicoplanin. These act as inhibitors to the second stage of bacterial cell wall synthesis and it is also evidenced that vancomycin selectively inhibits ribonucleic acid synthesis by altering the permeability of the cell membrane (Watanakunakorn, 1984). Linezolid (Figure 4) is a

newer antibiotic that is the first available oxazolidinone antibacterial agent which disturbs the protein synthesis by interfering with the binding of messenger ribonucleic acid (mRNA) to ribosomal ribonucleic acid (rRNA) during the initiation stage of translation (Shinabarger *et al*., 1997). It is active against MRSA and is a potent option to vancomycin which normally causes renal toxicity in some patients and is poorly absorbed when intravenously administered (Tsiodras *et al*., 2001). Daptomycin, a cyclic lipopeptide antibacterial agent is another drug considered potent in the treatment of MRSA infection and it is believed to have a dual mechanism of action, one of which is the inhibition of cell wall synthesis and the other is to establish multiple ion gradients across the cytoplasmic (Streit *et al*., 2005).

Antibiotic resistance is one of the world's most pressing public health problems because there is a high proportion of resistance worldwide (WHO, 2014; Cornaglia, 2009). There is thus an urgent need to discover new generation of antimicrobial compounds which have different chemical structures and perhaps novel mechanisms of action to treat the new emerging infectious diseases.

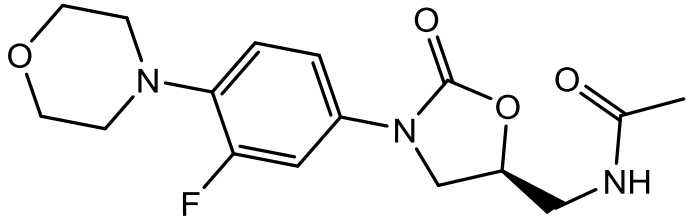


Figure 4: Antibiotic – Linezolid

1.2.2 Tuberculosis

Tuberculosis (TB) is the second most infectious disease after HIV/AIDS with a high morbidity and mortality (WHO, 2012). It is a contagious bacterial infection that mainly affects the lungs, but can spread to other organs. TB is caused by *Mycobacterium tuberculosis*, an obligate aerobic pathogen that is spread by coughs or sneezes of infected persons and be inhaled by uninfected subjects. On first contact with the pathogenic bacteria, also known as primary TB infection, the person may not develop the symptoms; however, the bacteria can remain dormant in the body. This is referred to as latent TB. About one-third of the world's population asymptomatically harbors the latent TB which, can be reactivated anytime (Koul *et al*., 2011). In most cases, however, the latent TB is activated when the body's immune system weakens causing characteristic symptoms of the disease to appear. Such symptoms may include chest pains which maybe accompanied by dyspnea, persistent cough (which may produce phlegm), or coughing up blood, night sweats, tiredness, fever, loss of appetite and drastic weight loss.

According to the updated WHO (2012) report about 8.6 million people suffered from TB, while 1.3 million died from it in 2012. This record is, however, a decline in comparison to the 1.4 million people who died from the scourge in 2011, and a further decline from the 1.7 million who died in 2009 with the highest number of deaths occurring in Africa (WHO, 2012 and 2010). As much as TB is more prevalent in the world today than it has ever been (Koul *et al*., 2011), the diagnosis and treatment strategies have helped in reducing deaths. However, the prevalence is compounded by the worldwide emergence of multidrug-resistant (MDR) strains which has seen an increase by 10 000 infections to approximately 450,000 new cases of MDR-TB emerging in 2012, with one third of the world's total population being infected with TB *bacilli* (WHO, 2014 and 2010). One in every 10 of those people will become sick with active TB in their lifetime (WHO, 2010). People living with HIV are at a much greater risk of contracting TB because HIV associated TB and multidrug-resistant-TB are harder to diagnose and cure (WHO, 2010), leading to a higher number of deaths as a result of the infection.

1.2.2.1 Treatment

The treatment of Tuberculosis is done in two phases. The treatment regimens for TB constitute a combination of four drugs (Figure 5) which include isoniazid, rifampicin, pyrazinamide, and either streptomycin, ethambutol or ethionmide as secondary drugs for the first two months. Then then a follow up retreatment (second phase) with isoniazid and rifampicin for a further four months is done. Although the combination regimen has been effective even against resistant forms (MDR-TB), non compliance to the treatment has resulted in some extensive multiple resistant strains (XDR-TB) of *M. tuberculosis* to surface as has been identified in 92 countries (WHO, 2014), which are even more challenging to treat. Hence the imperative need for the discovery of new anti-TB drug regimens.

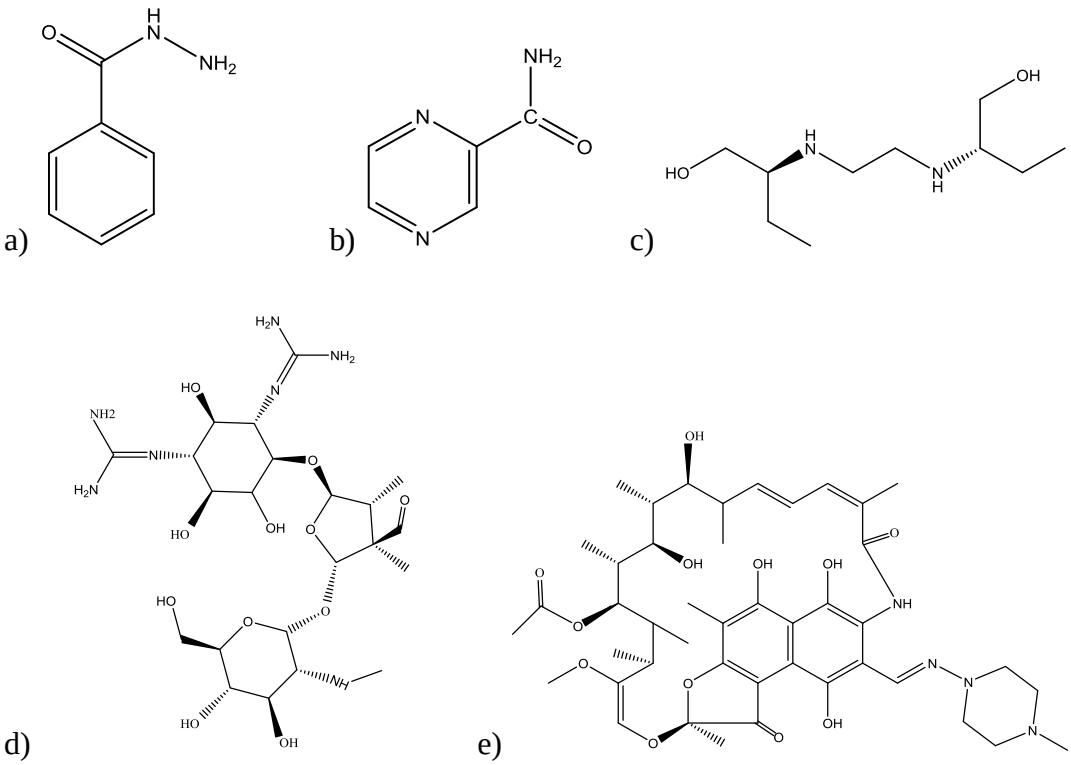


Figure 5: Drugs used for treatment of Tuberculosis. (a) Isoniazid. (b) Pyrazinamide. (c) Ethambutol. (d) Streptomycin. (e) Rifampicin

1.3 PARASITIC INFECTIONS

Parasitic diseases are infection caused by parasites. Parasites are organisms that live in another organis (host) often causing damage to it. They can either live inside the body of the host as endoparasites, or live on the outer surface of the host body as ectoparasites. Endoparasites such as helminths and protozoa constitute the major causes of diseases to humans in most affected regions of the World. Helminths can be in the form of roundworms (nematodes), flatworms (trematodes) and tapeworms (cestodes), while there are many genera of protozoans which are pathogenic to humans. In this thesis, the focus will be on helminths that belong to the genus *Schistosoma* and protozoans of the genera *Leishmania*, *Trypanosoma* and *Plasmodium*.

1.3.1 Schistosomiasis

Schistosomiasis, also called bilharzia is one of the tropical diseases caused by digenetic trematodes of the genus *Schistosoma*. It is prevalent in about 74 nations mostly those occurring in tropical and sub-tropical regions of the World, with Africa having the largest disease cases (WHO, 2013; Chitsulo *et al*., 2000). According to the WHO report (2013), over 240 million people were infected worldwide (highest rates in children) in 2012, with highest prevalence in the tropical and sub-tropical regions (Gryseels *et al*., 2006). Sub-saharan Africa in particular records more than 200 000 deaths annually due to schistosomiasis (WHO, 2013). The infection thrives mostly in densely populated and poor communities which have inadequate sanitation (WHO, 2013). The World Health Organisation actually describes it as 'a disease of poverty'. Schistosomiasis is highly prevalent even though it has variable and low morbidity (Gryseels *et al*., 2006).

There are five main members of this genus that cause schistosomiasis, which is in two forms. One is the intestinal schistosomiasis caused by *Schistosoma mansoni, Schistosoma japonicum, Schistosoma mekongi* and *Schistosoma guineensis*, and the other is the urogenital schistosomiasis mainly caused by *Schistosoma haematobium* found in Africa and the Arabian Peninsula (Gryseels *et al.*, 2006).

Transmission of schistosomiasis occurs through the skin. Thus infectious larval forms (cercarie) of the parasitic blood flukes when released by freshwater snails penetrate the

skin of people who have been exposed to the contaminated water. While in the skin the cercarie transform into schistosomula and then migrate to the liver where they mature into adult worms. The microscopic adult worms live in the veins where the females produce eggs which are then shed through the intestines and the urinary tract (Coultas and Zhang, 2012). Most of the eggs they lay are trapped in the tissues and the body's reactions to them cause massive organ damage, to which the schistosome maturation and fecundity depend on this immune response (WHO, 2013; Pearce and MacDonald, 2002). Meanwhile, some of the eggs are excreted in the feaces or urine to continue their life-cycle, and they can remain viable for up to 7 days once excreted.

1.3.1.1 Treatment

Schistosomiasis is one of the greatest neglected tropical diseases and is currently being treated and managed with Praziquantel, as recommended by the World Health Organisation (WHO, 2013). This drug is safely co-administered with albendazole or ivermectin, in areas where these drugs have been used separately for preventive chemotherapy. Praziquantel is an acylated quinoline-pyrazine (Figure 6), which has little or no effect on larvae and immature worms, but has a broad spectrum of activity against all mature human trematodes and cestodes (Doenhoff and Pica-Mattoccia, 2006). The effect that Praziquantel is believed to have on the worm, is that it increases the cell permeability to calcium in schistosomes. This then causes strong contractions and paralysis of the worm's musculature, leading to detachment and dislodgement of the suckers from the blood vessel walls. The molecular target of Praziquantel has however, remained unknown even though it has been used for over 20 years (Aragon *et al*., 2009).

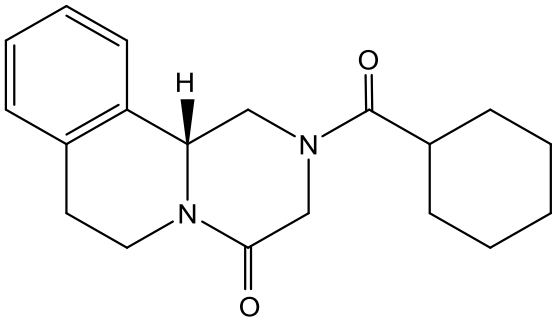


Figure 6: Common drug for schistosomiasis - Praziquantel

1.3.2 Protozoal Infections

Protozoal infections are caused by single celled parasites called Protozoa. These parasites predominantly occur in the tropical and sub-tropical regions of the world to cause a lot of debilitating diseases (Wright and Phillipson, 1990). The most widespread and common protozoal diseases include leishmaniasis, trypanosomiasis and malaria which is the most troublesome.

1.3.2.1 Leishmaniasis

Leishmaniasis is caused by parasitic protozoa that belong to the genus *Leishmania*. It occurs in about 88 countries, many of which are in tropical and sub-tropical regions. Members of this genus are intracellular pathogens which affect macrophages and dendritic cells. The parasite is transmitted by the bite of a blood-sucking female sandfly of the genus *Phlebotomine* (family: Psychodidae). Of the five hundred known species of *Phlebotomus*, only about thirty are vectors of leishmaniasis (WHO, 2012; Anderson, 2003).

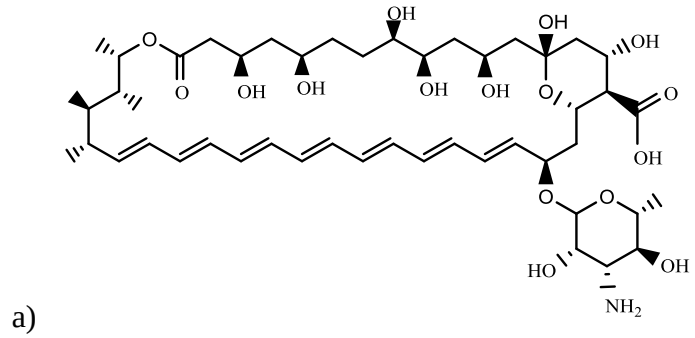
There are four main types of leishmaniasis. These include visceral leishmaniasis, cutaneous leishmaniasis, diffuse cutaneous leishmaniasis and mucocutaneous leishmaniasis. Visceral leishmaniasis, also called kala-azar is caused by *Leishmania donovani* and is the most fatal of the four types. It is characterized by weight loss, high temperatures, anemia and hepatosplenomegaly. The disease develops in a short period of time such that, patients can die within months of getting infected unless they receive treatment. It is widespread, occurring in many parts of the world and affects millions of people (Tasdemir *et al*., 2005).

1.3.2.1.1 Treatment

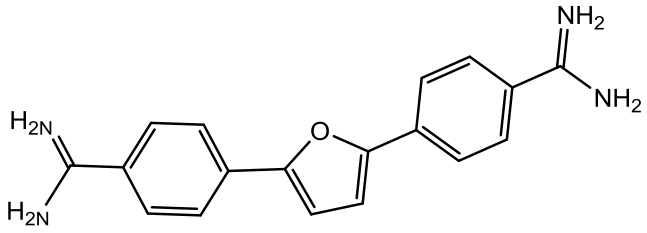
Leishmaniasis is a difficult disease to manage and treatment is quite limited. The drugs normally used for treatment include antimony derivatives, diamidines and amphotericin B (

Figure 7). For instance visceral leishmaniasis can be treated with antimonides such as sodium stibogluconate (Pentostam®) and meglumine antimoniate (Glucantim®). However, there is growing resistance against sodium stibogluconate. In addition, the

period of treatment is relatively long and painful, and it can cause undesirable side effects (Witschel *et al.*, 2012). Most of the anti-protozoal drugs are inadequate because of their toxicity, lack of efficacy and inability to elimimnate all life cycle stages of the parasites from the host (Tasdemir *et al*., 2005).



a)



b)

Figure 7: Drugs for Leishmaniasis. a) Amphotericin B. b) Diamidine furamidine

1.3.2.2 Trypanosomiasis

Trypanosomiasis is a vector-borne parasitic disease caused by pathogenic protozoa belonging to the genus *Trypanosoma.* It manifests in many forms depending on the *Trypanosoma* species involved. The most common is the human African Trypanosomiasis, also known as African sleeping sickness, which is transmitted to humans by a bite of various species of tsetse flies belonging to the genus *Glossina*, which are endemic only to sub-saharan Africa (WHO, 2013), but have spread to other parts of Africa that have accommodated the species with favourable conditions for survival . Sleeping sickness is thus caused either by *Trypanosoma brucei gambiense* or *Trypanosoma brucei rhodesiense.* The former occurs mainly in west and central Africa

and accounts for 95% infections (WHO, 2012). Meanwhile, the latter occurs in eastern and southern Africa, representing 5% of cases. Another type of trypanosomiasis is caused by *Trypanosoma cruzi* and it occurs in the Latin American countires. It is transmitted to humans by blood-sucking triatomine bugs endemic to those countries. It is called American trypanosomiasis or Chagas disease and is characterized by neurological disorders, including dementia, damage to the heart muscle leading to cardiac failure, sometimes dilation of the digestive tract and weight loss in its chronic form.

1.3.2.2.1 Treatment

There are four registered drugs of choice for the treatment of African sleeping sickness. These are pentamidine and suramin which are used in the early stage of the infection, that is when the parasites are only restricted to the blood and lymph (Witschel *et al*., 2012). Pentamidine is used for the treatment of *T. brucei gambiense* while Suramin for the treatment of *T. brucei rhodesiense*. The other two drugs are Melarsoprol (Figure 8) and Eflornithine which are used in the advanced-stage of the disease, which includes the parasite's invasion into the central nervous system (CNS) (Hubert and Barrett, 2001). The two latter are most effective for complicated infections against all *Trypanosomal* infections. Melarsoprol is an organoarsenic drug that targets the parasite by crossing the blood-brain barrier. It is however, very toxic and quite difficult to administer because it is only by intravenous means. Because of its toxicity, Melarsoprol causes some serious unwanted effects among others encephalitis in about 10% of patients and about 5% die as a result (Witschel *et al*., 2012; Wright and Phillipson, 1990). With these limitations, there is need for more less toxic drugs.

Meanwhile, Chagas disease caused by *T. cruzi* has two first line drugs available for treatment. These are Nifurtimox and Benznidazole (Figure 8), which like Melarsoprol have limitations such as toxicity, poor drug activity, and possible emerging parasite resistance (WHO, 2012). On top of that, they are also poorly tolerated and the length of time needed to complete the course is long (Witschel *et al*., 2012). Furthermore, treatment is really only effective when given during the acute stage of the disease, which maybe detrimental to the patient. There has been recently introduced a combination

treatment of nifurtimox and eflornithine even though it is only effective against *T. brucei rhodesiense* (WHO, 2012).

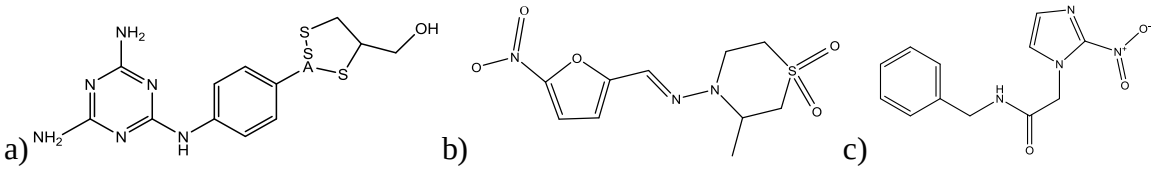


Figure 8: Common drugs for trypanosomiasis. a) Melarsoprol. b) Nifurtimox. c) Benznidazole

1.3.2.3 Malaria

Malaria is another infectious disease that remains a serious global health problem (Wright, 2010). At least one in every five (20%) children is affected by the disease, such that an African child suffers from malaria about 2 to 5 times every year (WHO, 2009). Being the most prevalent and deadly parasitic disease, some successes in terms of transmission reduction have been recorded. There has been a reduction of malaria cases worldwide as can be observed by World Health Organisation figures from 2011 to 2012. According to the WHO (2014) estimates, malaria cases reduced from about 219 million in 2011 to about 207 million in 2012, and the mortality rates were recorded at about 627 000 during the same period. Malaria is prevalent in over 100 countries in tropical and sub-tropical regions of the world as can be seen in Figure 9A (WHO, 2010).

In Africa, malaria is not only a health problem, but also an economical and food security challenge as it consumes up to 40% of public health expenditure in most affected areas (Kitua *et al*., 2011). In Zambia, as it remains the number one killer disease, especially to children under the age of five, pregnant mothers and immune compromised individuals, with the highest transmission rate falling within the season of cultivating the staple food (between the months of November and early April), the disease is a burden and household food security is heavily affected.

The clinical manifestations of malaria are caused by the blood stage parasites. They include periodic episodes of chills, fever followed by intermittent sweating which are usually accompanied by headache, nausea and vomiting. These symptoms come about when some toxic elements and hemozoin pigments accumulate in the infected erythrocytes during the parasites' development. When these erythrocytes lyse, they release into the blood stream invasive merozoites and the toxic elements which induce cytokines and other tumor necrosis factors that cause the symptoms. Thus the destruction of the red blood cells leads to anemia and hypoxemia (Kaur *et al*., 2009). The effects include hypoglycemia and cerebral complications, which if left untreated can cause death.

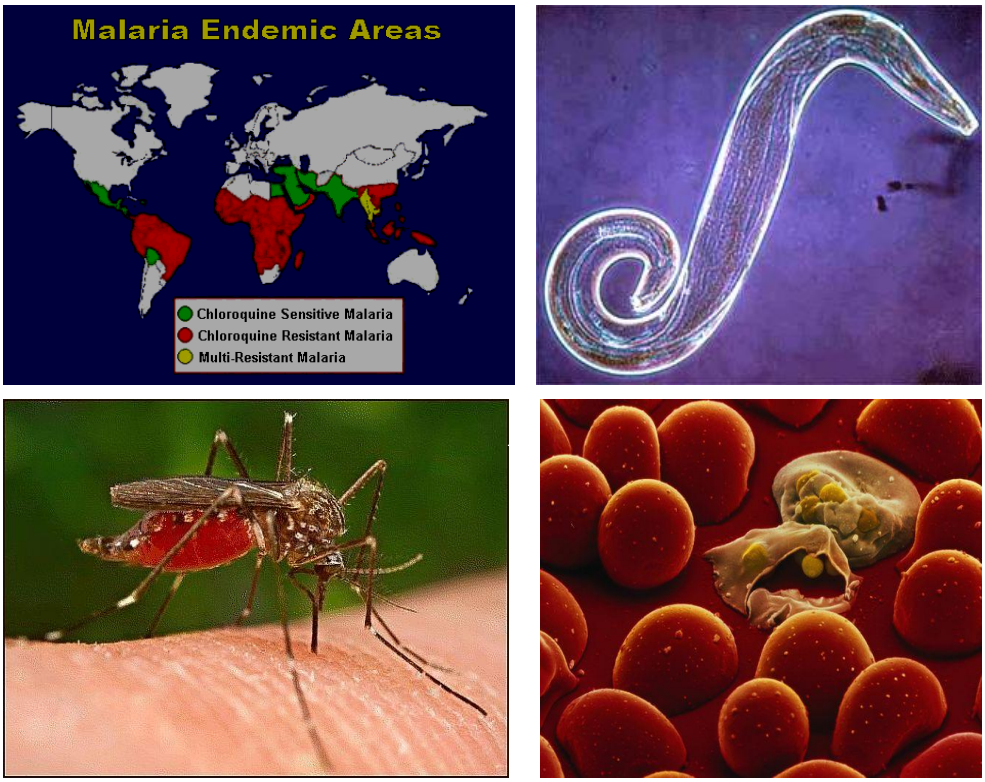


Figure 9: A) World distribution map of Malaria. B) *Plamsodium* species. C) A mosquito having a blood meal. D) Schizontes infected erythrocytes.
(http://www:traveldoctor.co.uk/malaria.htm;
http://www.primehealthchannel.com/mosquito bites pictures
http://www.henry4school.fr/Body_and_Health/health/malaria.htm)

1.3.2.3.1 Plasmodium

Malaria is caused by single-celled organisms that penetrate host tissues to obtain food. These are parasitic organisms called protozoa which belong to the phylum Apicomplexa, which holds more than 5,000 species (Surolia *et al*., 2002). The most documented apicomplexans are malaria parasites of the genus *Plasmodium* (Figure 9B), of which five species cause malaria. These include; *Plasmodium falciparum, Plasmodium vivax, Plasmodium ovale, Plasmodium malariae* and *Plasmodium knowlesi*. The most common ones being *P. falciparum* which causes malignant malaria and *P. vivax* causes benign malaria, even though *P. falciparum* is the most predominant and deadly parasite causing the most malaria-linked deaths especially in sub-Saharan Africa (Wells *et al*., 2009; WHO, 2012).

1.3.2.3.2 The life cycle

The life cycle of the *P. falciparum* is completed in two hosts, the human host for the asexual stage and the mosquito host for the sexual stage (Figure 10). The parasite is transmitted into the host through a bite by a female mosquito, mostly the *Anopheles gambiae* and other species, in which hundreds of sporozoites are released into the bloodstream of the host to infect the hepatic parenchymal cells. The invasion of the hepatocytes is referred to as the liver stage malaria. In the liver the sporozoites develop into merozoites and a period of massive growth ensues to produce more than 10,000 per liver schizont (Singh *et al*., 2009; Spalding and Prigge, 2008). This liver stage also called the exoerythrocytic stage is asymptomatic and lasts between 2-16 days after the bite, depending on the *Plasmodium* species. It takes about 6-7 days in *P. falciparum* (Tasdemir *et al*., 2011). Upon maturation, the merozoites invade erythrocytes where they periodically replicate asexually while, at the same time progressively breaking down the red blood cells (Figure 9D). This causes episodes of fever, chills and anaemia in the infected individual. This is the symptomatic stage and the cycle is completed within 48 hrs for *P. falciparum* and *P. vivax*, 72 hrs for *P. malaraie* and 24 hrs for *P. knowlesi* (Wells *et al*., 2009). In severe cases however, blood vessels in the brain get obstructed by infected red blood cells to cause the deadly cerebral malaria. In all this, some merozoites differentiate to form male and female gametocytes, which are then taken up by the female

mosquito during a blood meal (Figure 9C) to continue its life cycle in its second host (the mosquito). In this form they can also live quiescently in the bloodstream for weeks.

Whilst in the mosquito gut, the female and male gametes develop and then fuse to form diploid ookinetes. These then, migrate to the midgut and pass through the gut wall to form oocyts. After a meiotic division, sporozoites are born, which migrate to the salivary glands waiting to be deposited into the human host to start the cycle over again.

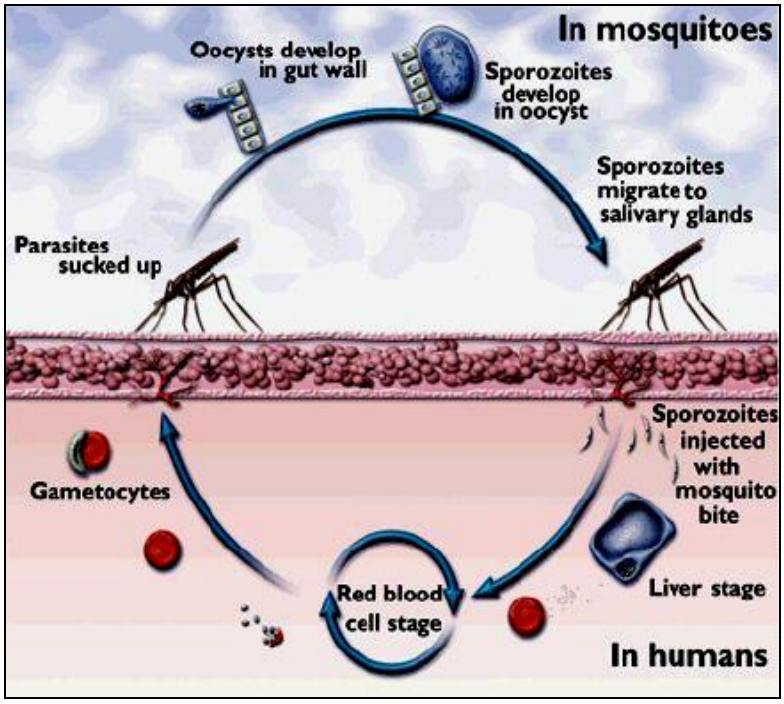


Figure 10: Malaria parasite's life cycle in the humans and in the *Anopheles* mosquito (Winzeler, 2008).

1.3.2.3.3 Malaria Prophylaxis

Malaria prophylaxis is not absolute as infection can still occur. This is because the complicated life cycle of the *plasmodium* parasite in two hosts has made it difficult to develop vaccines against malaria (Wells *et al*., 2009). Thus protection against infected mosquito bites would be the best option, as there is no long term prophylaxis available yet. However, there are some vaccines that are currently under phase 3, phase 2 and phase1 clinical trials (WHO, 2011). Malaria prophylaxis can be divided into two categories; suppressive prophylaxis and causal prophylaxis. The former is when drugs

eradicate and suppress blood stage parasites from causing symptoms, while in the latter; parasites are eradicated at liver stage, thus preventing the symptomatic blood stage infection.

Suppressive prophylaxis

The World Health Organization (WHO, 2010) recommends the combination of Atovaquone with proguanil as suppressive prophylaxis for travelers. Atovaquone is very effective but quite expensive to manufacture, hence it may not be susutainable to poor countries where malaria is most prevalent (Leitao and Rodriguez, 2010). Other chemoprophylactics that have been used include chloroquine, mefloquine and doxycycline (Well *et al*., 2009).

Causal prophylaxis

The only licensed drugs available to kill dormant liver stages and gametocytes for causal prophylaxis are 8-aminoquinolines, in particular Primaquine and tefenoquine (Leitao and Rodriguez, 2010). Since the parasitic load in the liver tends to be low, the population would take a bit longer to multiply to massive loads thereby reducing the chances of drug resistant forms from developing. However, some of the 8-aminoquinolines are reported to be toxic, poorly complied to when taking them, and to add on causes hemolytic anaemia in patients with glucose-6-phosphate dehydrogenase (G6pDH) deficiency (Leitao and Rodriguez, 2010; Singh *et al*., 2009; Wells *et al*., 2009). This enzyme deficiency is a red cell polymorphism that is common to populations in Africa, Asia and the Mediterranean regions; in essence the malaria bug-ridden regions, because it is associated with some form of immunity against the malaria disease (Ruwende *et al*., 1995; Allison and Clyde, 1961). Primaquine is effective for liver stage parasites and radically cures *P. vivax* infection (Winzeler, 2008). Mefloquine is a drug with a long half-life and can thus provide effective prophylaxis when administered weekly; but is not well tolerated in that it has unpleasant side effects of gastrointestinal and psychiatric effects (Well *et al*., 2009).

1.3.2.3.4 Treatment

Malaria has been treated with a wide range of drugs. There have been successes in the treatment for as long as resistant parasitic strains have been at bay. The types of drugs that have had success stories include quinine and its derivatives such as chloroquine; antifolates such as sulfadoxine/pyrimethamine and the current artemisinin in combination with other agents, lumefantrine being the most common. However, some earlier generation medicines like chloroquine and antifolates such as sulfadoxine/pyrimethamine have developed some resistance against the parasites, but they are still being used in some regions. For instance, the antifolate, sulfadoxin/pyrimethamine is still being used in Zambia in rural health centers and also for prophylaxis in pregnant mothers. Their continued use is however, is risky because the development of resistant strains may not be noted easily in areas where healthcare provision is already inadequate (WHO, 2010).

Quinine

Quinine (Figure 11A) was the first widely used antimalarial drug for complicated malaria for some time (Wells *et al*., 2009). It is a natural product extracted from the bark of the trees of the genus *Cinchona* L. (Rubiaceae). Members of the genus which are sources of quinine include; *Cinchona calisaya* Wedd., *C. ledgeriana* Moens ex Trimen and *C. pubescens* Vahl. amongst others (van Wyk and Wink, 2004). The use of the *Cinchona* bark has been known as far back as the 17th century, and since then, it has been the only source of quinine (Adams *et al*., 2011). Even though, resistance has been reported, it has remained useful in the treatment of complicated malaria. More importantly, quinine has been used as a scaffold for the production of other successful synthetic derivatives such as Chloroquine and Mefloquine, which are also still being used in some regions. Other derivatives include 8-aminoqionoline primaquine and 4-aminoquinoline chloroquine which are also used for prophylaxis. In addition chloroquine-like drugs such as lumefantrine, piperaquine, pyronaridine, amodiaquine, mefloquine and halofantrine have been produced and are being used mostly in combination with other drugs (Wells *et al.*, 2009).

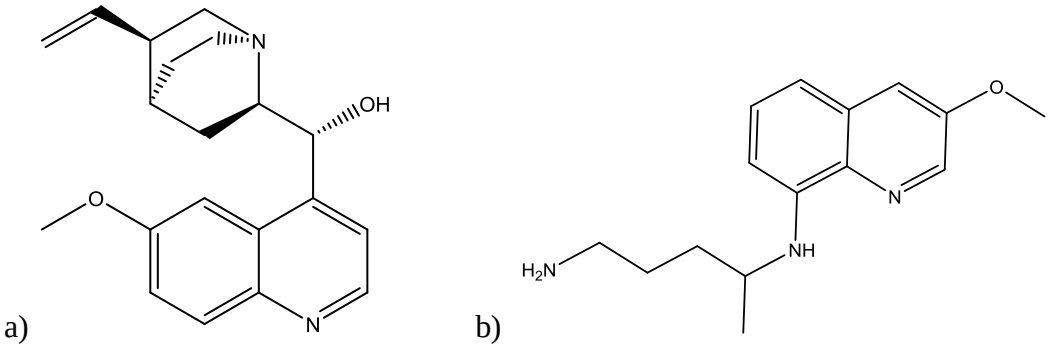


Figure 11: Quinine and its derivatives (a) Quinine. (b) Primaquine

Fansidar

Fansidar (SP), a drug that contains sulphadoxine (slow eliminated sulphonamide) and pyrimethamine (a slow-acting 2, 4-diamino-sulphadoxine) which eliminates the blood schizontes (

Figure 12) has been used widely. Unfortunately, resistance for this drug arose quite rapidly (Wells *et al.*, 2009), even though it is still being used, mostly for prophylaxis. These two drugs belong to the group of antifolate drugs, which interfere with nucleic acid synthesis, by blocking enzymes involved in the synthesis of folate in the cytoplasm of the parasite (Brown, 1971; Ferone, 1970).

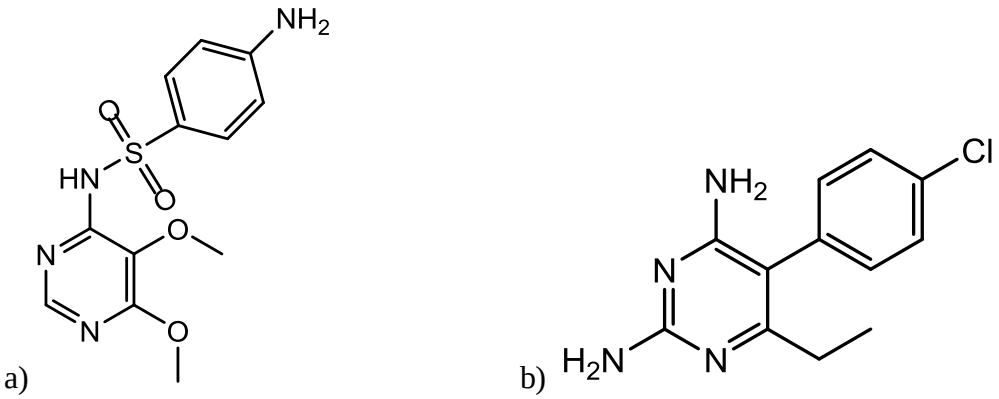


Figure 12: Antifolate antimalarial drugs. (a) Sulfadoxine. (b) Pyrimethamine

Malarone

Malarone is another combination drug of choice which constitutes proguanil and atovaquone (Figure 13) and is used to treat acute uncomplicated malignant malaria. It is also used for causal prophylaxis because it is effective against both drugs sensitive and drug resistant prarasitic strains. Malarone works as a blood schizonticide and transmission blocker in particular; affecting the biosynthesis of pyrimidines required for nucleic acid replications (Wells *et al*., 2009). The asymptomatic liver stage has emerged as a good causal prophylactic target because the interference of this phase would prevent the symptomatic stage, hence reducing the chances of parasite transmission because the production of gametocytes is interrupted. Additionally, the low parasitic load with limited multiplication significantly reduces the likelihood for drug resistant strains to develop. One further area which is important for targeting the liver stage is the elimination of schizontes for *P. vivax* and *P. ovale*, which remain dormant in the hepatocytes for several years and they usually cause relapses. Theses are referred to as hypnozoites. Malarone unfortunately has not been found to be effective against these *Plasmodium* species.

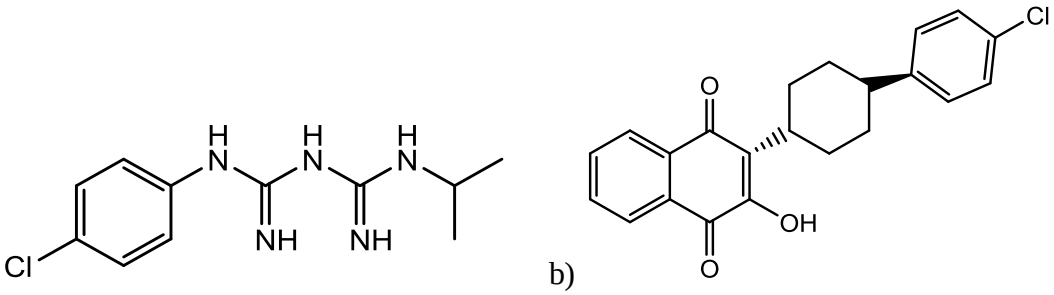


Figure 13: Prophylactic malarial drugs. (a) Proguanil. (b) Atovaquone

Artemisinin combination therapies

Artemisinin is sourced from the aerial parts of *Artemisia annua* (commonly known as sweet wormwood) which belongs to the family Asteraceae. Artemisinin was first isolated in 1972 and characterized in 1979 (Golenser *et al*., 2006). It is the current effective treatment for malaria, however, in combination with other chemical moieties. Thus artemisinin-based combination therapies (ACTs) (

Figure 14) are the first line treatment options recommended by the World Health Organization in 2005 (WHO, 2009). The five ACTs that are recommended include; artemether-lumefantrine (Coartem), artenusate-mefloquine, artesunate-amodiaquine (Carsucam or ASAQ), artesunate-SP and DHA-piperaquine (EUrartesim or Artekin). Artemisinin (qinghaosu) is a sesquiterpene trioxane lactone which contains an endoperoxide bridge known to be essential for activity. However, the most effective antimalarial principle for the treatment of *P. falciparum* is in the form of dihydroartemisin (DHA) and its semisynthetic derivatives artemether and artesunate (White, 2008). Artemisinin in its pure form has poor oral availability, poor solubility both in water and oil, and is less active (Held *et al*., 2011). Meanwhile, when reduced, its active form, DHA is generated. This form is very potent as it is readily absorbed, is fast acting and can reduce the parasite load by a factor of approximately 10, 000 per asexual cycle or below detectable levels compared to 100 –1,000-fold for other antimalarials (WHO 2010; Willcox, 2009). The rapid parasite load reduction in turn reduces the rate of parasite transmission and results in the shortest fevr clearance times (Well *et al*., 2009), a characteristic that can be used as an indicator for artemisinin resistance (White, 1997). Other than its rate of activity, DHA, exhibits an unusual broad spectrum of activity against all the asexual blood stages of the parasite from young rings to schizonts and to gametophytes in *P. faciparum* malaria (Wells *et al.,* 2009; Golenser *et al*., 2006).

With all the rich activity profile for DHA, it still cannot be used as a monotherapy, because it has a very short half-life and short elimination half-time (about an hour), hence not all the parasites are likely to be eliminated within that time. This factor unfortunately increases the chances of recrudescence (Willcox, 2009; Golenser *et al*., 2006). Consequently, to continue keeping DHA's profile viable, a second slow-acting drug is

usually combined with it to completely eliminate all the remaining parasites including possible arisen artemisinin-resistant ones, thus preventing the re-emergence of the disease and that of resistance (WHO, 2010; Wells *et al.*, 2009).

The mechanism of action of artemisinin and its derivatives is still not very clear. However, there are several targets that have been suggested for the mode of action. They have been attributed to interference of the parasite transport proteins, in particular the only plasmodial sarcoplasmic/ endoplasmic calcium ATPase gene (SERCA-*pf*ATPase6) (Chaponda *et al*., 2009; Golenser *et al*., 2006), and disruption of parasite mitochondrial function by inhibiting the respiratory chain (Krungkrai *et al*., 1999; Li *et al*., 2005), modulation of host immune function, inhibition of angiogenesis and heme polymerization.

Artemisinin combination therapies, like other antimalarial drugs have not being spared from parasitic resistant strains emerging. Four Southeast Asian countries (the Thai-Cambodian border, Myanmar, Thailand and Vietnam) have reported emergency of resistance (WHO, 2010; Al-Adhroey *et al*., 2010). To add on, the production of artemisinin is not cost effective as the yields are very low, less than 2% of dry plant material. This inevitably raises isolation and purification costs (Weathers *et al*., 2011). With the highest malaria mortality rate being recorded in countries with high poverty levels, it therefore, becomes an expensive drug both to manufacture and to use. It is therefore, imperative to discover more drug targets and agents to manage the disease before the present drugs become resistant and lose efficacy.

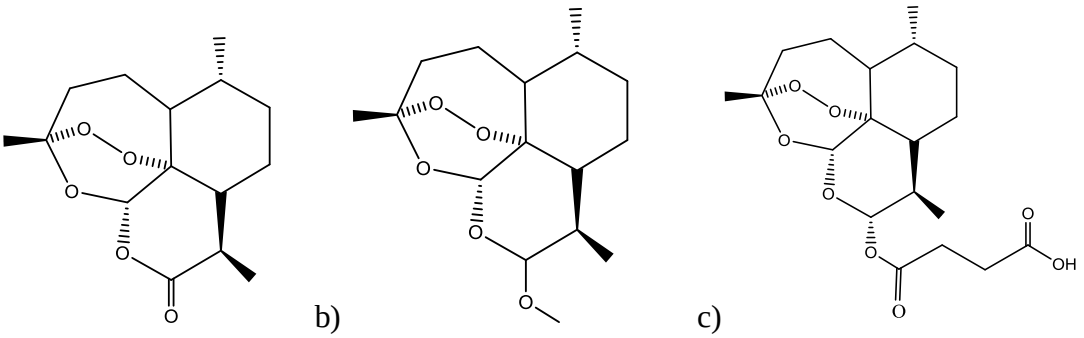


Figure 14: Blood stage malaria drugs. (a) Artemisinin. (b) Artemether. (c) Artenusate

1.3.2.3.5 Prevention

Malaria is treatable and is also preventable. The prevention of malaria can be at different levels. Firstly, personal protection against mosquito bites presents the first line of malaria prevention. This can be done by applying mosquito repellants to the body, spray insecticide in the room where you are or close windows before dusk. Secondly, at community level, vector control is the main way to reduce malaria transmission (WHO 2011). The insecticide-treated mosquito bed nets (ITNs) and indoor residual spraying (IRS) are two main forms of vector control that have so far been quite effective at community level (Ranson *et al*., 2011). Nowadays, there are even insecticide-treated mosquito window laces and mosquito repellant wall paint (personal observation). Of the ITNs for public health distribution programmes, the long-lasting insecticidal nets (LLINs) are the preferred and recommended ones by WHO (2011), the custodians of health matters worldwide. These nets are mainly distributed for free to vulnerable individuals, who include children under the age of five and pregnant mothers.

The other vector control intervention is the indoor residual spraying (IRS) with insecticides. It is a powerful way to reduce the transmission of parasites since the vectors are removed from the environment. This method is effective beyond 3 months, depending on the insecticide used and the type of surface on which it is sprayed (WHO, 2011). For instance, DDT (most commonly used) is one of the insecticides used and it can be effective for 9–12 months in some cases. Another class of insecticides currently being depended on is the pyrethroids (Ranson *et al*., 2011). There are however, challenges of resistance development by vectors to insecticides just like the parasites are developing resistance to antimalarial drugs (Corbel *et al*., 2012). There is therefore, need for the development of other classes of insecticides to lessen seriousness of the malaria problem

should the current pyrethroids become ineffective. With these prevention measures, Zambia has recorded a major reduction in malaria cases.

1.3.2.3.6 Fatty acid biosynthetic pathway II (FAS II) as drug target

In as much as successes have been recorded in reducing malaria, the ever evolving for survival parasites still pose a great challenge to the eradication of the disease. Thus the search for new drugs to prevent and treat malaria is still on. As such, the discovery of the apicoplast, a non photosynthetic plastid organelle of cyanobacterial endosymbiont origin, brought in a new prospect for drug development against *Plasmodium falciparum* (Surolia *et al*., 2004; Waller *et al*., 1998). This organelle which is essential for the functioning of the malaria parasite is the site of several vital anabolic processes such as cell growth, differentiation and homoeostasis (Surolia *et al*., 2002). A fatty acid biosynthetic pathway different from the known traditional one (FAS I) was discovered to occur in *Plasmodium*. This pathway called the type II or dissociation fatty acid biosynthesis (FAS II) occurs in the apicoplast of the *Plasmodium* (Waller *et al*., 1998; Ralph *et al.*, 2001; Surolia and Surolia, 2001). FAS II, mainly present in prokaryotes, plants, and some protozoans, is markedly different from FAS I (Vaughan *et al.*, 2008), which is present in eukaryotes including humans, yeast, and some mycobacteria. The type II FAS consists of seven small, monofunctional enzymes, each encoded by separate genes and each catalysing one single reaction, while type I (or associative) FAS consists of a single multifunctional protein derived from a single gene in which various domains catalyze different reactions of the biosynthetic pathway. Thus, the fundamental differences between the human type I and the plasmodial type II FAS pathways make the latter a striking target for antimalarial drugs, just as the FAS enzymes have been studied as targets for antimicrobials (Perrozo *et al*., 2002).

The *de novo* FAS-II pathway was found to be important for the survival of the *plasmodium* parasite in the late liver stage, particularly in its transition from liver to blood stage (Tasdemir *et al*., 2010; Singh *et al*., 2009; Yu *et al*., 2008). It thus emerges to be the practical drug target for causal prophylaxis and perhaps vaccines which have not been successfully developed. This was evidenced in an experiment carried out by Vaughan *et al*. (2008), in which the deletion of critical elongation enzymes such as FabB/F and FabZ

in *Plasmodium yoelii* caused the parasites not to complete its liver stage development. In another similar work, the role of FabI in *Plasmodium berghei* caused a delay in development of parasites to develop into blood stages. Of the seven enzymes involved in the FAS II pathway, FabI has been the widely studied because it is the rate-limiting enzyme and it catalyzes the final enzymatic reaction in the fatty acid elongation process.

Figure 15 illustrates the FAS II pathway in *P. falciparum*. In brief, the fatty acid synthesis is initiated by the carboxylation of acetyl-CoA to malonyl-CoA by acetyl-CoA carboxylase (ACC). Malonyl-CoA is then transferred to acetyl-carrier protein (ACP) by FabD (ACP transacylase) enzyme. The first step in the elongation pathway is the condensation of malonyl-ACP with acetyl-CoA catalyzed by FabH (β-ketoacyl-ACP synthase III) to form acetoacyl-ACP. The second step is the reduction of acetoacyl-ACP by FabG (β-ketoacyl-ACP reductase) in a NADPH-dependant reaction to form β-hydroxyacyl-ACP. A dehydration process catalyzed by FabA (β-hydroxydecanoyl-ACP dehydratase) or FabZ (β-hydroxyacyl-ACP dehydratase) follows to form *trans*-2-enoyl-ACP. The final and rate-determining step in the elongation process is another reduction catalyzed by FabI (enoyl-ACP reductase) in a NADH-dependent reaction. It reduces *trans*-2-enoyl-ACP to acyl-ACP.

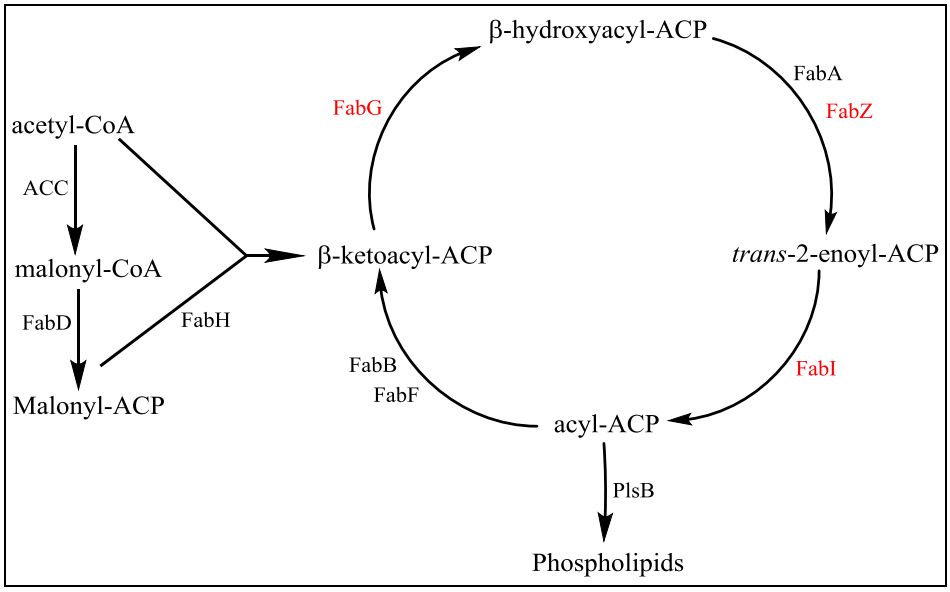


Figure 15: Type II fatty acid biosynthetic pathway system of *Plasmodium falciparum*.

Subsequent cycles are started by the condensation of malonyl-ACP with acyl-ACP by either FabB (β-ketoacyl-ACP synthase I) or FabF (β-ketoacyl-ACP synthase II). The cycles then continue until the required length of fatty acid chain is achieved.

The biosynthetic pathway has shown that, there are more enzymes and stages of parasite growth that can be targeted to prevent the parasites reaching the blood stage. For our study we selected three elongation enzymes (FabI, FabB and FabZ) as targets for enzyme spectrophometric inhibition assays.

1.3.2.3.7 Traditional plants and their use against malaria

The use of medicinal plants by traditional healers to treat illnesses is an important part of primary healthcare in most rural communities in Africa (Ndubani and Hojer, 1999). This practice has been recorded from ancient times and many different plant species (about 400) have been used and are still being used for the treatment of fever which is associated with malaria (Kaur, 2009; Ndubani and Hojer, 1999) and for malaria itself as compiled by Neuwinger, (2000). Other plant species are also used as mosquito repellent. The most successful conventional malaria drugs of all times have been sourced from plants. These include quinine, from which derivatives like chloroquine were generated and the present artemisinins which are successfully used in combination with other drugs to treat blood stage malaria.

There are several members of the genus *Ficus* L. (Moraceae) that have been reportedly used against malaria. For instance, the aerial parts of *F. asperifolia* Miq. and *F. sycomorus* L. (mixed with *Detarium microcarpum* Guill and Perr. and *Piliostigma thonningii* Schumach, Milne-Redh.) have been used to calm fevers caused by malaria. These are steeped in water for bathing (Neuwinger, 2000). The decoction of the leaves, bark and roots of *F. polita* vahl subsp. *polita* (synonym = *F. megapoda*), *F. melleri, F. pyrifolia* are also used for fevers caused by malaria. The root decoction of *F. natalensis* and the aerial parts is drunk to alleviate malaria symptoms (Randrianarivelojosia *et al*., 2003).

Some of the plant species reportedly used against malaria by the Masai people of Kenya include: *Acacia tortilis* (Forssk.) Hayne (Fabaceae), *Fuerstia africana* T.C.E Fr. (Lamiaceae), *Manilkara discolor* (Sond.) J.H. Hemsl (Sapotaceae), *Pentas lanceolata* Deflers (Rubiaceae), *Sericocomopsis hilderbrandtii* Schinz (Amaranthaceae), for which *in vitro* antimalarial and cytotoxity activities have been documented (Kigondu *et al*., 2011).

1.4 THE GENUS *FICUS*

The genus *Ficus* L. belongs to the family Moraceae. It is one of the most populous species in number of all plant genera (Lansky and Paavilainen 2011). It has over 750 different species, of which 500 occur in Asia and Australia, 150 reportedly occur in tropical America and 100 are in Africa and Madagascar. Of the 92 documented in sub-saharan Africa, Zambia has 34 indigenous species (Fowler, 2007; Klopper *et al*., 2006; Phiri, 2005; Burrows and Burrows, 2003; Leister, 2000; Launert and Pope, 1991). Figs are all perennial trees or shrubs, mainly evergreen but with some deciduous. They are divided into three categories depending on their growth form. They can grow as rock-splitters, stranglers or free standing terrestrial trees. They are characterized by latex that exudes from the bark, branches, leaves and fruits on injury. The leaves are mostly entire, some rarely lobed with irregular margins. The other characteristic feature of the *genus*, which runs in the whole family Moraceae is the fruit termed the syconium. This is a closed receptacle which forms a hollow sphere with inner surfaces lined with male and female flowers (dioecious figs) or flowers of a single sex (monoecious figs). Monoecious figs are essentially females which are also the edible ones (Lansky and Paavilainen 2011). The existence of the syconium is a feature that makes figs unique, and interestingly, each *Ficus* species has co-evolved with its specific wasp pollinator (Burrows and Burrows, 2003).

1.5 FOLKLORIC USE OF THE GENUS *FICUS*

Many people still use plant traditional medicine to treat a variety of diseases caused by different pathogens. The genus *Ficus* is no exception as it has worldwide use, because members of this genus are one of the earliest sources of cultivated medicines and food by

both humans and animals (Lansky and Paavilainen, 2011; Ipulet, 2007). The members of the genus *Ficus* are used to treat many health conditions, as well being used for food. For instance, the fruits, fresh leaves and young stems are reportedly eaten as vegetables (Aref *et al*., 2010). Some members have also been used domestically for ropes, as dye for leather, and also as lime for bait to hunt down birds (Burrows and Burrows, 2003). Another use has been of ecological significance in that they can be indicators of ground water (Ipulet, 2007). Thus the occurrence of *Ficus* species in an area may indicate high ground water table. Mostly of interest in this research, is their ethnomedicinal use for curative purposes.

There are many *Ficus* species reportedly been used for food, some of which are the fruits and leaves of *F. dicranostyla* Mildbr., *F. sur* Forssk. (synonym: Thunb.), *F. natalensis* Hochst., *F. sycomorus* L. and *F. wakefieldii* Hutch. Edible fruits are chewed for dyspepsia, while leaves (also edible) or bark and root infusions are used in the treatment of infectious diseases, abdominal pains and diarrhoea (Kuete *et al*., 2011).

Many reports however, come from the use of the genus as folk medicines throughout the continent of Africa and a few for domestic purposes (Rubnov, 2000; Burrows and Burrows 2003; Ipulet, 2007). As folk medicine, they have been used as astringents, laxatives, antihelmintics (de Amorin *et al*., 1999), for skin inflammations and warts. For example, the milky latex mixed with the bark of *F. sycomorus* and *F. wakefieldii* have antifungal properties which are used against some skin infections such as ringworms (Burrows and Burrows, 2003). In Ghana, the bark infusions of *F. asperifolia* Miq. are reportedly used for washing sores and ulcers and applied to circumcision wounds, while the rough leaves are used for scraping patches of ringworm before further treatment (Annan and Houghton, 2008). Decoctions of the bark of *F. virgata* are used in treating various skin diseases and ulcers (Abdul *et al*., 2008). It is also said to be effective in the treatment of piles, asthma, gonorrhea, hemoptysis, and urinary diseases. Decoctions of some *Ficus* fruits (*F. carica L.* and *F. natalensis*), the barks (*F. trichopoda* Baker, *F. asperifolia, F. craterostoma* Warb. ex Mildbr. & Burret., *F. exasperata* Vahl, *F. thonningii* Blume (synonym: *F. iteophylla* Miq.)), leaves (*F. sagittifolia* Warb. ex Mildbr. & Burret and *F. populifolia* Vahl), roots (*F. sur* Forssk. (synonym: *F. capensis*)) and the

milky latex of *F. sycomorus* subsp. *gnaphalocarpa* are reportedly used against sore throats and coughs (Burrows and Burrows, 2003; Neuwinger, 2000). In Senegal, the maceration of the leaves of *Ficus dekdekena* are used to treat tuberculosis, while in Cameroon the 1:1 decoction of *Ficus chlamydocarpa* and *Ficus cordata* is also used traditionally and indiscriminately used in the treatment of filaris, diarrhoeal infections and tuberculosis (Kuete *et al.,* 2008). In view of the ayurvedic system of medicine the bark decoction of *F. virgata* Reinw. ex Blume and *F. racemosa* L. is gargled to cure mouth ulcers (Abdul *et al*., 2008; Mahota and Chaudhary 2005). The stem bark of *F. ingens, F. lutea, F. polyphlebia, F. virgata, F. sycomorus, F. thonningii, F. sur*, and *F. umbellata* have been used for stomach troubles; diarrhea, dysentery, colds and as an astringent (Al-Musayeib *et al*., 2012; Teklehaymanot and Giday, 2007; Burrows and Burrows, 2003).

Some non-communicable diseases such as diabetes have also been managed by traditional medicines obtained from the genus *Ficus* (Aref *et al.*, 2010). For instance decoctions of the bark and fruits of *F. virgata* and *F. glumosa* Delile are well known to be useful in diabetes (Abdul *et al*., 2008; Neuwinger, 2000). Abdul *et al*., (2008) also reports how the bark of *F. virgata* is antiseptic, antipyretic and vermicidal hypoglycemic. The use of leaves, stem bark and root bark of some species like *F. sycomorus* have been extended to treat conditions such as mental illnesses, liver problems, hepatitis and sickle-cell anemia symptoms (Hubert *et al*., 2011). Koné *et al*., 2004 reports the use of the leaves of *F. thonningii* for rheumatism and the stem bark of *F. vallis-choudae* Delile to treat heart problems in Cˆote-d'Ivoire. The aerial and root parts of *F. natalensis* (mixed with *Sporobolus indicus* (L.) Br.), *F. sycomorus* (mixed with *Bridelia micrantha* Hochst.) and *F. sur* are reported to be used for jaundice and as snake bite antidote (Mpiana *et al*., 2008; Burrows and Burrows, 2003; Neuwinger, 2000). The paste of the bark of *F. racemosa* and fresh leaves of *F. trichopoda* has been used to cure swellings of foot and hands (Mahota and Chaudhary 2005; Neuwinger, 2000). The leaf decoction of *F. vallis-choudae* (mixed with *Ipomoea argentaurata* Hallier f.) is reportedly used against hyperthermia (Neuwinger, 2000).

For maternal health, *F. natalensis* has been reportedly used as an ecbolic (an agent that induces contractions of the uterus) during labour and also to induce lactation. While the fruit of *F. sycomorus* subsp. *gnaphalocarpa* mixed with *Cordyla pinnata* (Lepr.) Milne-Redh. bark are used for oxytocic activity during child birth (Neuwinger 2000). Meanwhile in Nigeria, the leaves of *F. exasperata* Vahl are also used for oxytocic activity, and *in vitro* uterine stimulatory effect was investigated (Bafor *et al.*, 2010).

In Zambia, some members of the genus *Ficus* have also been used for different illnesses as summarized in (Table 1). However, the identification of most of these *Ficus* plants in folklore use does not go up to species level. This has been observed by Fowler (2007) that a great number of plant concoctions are prescribed for the same disease. In addition the same vernacular name is applied to more than one species, calling for caution in their use and one of the reasons this study was undertaken.

Table 1: Summary of the traditional medicinal uses of *Ficus* species in Zambia as documented by Fowler (2007).

Species	**Traditional medicinal use**
F. burkei	Bleeding, constipation, colds, cuts and wounds, eye infections, varicose veins, tonsillitis and sore throat
F. sycomorus	Chest complaints, coughs, dysentery, diarrhoea, swollen glands, inflammation, tonsillitis and sore throat, sores and skin rashes, ulcers, TB (lymph nodes), to stimulate lactation
F. natalensis	Colic, influenza, syphilis, skin rashes, midwifery, to induce lactation
F. sur	coughs, eye infections, gonorrhoea, gynaecological ailments, infertility, impotence, TB, tonsillitis and sore throat, pneumonia, nausea and anti-emetic, tongue and mouth, bladder and kidney
F. glumosa	Eye infections, induce lactation
F. exasperata	Eye infections, tonsillitis and sore throat, tongue and mouth, tonic, stimulant, worms
F. dekdekana	Stomach ailments
F. vallis-choudae	Leprosy
F. ingens	Anaemia
F. capensis	Antipyretic
F. gurkei	To increase lactation

With all the acclaimed traditional uses of the genus *Ficus*, there has also been a number of scientific works done to validate and document the pharmacological properties that the plants exhibit. The genus has been well documented for its biological activities like antioxidant (Li *et al*., 2004), anticancer (Mradu *et al*., 2012), antidiarrhoeal (*F. lutea*, *F. cordata* Thunb. subsp. *salicifolia* (Vahl) C.C. Berg. (syn: *F. religiosa* Forssk.) *F. sycomorus*), antibacterial *(F. ingens*), antifungal (Kuete *et al*., 2009), antiplasmodial (F. sycomorus, *F. polita*), antiulcer, gastroprotective and wound healing activity in experimental animals (Thakare *et al*., 2010; Kuete *et al*., 2009; Lansky *et al*., 2008).

1.6 THE *FICUS* SPECIES SELECTED FOR THIS STUDY

1.6.1 *Ficus ingens* (Miq.) Miq.

Ficus ingens (Figure 16) is commonly known as the red-leaved fig, while in Zambia it is locally known by different names such as *Mupata* (Bemba*), chilembalemba* (Kaonde), *mutate* (Lozi) or as *muteba*. In Zambia, it has a continuous distribution in four provinces namely; The Copperbelt, Northern, Eastern and Southern provinces.

Figure 16: *Ficus ingens* leaves and fruit (*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com).

1.6.2 *Ficus lutea* Vahl (Giant-leaved fig)

Ficus lutea (Figure 17) also known as the giant-leaved fig is a large tree measuring up to 20 m tall. The distribution in Zambia covers Copperbelt, Eastern, Northern and Southern provinces.

Figure 17: *Ficus lutea* leaves and fruit (*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com).

1.6.3 *Ficus natalensis* Hochst. subsp. *natalensis*

Ficus natalensis (Figure 18) is also known as the natal fig, while in Zambia it is locally called *Kanyanguni* and *Mutaba* (Bemba) and then called *kachele* (Chewa). In Zambia, it is distributed throughout the Copperbelt, Luapula and North-Western provinces. This species is a small to large tree, up to 20 m tall and 30 m wide, epiphytic or a rock splitter, eventually a free-standing tree, evergreen or deciduous, with a few abundant aerial roots (Burrows and Burrows, 2003).

Figure 18: *Ficus natalensis* subsp. natalensis leaves and fruit
(*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com)

1.6.4 *Ficus ovata* Vahl.

Ficus ovata shown in Figure 19, also known as the rough-barked fig is locally called *chilemba* (Bisa, Kunda). In Zambia it occurs in the Copperbelt, Luapula and Northern provinces. It is a small to medium evergreen tree, upto about 25 m with a spread of approximately 15 m, terrestrial. Initially it occasionally grows as a hemi-epiphyte and lacking aerial roots.

Figure 19: *Ficus ovata* leaves and fruits (*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com).

1.6.5 *Ficus sansibarica* Warb. subsp. *macrosperma* (Mildbr. and Burret) C.C. Berg

Common names of *Ficus sansibarica* include Zanzibar fig, Angola fig and knobbly fig (Figure 20). In Zambia, with the tribes in brackets, it is called chitabataba (Kaonde), *musoko* (Tabwa), *mutaba* (Bemba) and its distribution in Zambia covers the Copperbelt, Lusaka, North-Western and Southern provinces.

It is a large tree with a wide-spreading crown, normally up to 20 m tall and 30 m wide but occasionally larger, hemi-epiphytic, becoming terrestrial, commonly unbutressed and with a few small aerial roots, but usually without (Burrows and Burrows, 2003).

Figure 20: *Ficus sansibarica* leaves and fruits
(*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com)

1.6.6 *Ficus sycomorus* L. subsp. *sycomorus*

Ficus sycomorus subspecies *sycomorus* is commonly known as the sycamore fig or mulberry fig (

Figure **21**). In Zambia, the species is known by local names; *Mukunyu* (Bemba, Bisa), *Chikujumba* (Chewa), *Chikunyu* (Bisa, Kunda), *Katema* (Kaonde, Lozi, Lunda, Tonga), *Musuera* (Luvale), *Nkuyu* (Tumbuka). It is widespread in Zambia.

It is a large, spreading tree up to about 30 m tall and 30 m wide (canopy covering), deciduous or evergreen; with a typically short, thick trunk extending to 3.5 m in diameter, old tree developing buttresses but lacking root sucker, branches wide-spreading, not developing aerial roots.

Figure 21: *Ficus sycomorus* subsp. *sycomorus* leaves and fruit
(*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com).

1.6.7 *Ficus sycomorus* subsp. *gnaphalocarpa* (Miq) C.C. Berg

Ficus sycomorus subsp. g*naphalocarpa* is the large-fruited sycamore fig (Figure 22). It is commonly known as the sycamore fig just like the subspecies *sycomorus*. It is also known by the same local names in Zambia. It is widespread in Zambia.

Figure 22: *Ficus sycomorus* subsp. *gnaphalocarpa* leaves and fruits (*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www. Plantzafrica.com).

1.6.8 *Ficus wakefieldii* Hutch

Ficus wakefieldii is commonly known as Wakefield's fig (Figure 23). In Zambia, it has different names according to the region. It is called Chikolo or Chitabataba (Lamba), Chilemba (Bemba), *Ituntu*, *mukunyu*, *mupulampako* (Bemba, Kaonde), *mutawa* (Chewa). Distribution in Zambia covers the Central, Copperbelt and Luapula provinces, being confined to high rainfall area zone.

Figure 23: *Ficus wakefieldii* leaves of a young branch
(*http://www.figweb.org/Figs_and_fig_wasps/index.htm* and www.Plantzafrica.com).
(http://www.zambiaflora.com/speciesdata/image -display.php).

1.7 CHEMICAL CONSTITUENTS IN THE GENUS *FICUS*

The chemical composition of some of the members of genus *Ficus* has been widely studied. Most members have been investigated pharmacologically and phytochemically because of their chemical constituents which, are used medicinally. A phytochemical review (see Appendix) shows that plants from this genus are rich sources of characteristic classes of secondary metabolites such as prenylated flavonoids and isoflavonoids, lignans, terpenoids (Figure 24d), alkaloids, coumarins, chromones, phenylpropanoids and tannins (Chen, *et al.*, 2010; Kuete, *et al.*, 2008). Other classes reported include steroids, triterpenoids, fatty acids, saponins and anthocyanins (Chawla *et al*., 2012). Sugars such as sucrose and lactose have also been isolated from *Ficus* species. Some of these classes will be discussed in the text. The chemical composition of latex is very complex as it is composed of proteins, alkaloids, starches, sugars, oils, tannins, resins, gums and other minor constituents (Richardo *et al.*, 2004).

1.7.1 Phenolic compounds

Phenolic compounds are ubiquitous in the plant kingdom and are thus the most studied, even though not exhaustively, phytochemicals in plant research (Mradu *et al*., 2012). They are chemically diverse secondary metabolites, which are characterized by aromatic rings with one or more hydroxyl functional groups. Phenolic compounds can be classified into different groups such as flavonoids, flavonones, flavones, anthraquinones and tannins (Figure 24).

Flavonoids such as catechins, epicatechins and epiafzelechins isolated from *F. ovata* are common to the genus (Kuete *et al*., 2009). Furthermore, isoflavones such as genistein, prunetin, and (2S)-naringenin were indentified in *F. polita*. The antimycobacterial activities of these three compounds have been shown against *Mycobacterium tuberculosis* H37RV *in vitro* and the determined MIC values were 35 µg/ml, 30 µg/ml and ≤ 2.8 µg/ml, respectively (Chen *et al.*, 2010).

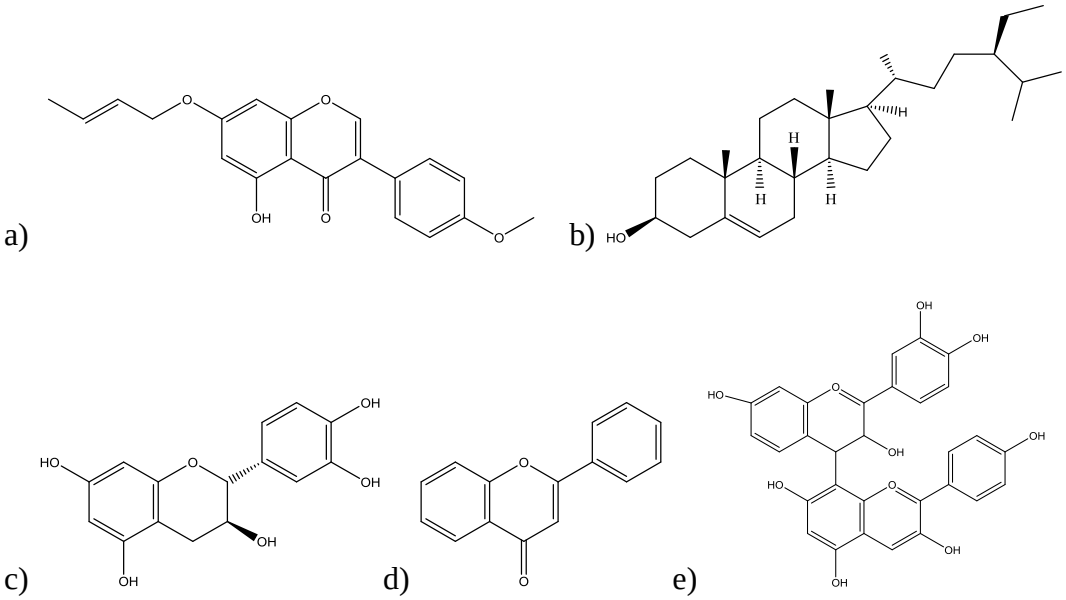


Figure 24: Some common constituents of *Ficus* species

a) prenylated flavonoid (7-γ,γ-dimethylallyloxy-5-hydroxy-4'-methoxyisoflavone) isolated from *Ficus sycomorus*; b) β-sitosterol isolated from *F. glomerata*; c) catechin isolated from *F. ovata* and sycomorus. d) Flavone; e) Tannin

1.7.2 Terpenoids

Terpenoids such as taraxeryl acetate and betulinic acid are common bioactive compounds that have been isolated from the genus, and these are known to have antiprotozoal properties (Hubert *et al*., 2011). In most of the *Ficus* species, some compounds have been identified as marker compounds, these include bergapten, fumaric acid, 1,4-dimethyl-7-isopropylazulene (guaiazulene), lupeol, oleanolic acid, psoralen, rutin, scopoletin (Flavonoids), α-terpineol, umbelliferone and xanthotoxin (Phytolab, 2010/2011).

1.7.3 Steroids

Another interesting class of bioactive compounds found in the genus *Ficus* are steroids. These include *ß*-sitosterol (Figure 24b) isolated from *F. polita* and *ß*-sitosterol-D-glucoside isolated from *F. glomerata* and *F. cordata* stem bark. Potent hypoglycaemic and antibacterial properties of *ß*-sitosterol-D-glucoside have been reported (Channabasavaraj *et al.,* 2008). Of the eight *Ficus* species under study, *F. sycomorus* is

the most widely studied, and as such many compounds have been isolated and identified from this species.

Amongst other groups of compounds that have been isolated from *F. sycomorus* root bark include tannins, alkaloids, sugars, saponins and anthracenosides which have been reported to exhibit antimicrobial activity (Zaku *et al*., 2009). Below is Table 2 of the secondary metabolites that have been isolated and identified within the eight selected *Ficus* species under study. The chemical structures are presented in the appendix (6.1).

Table 2: Chemical constituents that have been isolated from the eight *Ficus* species under study.

Chemical compound	**Species**	**Area of plant collection**	**parts**	**References**
Flavonoids				
Rutin (quercetin-3-0-α-L-rhamnopyronosyl (1→6)β-D-glucopyranoside	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al*., 2010
Isoquercitrin (quercetin-3-0-β-D-glucopyranoside)	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al*., 2010
Quercetin 3,7-0-α-L-dirhamnoside	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al*., 2010
Quercetin-3-0-β-D-galactopyronosyl (1→6) glucopyranoside	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al.*, 2010
3-methoxyquercetin –catechin	*F. gnaphalocarpa*	N.I	B	Hubert *et al*., 2011
Epicatechin	*F. gnaphalocarpa*	N.I	B	Hubert *et al*., 2011
Quercetin	*F. gnaphalocarpa*	N.I	B	Hubert *et al*., 2011
	F. sycomorus	El-Qualubia Governorate	L	El-Sayed *et al*., 2010
Quercitrin	*F. gnaphalocarpa*		B	Hubert *et al*., 2011
Cajanin	*F. ovata*		B	Kuete *et al*., 2009
Terpenoids				
3-friedelanone	*F. ovata*	Mt. Kala, Cameroon, 2004	B	*Kuete et al., 2009*
Taraxeryl acetate	*F. ovata*	Mt. Kala, Cameroon, 2004	B	Kuete *et al.*, 2009
Betulinic acid	*F. ovata*	Mt. Kala, Cameroon, 2004	B	Kuete *et al.*, 2009
	F. *gnaphalocarpa*	N.I	B	Kamga *et al.*, 2010
A-amyrin	*F. sycomorus*		La	Lansky and Paavilainen, 2011
Bergapten	*F. sycomorus*		La	Lansky and Paavilainen, 2011
Imperatorin	*F. sycomorus*		La	Lansky and Paavilainen, 2011
Xanthotoxin	*F. sycomorus*		La	Lansky and Paavilainen, 2011

Oleanoic acid	*F. ovata*	Mt. Kala, Cameroon, 2004	B	Kuete *et al.*, 2009
Steroids				
Gallic acid (3,4,5-trihydtxybenzoic acid)	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al.*, 2010
ß-sitosterol-3-0-β-D-glucopyranoside	*F. sycomorus*	El-Qualubia Governorate	L	El-Sayed *et al.*, 2010

Chemical compound	**Species**	**Area of plant parts collection**	**References**
Essential oils			
2-Hexen-1-ol benzoate	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Ephytol	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Benzyl tiglate	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
E-Menth-2-en-1-ol	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Z-Menth-2-en-1-ol	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
β-Selinene	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
1,2-Dimethoxy-4-ethylbenzene	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Geranyl acetone	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
1-Octen-3-ol	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Caryophyllene oxide	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Octacosane	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Heptacosane	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Isophytol	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
6,10,14-Trimethyl-2-pentadecanone	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
α-Ionone	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
n-Nonanal	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Limonene	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Benzyl benzoate	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Oleic acid	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
6-Methyl-5-hepten-2-one	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Benzaldehyde	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Neral	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
Linalool	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
β-Ionone	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
α-Pinene	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
β-Caryophyllene	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009
3,4-Dimethyltoluene	*F. ovata*	Nigeria	L	Sonibare *et al.* , 2009
Octadecanoic acid	*F. ovata*	Nigeria	L	Sonibare *et al.*, 2009

N.I- Place and year of collection not indicated; B- stem bark; L- leaves; La- latex; R- roots.

1.8 AIM

The main aim of this thesis was to evaluate the medicinal potential of members of the genus *Ficus* growing in Zambia. The *Ficus* species are used indiscriminately against infectious diseases including microbial and parasitic infections as well as a few non communicable diseases like gynaecological conditions, diabetes and high blood pressure. To achieve this global picture, this project will collect, extract and fractionate eight *Ficus* species that occur in high rainfall areas in Zambia , namely *F. ingens, F. lutea, F. natalensis, F. ovata, F. sansibarica* subsp. *macrosperma, F. sycomorus* subsp. *gnaphalocarpa, F. sycomorus* subsp. *sycomorus* and *F. wakefieldii.* Information on their ethnobotanical use will be gathered from the users. The resulting extracts and subextracts of different plant parts will then be:

- phytochemically profiled using chromatographic methods such as thin layer chromatography (TLC), proton Nuclear Magnetic Resonance (^{1}H NMR) and high performance liquid chromatography (HPLC) fingerprinting;
- screened *in vitro* against a panel of fungi and bacteria, as well as *Mycobacterium tuberculosis, Schistosoma mansoni, Leishmania donovani, Trypanosoma brucei gambiense, Trypanosoma cruzi* and *Plasmodium falciparum;*
- screened *in vitro* against recombinant FAS-II elongation enzymes, FabG, FabI and FabZ as potential targets in liver stage malaria parasites. Hence, a bioactivity guided isolation of the active principles will be attempted.

2. MATERIALS AND METHODS

2.1 HEALTH AND SAFETY

All the procedures and experiments in this thesis were performed in level two (2) laboratories and were carried out in accordance with the provisions of the Health and Safety Act, of the University College London - School of Pharmacy. Risk assessment was carried out for which Control of substances hazardous to health regulations (CoSHH) forms were completed and the outcome was favourable to enable us work in reasonably practicable and safe working environment conditions.

2.2 PLANT MATERIALS

2.2.1 Plant collection

The plant materials were collected in Zambia, during the month of December 2009. This is the fruiting period for all but one selected *Ficus* species. The aerial plant parts and roots were air dried for several days and stored at room temperature in a dark cupboard until required for extractions.

Different plant species of *Ficus* were collected from the high rainfall and riverine areas of Zambia. These include plants that are native to the north of the Zambezi River, namely *F. ovata* Vahl. and *F. wakefieldii* Hutch. The other *Ficus* species collected are widely spread throughout the country, such as *F. natalensis* Hochst. subsp. *natalensis*, *F. sansibarica* Warb. subsp. *macrosperma* (Mildbr. and Burret) C.C. Berg, *F. lutea* Vahl, *F. ingens* (Miq.) Miq., *F. sycomorus* subsp. *gnaphalocarpa* (Miq) C.C. Berg and *F. sycomorus* L. subsp. *sycomorus*. The plants were identified by a taxonomist, Professor Patrick Phiri of the Copperbelt University, Zambia. Voucher specimens have been deposited at the University of Zambia herbarium (UZL) under accession numbers ranging between 20904 and 20911 (Table 3).

Table 3: Localities, date of collection and plant parts of eight *Ficus* species.

Plant species	Date of collection	Ecology of Place of collection	GPS reading	Voucher number
F. ingens	17/12/2009	Juvenile plant as an epiphyte	Lat:$12^0$50.7' S, Log:$28^0$21.6'E Alt: 1049m	20909
F. lutea	17/12/2009	Juvenile plant as an epiphyte	Lat:$12^0$50.7' S, Log:$28^0$21.6'E Alt: 1049m	20908
F. natalensis	17/12/2009	tree on termite mounds	Lat:$12^0$48.05'S, Log:$28^0$14.28'E Alt: 1050m	20910
F. ovata	16/12/2009	Tree on dambo soil	Lat:$12^0$50.9' S, Log:$28^0$21.6'E Alt: 1050m	20906
F. sansibarica	16/12/2009	Mesic miombo woodland on termite mounds	Lat:$12^0$50.9' S, Log:$28^0$21.6'E Alt: 1050m	20905
F. sycomorus subsp. *gnaphalocarpa*	26/12/2009	Tree in acacia-combretum woodland	Not taken	20904
F. sycomorus subsp. *sycomorus*	16/12/2009	Not specific	Lat:$12^0$50.9' S, Log:$28^0$21.6'E Alt: 1050m	20911
F. wakefieldii	16/12/2009	mesic miombo woodland on termite mounds	Lat:$12^0$50.9' S, Log:$28^0$21.6'E Alt: 1050m	20907

2.2.2 Plant Extraction and partitioning

There are several methods of extracting plant constituents. In this study, cold continuous maceration on a magnetic stirrer was used throughout the project. This method allows for chemical constituents to be extracted from plant material by continuously stirring ground material in a solvent. The major advantage of this method is that it is exhaustive, especially when you use a solvent such as an alcohol, methanol being the most common, which is a good all-purpose solvent for preliminary extractions (Waksmundzka-Hajnos *et al*., 2008; Heinrich *et al.,* 2004; Harbone, 1998). Almost all constituents get extracted. However, some may not as they maybe insoluble in cold solvent, in which case a little heating can be applied. An additional advantage is the low cost of equipment and solvents required. Thus, for an exhaustive extraction, solvents in which both nonpolar and polar constituents could dissolve were used in this study, plus the technique of continuous maceration.

The experimental procedure was as follows: twenty grams (small scale) and about 320 g (large scale) of the dried plant materials (leaves, stem barks and roots) were ground to a fine powder. This was done to increase the surface area of the plant materials in order to reduce the time of extraction. The ground plant materials were then extracted with 200 ml methanol (MeOH) (Fisher, HPLC grade) for small scale and twice with 800 ml for large scale by continuous maceration on a magnetic stirrer for 24 hrs and 48 hrs (for each run) respectively at room temperature. The total extraction time for large scale, thus lasted 96 hrs, with a total volume of solvent used coming up to 3.2 liters. The extract was then filtered by vacuum using a Buchner funnel lined with Whatmann No.1 filter paper, and the filtrate evaporated to dryness using a rotary evaporator (Heidolf – Laborota 4003) at 32^{o}C. This is what will be referred to as the crude methanol extract (CR-MeOH). The samples were stored at -21^{o}C, until screened for biological activity.

The CR-MeOH extracts were further partitioned to provide a coarse separation of subextracts according to polarity. Thus a solvent-solvent partition extraction was performed according to Searle and Molinski (1994). Three (nonpolar, mid-polar and polar) subextracts were obtained from this process (Figure 25). The CR-MeOH extract was dissolved in 100 ml of 90% methanol and then partitioned three times against 100 ml n-hexane (Fisher, HPLC grade). The resulting n-hexane subextracts were combined and evaporated to dryness. The aqueous-methanolic layer was then diluted to 70% MeOH to create distinct layers of two immiscible solvents (Figure 26). It was further partitioned three times with 100 ml chloroform (Fisher, HPLC grade). For large scale partitioning, 62.83g dried CR-MeOH extract of *F. sansibarica* was dissolved in 1.5L of 90% MeOH. It was partitioned three times against 1.5L n-hexane. The residue was then diluted to 70% MeOH and further partitioned three times with 1.5L $CHCl_3$. The $CHCl_3$ subextracts were combined and evaporated to dryness, while the aqueous-methanolic fraction was freeze dried. All subextracts were stored at -21^{o}C until work-up.

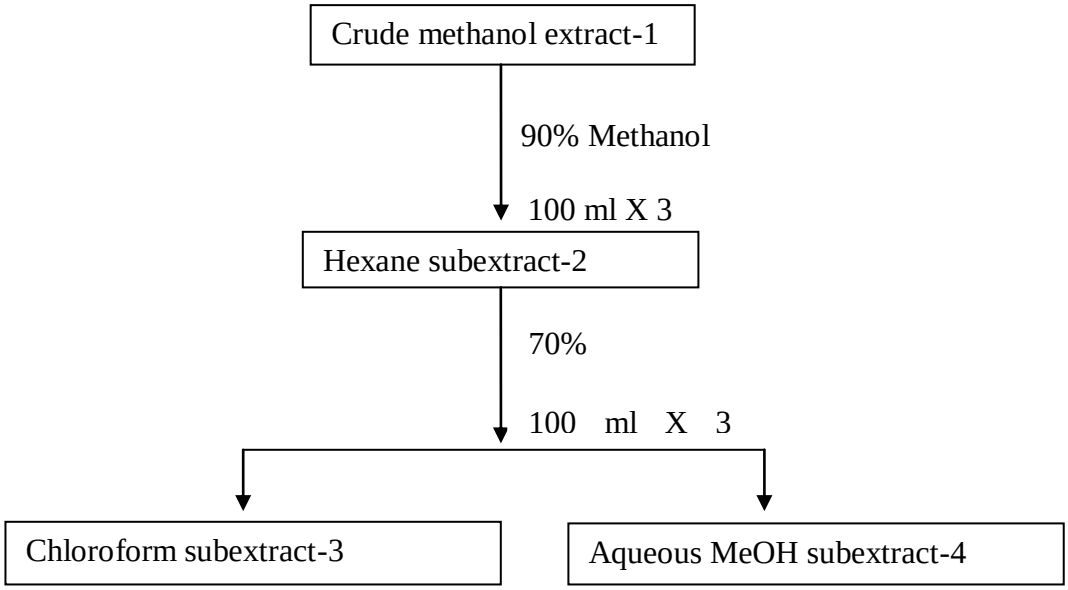


Figure 25:Liquid-liquid partitioning (Modified Kupchan extraction) scheme

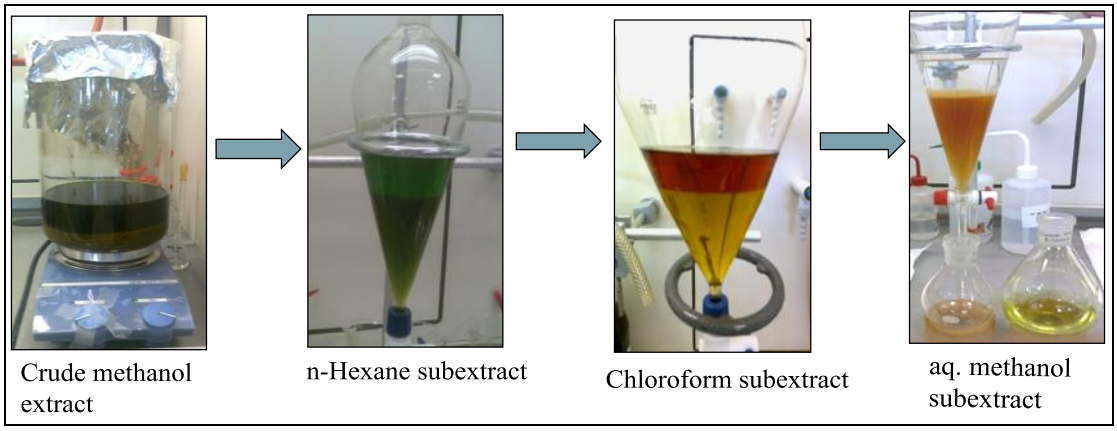


Figure 26: The liquid-liquid partitioning (modified Kupchan method).

2.3 PHYTOCHEMICAL METHODS

Phytochemical methods were employed for the extraction, isolation, purification and characterization of the plant extracts. Most of the techniques used were chromatographic and spectroscopic techniques. In addition to the aboce procedures, chemical profilling by TLC, ^{1}H NMR and HPLC was performed. The biological assays together with the chemical profilling were applied to create a phytopharmacological profile of the selected *Ficus* species.

2.3.1 Chromatographic techniques

Chromatography is a technique used for the separation of mixtures. It works on the principle of the different affinity towards the stationary phase and the mobile phase that the various constituents in the mixture have. Thus, the various constituents travel at different speeds along the stationary phase, causing a separation based on differential partitioning between the mobile and stationary phases. There exist several chromatographic techniques that can be used to isolate and purify chemical constituents of interest from crude extracts. However, compounds are isolated and purified largely on the basis of polarity, size, charge and specific affinity to the stationary phase. The techniques used in this project include; size exclusion gel chromatography, thin layer chromatography (TLC), vacuum liquid chromatography (VLC), gravity based open column chromatography (OCC), solid phase extraction (SPE) and high performance liquid chromatography (HPLC).

2.3.1.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) is a technique that can be used to analyze and separate plant extracts qualitatively and quantitatively. It is often used to provide the first characteristic fingerprints of an extract as it shows the different constituents of a crude mixture and also the purity of an isolated compound (Waksmundzka-Hajnos *et al*., 2008). It operates on the principle of different migration properties of compounds in different solvent systems. It is also defined as the partitioning of a substance between two immiscible phases (Evans, 2009). The stationary phase is a thin layer of adsorbent material, mostly silica gel, which is immobilised on an aluminium plate. On it as well, a fluorescent coating (F_{254}) which allows visualization of UV active compounds when

viewed under long- and short-wave UV light (254 nm and 366 nm) is placed. For the visualization of colourless compounds, staining reagents are used. The mobile phase is a mixture of solvents into which one end of the plate is dipped. Because of capillary action the solvent mixture migrates to the top of the plate carrying with it different components of the extract migrating at different paces. The type of compounds that will travel the fastest to the top of the plate depends on the strength of the solvent mixture. For a mixture dominated with polar compounds, the solvent mixture would need to have polar properties and vice versa. The migration of the compounds in a mixture can be described by their R_f values defined by the equation below.

$$R_f = \frac{Distance\ travelled\ by\ the\ compound}{Distance\ travelled\ by\ the\ solvent\ front}$$

In this research, TLC was performed on the plant extracts and subextracts (CR-MeOH, hexane, $CHCl_3$ and aq.MeOH) of *Ficus* as follows;

One milligram (1 mg) of the dry sample was dissolved in 50 µl of appropriate solvent. Then 5 µl of the 20 mg/ml sample was applied 2 cm from the bottom on silica gel 60F_{254} pre-coated TLC plate (Merck 105554.0001). It was then placed in a tank saturated with an appropriate mobile phase as shown in and quaternary amine compounds.

Table 4. After the separation was completed, the plate was air dried and viewed under short wavelength UV_{254} nm and long wavelength UV_{366} nm (UV cabinet, Camag) to determine absorbance and fluorescence properties of the compounds in the extracts. Substances that quench fluorescence appeared as dark spots in short wavelength UV_{254} nm and they were pencil marked on the left ([). The substances that fluoresce in long wavelength UV_{366} nm appeared as bright spots and were marked on the right side (]).

The plates were sprayed with different detection reagents to visualize the characteristic compounds. The main reagent spray used was 4 % (W/V) vanillin (Sigma-Aldrich, V110-4, UK) in sulphuric acid (Sigma-Aldrich 32,050-1, UK) (VS), and thereafter heated (Hotplate, VWR) for about two minutes at 110 ^{o}C. VS reagent is a universal spray reagent which detects many natural products like components of essential oils such as terpenoids, phenylpropanes derivatives and phenols. Other natural products that can be

detected are some phenolic compounds and non polar compounds. The other reagent sprays used included natural product (NP) spray composed of 1% (W/V) methanolic diphenylborinic acid 2-aminoethyl ester (Acros Organics, 155400500) followed by 5 % (W/V) ethanolic polyethylene glycol (PEG) 4000 grade (Fisher, P/3680/53) to visualize flavonoids and related polyphenols under UV 366 nm wavelength. Dragendorff's reagent (DRG) which consisted of a 1:1 mixture of 0.85g bismuth nitrate dissolved in 10 ml acetic acid and 40 ml water and 8 g potassium iodide dissolved in 20 ml water was used to visualize alkaloid, heterocyclic nitrogen and quaternary amine compounds.

Table 4: TLC plate development conditions.

Plant extract	**Solvent extract dissolved in**	**Mobile phase**	**Detection spray reagent**
Crude MeOH	Methanol	EtOAc : MeOH: H_2O (40.5 : 5.5 : 4)	4 % Vanillin/Sulphuric acid
Hexane	Chloroform	Hex : EtOAc (8 : 2)	4 % Vanillin/Sulphuric acid
Chloroform	Chloroform	$CHCl_3$: EtOAc (6 : 4)	4 % Vanillin/Sulphuric acid Natural product spray *
Aqueous MeOH	Methanol	EtOAc : MeOH: H_2O (40.5 : 5.5 : 4)	4 % Vanillin/Sulphuric acid

*NP/PEG

2.3.1.2 Solid-Phase Extraction (SPE)

Solid-phase extraction (SPE) is another technique that was greatly used in the isolation of chemical constituents. It is mostly used for small amounts of extracts which have fairly few chemical components. SPE is an alternative technique to liquid-liquid extraction which operates on the principle of liquid-solid extraction. It is effective for small samples because it exhibits high selectivity and faster elution profile and minimization of solvent consumption (Waksmundzka-Hajnos *et al*., 2008; Hostettmann *et al*., 1998). The sample is usually dissolved in an appropriate solvent and passed through a small bed of adsorbent (solid phase) of very consistent particle size and shape to maximise separation efficiency. The compounds are eluted with stepped changes of small volumes of solvent depending on the stationary phase (Hostettmann *et al*., 1998). For normal phase, less polar to increasing polar eluents such as pentane, hexane, dichloromethane to chloroform can be used to extract non polar constituents. Meanwhile, hydrophobic and polar constituents are extracted from the aqueous solutions in reversed-phase SPE, in which more polar eluents

to decreasing polarity in the order of water, methanol and acetonitrile are used to achieve fractions enriched in specific chemical classes. Elution occurs upon the addition of an organic solvent that effectively disrupts the interactions between the adsorbent and the target constituents. Prior to the isolation, the column is activated by conditioning and equilibrating it with the solvent which will be used first to elute the sample. In reversed-phase, a polar organic solvent such as methanol is used to condition the sorbent, and then in a gradient equilibrate with water. While in normal phase, a polar organic solvent is used for conditioning, followed by equilibration with a less polar solvent like hexane or chloroform depending on what you are eluting.

In this thesis, the extract was dissolved in minimal solvent before it was applied evenly on top of the column under vacuum. For reversed-phase SPE, the Strata C18-E (55µm, 70A) 10g/60 ml Giga tubes by Phenomenex were used. The Strata SI-1 silica (55µm, 70A) 10g/60 ml Giga tubes were used for normal phase SPE. Fifty milliliters of increasingly polar (10 % gradient) mobile phase (100 % hexane to 100 % ethyl acetate) were eluted for normal phase SPE. Methanol (100 %) was used as the final wash. RP-SPE used a stepped up gradient from 100 % water to 100 % MeOH in 10 % increments. The collected fractions were subjected to TLC in which the similar fractions by TLC analysis were pooled, and then dried using a rotary evaporator at 34^{o}C. Aqueous fractions were frozen overnight before being freeze dried.

2.3.1.3 Vacuum-liquid chromatography (VLC)

Vacuum liquid chromatography (Figure 27A) is a quick and inexpensive technique used to fractionate large amounts of plant extracts. It works with the same principle as that of SPE, in which the chemical components are fractionated based on an elution polarity gradient. There are two types of VLC depending on the polarity of the constituents of interest in the crude mixture and the stationary phase chosen. These are normal-phase silica VLC and reversed-phase C18 VLC. In this thesis we used reversed-phase C18 because the chemical constituents of interest were mostly polar compounds.

The reversed-phase C18 stationary phase was firmly packed in a column which was then equilibrated with stepped up gradient solvent from 100 % methanol to 100 % water. The plant extract was dissolved in minimal methanol and then loaded onto the column.

Aliquots of the mobile phase were added to the column to elute the fractions in a stepped up gradient from 100 % water to 100 % methanol in 10 % increment of the methanol. The collected fractions that were similar in R_f values and colour spot by TLC analysis were pooled and dried on a rotary evaporator.

2.3.1.4 Size Exclusion Chromatography

Size exclusion or gel filtration chromatography is a useful technique for preliminary or final step in the isolation process of natural products (Hostettmann *et al.,* 1998). It is commonly performed as open column chromatography (OCC) on solid phase supports such as sephadex (Figure 27B). The most commonly used is Sephadex LH-20, a hydrophilic beaded dextran that is cross-linked by glycerin-ether bonds. It is hydroxypropylated and therefore exhibits both hydrophilic and lipophilic characteristics which allow separation of non-polar and polar compounds in the same run, as well as the separation of compounds according to their molecular weights. It is therefore, ideal for the separation of large polymeric molecules.

In our experiments, a weighed amount of sephadex LH-20 (GE Healthcare, 17-0090, UK) was soaked in methanol overnight to allow the gel to swell before packing the column. The sephadex slurry was then carefully poured into the column in a continuous flow to avoid bubbles and uneven settling. Meanwhile, the extract was dissolved in minimal solvent, usually in the packing solvent, and then applied gently on top of the column. Cotton wool was placed on top of the column in order to secure the surface so that the eluting solvent does not disturb the surface of the column when being poured onto it. The column was run isocratically using MeOH, at a flow rate of about 1 ml/min at the beginning of the run, and when there were distinct bands showing in the column, the flow rate was increased to about 5 ml/min. Fractions were collected in amounts between 10-50 ml. The fractions were then subjected to TLC analysis, and the similar ones were pooled, dried and stored at -20^{o}C until workup.

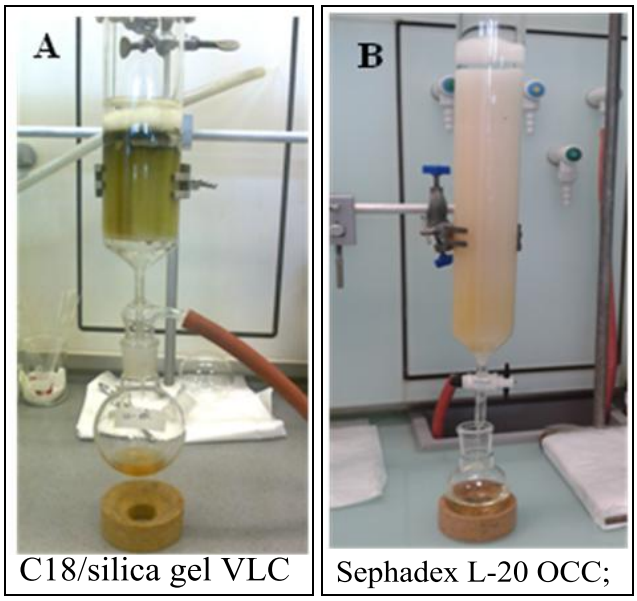


Figure 27: Chromatography apparatus and set up

2.3.1.5 High Performance Liquid Chromatography (HPLC)

HPLC is a high-resolution technique, with efficient and fast separation (Waksmundzka-Hajnos *et al.*, 2008; Heinrich *et al.*, 2004). It is the most widely used technique for the analysis of classes of compounds which are non-volatile such as phenolics, higher terpenoids, alkaloids, lipids and sugars which are soluble in organic solvents (Kalili and de Villiers, 2011; Qiao *et al.*, 2011; Harbone, 1998). It can also be used for fingerprinting of biologically active extracts, or monitoring chemical reactions of some metabolites in organic synthesis of pharmaceutically potent compounds. The most widely used stationary phase in HPLC is C_{18} (reversed-phase), generally using water/methanol or water/acetonitrile mixtures as the mobile phase. The mobile phases can be run in gradient elution mode, in which the concentration of a particular solvent is increased sequentially over a period of time, starting, for example, with 100% water and increasing to 100% acetonitrile over a set time. Another mode is the isocratic elution mode, in which a constant composition of solvent is maintained for a set period of time throughout the experiment (Heinrich *et al.*, 2004). There are two types of HPLC analyses that can be performed depending on the required results. These include analytical HPLC for the qualitative and quantitative determination of compounds, and preparative HPLC which is mainly used for the isolation and purification of targeted compounds (Waksmundzka-Hajnos *et al.*, 2008). The advantages of HPLC are the excellent resolution, speed and

reproducibility. The major disadvantage is the expense, especially of the preparative column.

The HPLC fingerprint of the phenolic composition of the crude methanol and aq. methanol subextracts of eight *Ficus* species was performed to identify the phenolic compounds that characterize the selected species. HPLC was determined using the Agilent 1200 series system equipped with a UV/Vis diode array detector (G1315D), quaternary pump (G1311A), autosampler (G1329A), fraction collector/autosampler thermostat (G1330B), thermostated column compartment (G1316A), refractive index detector (G1362A) and analytical fraction collector (G1364C). Samples were prepared at a concentration of 1 mg/ml in MeOH, and filtered through syringe filters with pore size Ø 0.45 µm before injection. The stationary phase used was a phenomenex $^{(R)}$ synergy polar-RP80A column (250 mm X 4.6 mm, I.D. 4 µm) protected with a Nova –pak $^{(R)}$ C18 guard column. The detector was set to scan from 190 to 400 nm at 40^{o}C. The binary mobile phase consisted of solvent A (0.2 % glacial acetic acid in water) and solvent B (methanol Fisher HPLC grade with 0.2 % glacial acetic acid). The solvents were acidified to maintain the pH of the crude sample inorder to enhance separation and to reduce peak tailing and prevent the deprotonation of the phenolic hydroxyl groups.

The conditions were set according to Giner *et al.* (1993). The gradient elution was set at 10-20 % B (0-5 mins), 20-50% B (5-65 mins), 80-100% B (75-80 mins) at a flow rate of 0.8 ml/min. The injection volume of each sample was 10 µl and the peaks were monitored at a short wavelength of 254 nm. A cocktail of 12 phenolic compounds were used as standards (50 µl of each compound at about 10 mg/ml was combined and used as a standard). The 12 phenolic standards that composed the cocktail included; (-) Epicatechin 90% (Aldrich 525952), Catechin hydrate (unhydrous), Quercetin (Li light and Co ltd, Colnbrook Bucks England), Rutin hydrate (Sigma 14 Ho5521), Gallic acid (Sigma G7384-100G), Quercitrin (Glucoside + rhamnoside), Kaempferol, Luteolin (Extrasynthese 69730 Genay France), Caffeic acid (Sigma C0625-29), Genistein (Genay Francs 69730), Apigenin-7-glucoside, and *Epi*-gallocatechin gallate (EGCG). The experiment was run and analysed by application of the Chemstation (Agilent) software.

2.3.2 Structure elucidation techniques

2.3.2.1 Nuclear Magnetic Resonance (NMR) Spectroscopy

2.3.2.1.1 Proton Nuclear Magnetic Resonance (^{1}H NMR)

Proton nuclear magnetic resonance (^{1}H NMR) is a spectroscopic technique mainly used for the identification of the hydrogen and carbon frameworks of organic compounds when applied to crude extracts of natural origin. This is all to aid in the structure elucidation of the organic compounds therein after a series of isolations. In this case certain chemical functional groups and classes such as, double bonds, aldehydes, aromatic structures, carboxylic acids, fatty acids, sugars and steroids can be determined. However, other spectroscopic techniques are applied to distinguish between chemical classes that are closely related and appear in the same region on the spectra.

NMR spectroscopy detects nuclei that have an overall net spin, *I*. The most useful nucleus for observation in NMR is that of the major isotope of hydrogen (^{1}H), which has two different possible states, a higher energy level and a lower energy level. The nuclei in a lower energy state can be brought to a higher state with electromagnetic radiation, this is said to be the nuclear magnetic resonance. The energy absorption gets detected as a signal plotted against the chemical shift (δ). The amount of energy that is necessary is related to the chemical binding properties of the atom.

In this study, ^{1}H NMR was used to get an overview of the chemical profile of the CR-MeOH extracts and the liquid-liquid partitioning subextracts (n-hexane, $CHCl_3$ and aq.MeOH) of the *Ficus* species. Thus, 20 mg of the dry plant extracts were dissolved in 500 µl of appropriate solvent according to the polarity and solubility of the extracts as shown in Table 5. The samples were transferred into NMR tubes and run on a Brüker 400 MHz equipment (128 scans at room temperature), and also at 500 MHz for purer compounds.

Table 5: Deuterated solvents used extracts and subextracts

Extract	**Solvent**
Crude methanol	deuterated methanol (CD_3OD)
Hexane	deuterated chloroform ($CDCl_3$)
Chloroform	deuterated chloroform ($CDCl_3$)
Aqueous methanol	deuterated methanol (CD_3OD)

2.3.2.1.2 Carbon Magnetic Resonance (^{13}C NMR)

Carbon magnetic resonance (^{13}C NMR) is complementary to ^{1}H NMR as it gives further information on the environment of the different functional groups and the number of carbons present and how these carbon are correlated to the hydrogens. In addition, ^{13}C NMR is used in the analysis of glycosides, one of which is showing the kind of bonds that link the sugar moieties and their configurations. These two techniques are said to be one-dimensional experiments as they detect more of the organic frame of the compounds, thus providing a very powerful means of structural elucidation. Another complimentary one-dimensional experiment is the Distortionless enhancement by polarization transfer (DEPT-35) which help to confirm the carbons that have protons attached to them, and the carbons that are quarternary (without any hydrogens attached to them).

2.3.2.1.3 Two-dimensional NMR experiments

Two-dimensional NMR experiments include; Correlation spectroscopy (COSY), Heteronuclear Multiple Quantum Coherence (HMQC) spectroscopy and Heteronuclear Multiple Bond Coherence (HMBC) spectroscopy.

COSY detects spin-spin couplings between protons that are close to each other (up to two germinal coupling, ^{2}J or three vicinal coupling, ^{3}J bond distances). It also detects interactions between two different nuclei like that of ^{1}H and ^{13}C. HMQC reveals one bond coupling between a carbon and proton (1-bond H-C), while HMBC shows correlations between protons and the carbon atoms that are two and three bonds away from each other; these couplings are referred to as ^{2}J and ^{3}J, respectively. HMBC spectra

are very informative and allow partial structure fragments to be constructed which can enable the full structure elucidation of the compounds.

In this thesis, the analysis of the extracts and subextracts was performed on an AVANCE 400 MHz spectrometer (Brüker) by ^{1}H NMR experiments with 128 scans. About 50 mg of sample (or the whole sample, if smaller amounts were isolated), were dissolved in 500 µl chloroform-d ($CDCl_3$) (Cambridge Isotopes Laboratories, DLM-7-100) or in 500 µl methanol-d_4 (MeOD) (Cambridge Isotopes Laboratories, DLM-24-10) and transferred into an NMR tube with 5 mm diameter and 17.8 cm length (Aldrich, Z27678). The Brüker Software Topsin 1.3 was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak (MeOD: δ 3.31, δ 4.8 (residual water peak), $CDCl_3$: δ 7.27).

For isolated metabolites, the structure elucidation was performed using a variety of one-dimensional (^{1}H NMR, ^{13}C NMR, DEPT) and two-dimensional (HMQC, HMBC, COSY and NOESY) experiments which were performed on an AVANCE 500 MHz spectrometer (Bruker). Analysis of pure compounds by ^{1}H NMR was performed with 512 scans, for ^{13}C NMR with 2 000 scans with a resonance frequency of 125 MHz and for DEPT-35 with 2 000 scans as well. HMQC and HMBC were both measured with 16 scans, while NOESY with 32 scans and 400 ms mixing. The Brüker Software Topsin 1.3 was used for spectra acquisition and processing. The spectra calibration was carried out on the residual solvent peak, as follows; MeOD: ^{1}H = δ 3.31, δ 4.8 (residual water peak), ^{13}C = δ 40; CDC_{l3}: ^{1}H = δ 7.27, ^{13}C = δ 77; pyridine-d_5: ^{1}H = δ 8.74, δ 7.58, δ7.22, ^{13}C = δ150.35, δ135.91, δ123.87; and for deuterium oxide (D_2O): ^{1}H = δ4.80.

2.3.3 Mass Spectrometry (MS)

Mass spectrometry (MS) is a technique that measures the molecular weight of a compound. It works by fragmenting small amounts of the organic compound and then recording the fragments according to their mass. Firstly, the compound is ionized to positive mode, then accelerated and deflected according to their mass to charge ratio (*m/z*) and finally detected. The lighter and the charged the fragmented particles are, the more they are deflected. Detection depends on the number of charges on the ions such that the resultant spectrum shows relative abundance of the separated ions according to their mass to charge ratio. In this study, a softer ionization technique, called the electrospray ionization (ESI) was used. This technique produces less fragmentation information for structure elucidation.

Mass spectra were acquired using an LCQ Mass spectrometer (Finnigan MAT) equipped with an electrospray ionization source (ESI). The operating parameters were as follows; the spray needle voltage was set at 4.5 kV and the spray was stabilized with a nitrogen sheath gas (700lb/in.^{2}). ES capillary voltage was 30V, helium was used as auxiliary gas (15lb/in.^{2}), and capillary temp was 200^{o}C, collision energy 45% of 5V for MS/MS. A syringe pump delivering 3 µl/min was used for the direct loop injections of pure standards dissolved in MeOH (about 0.3 µg/l). MS experiments were performed in negative mode. The negative MS^2 spectra were obtained with a CE-nano-ESI-$(MS)^2$ with 35 collision-induced dissociation (CID). The ESI (low resolution) and accurate mass (high resolution) experiments were applied to isolated compounds. High resolution accurate mass spectra were acquired using a micromass Q-TOF Ultima Global Tandem Mass spectrometer (Micromass). Samples were measured under electrospray ionization mode using 50% acetontrile (ACN) in water and 0.1% formic acid as solvent.

2.4 BIOLOGICAL ACTIVITY ASSAYS

All the extracts and subextracts obtained from the initial extractions were subjected to activity screenings which included both *in vitro* biochemical and biological assays.

2.4.1 Antimicrobial activities

There are several methods that can be employed for antimicrobial assays. The most common types for susceptibility testing include broth dilution and agar-based methods. Of the latter type, the agar disc diffusion method is the most common. It involves the application of the extract onto a filter paper disc, which is then placed onto solid medium seeded with the test microorganism. It is a simple, reliable, inexpensive and easily adaptable assay (Singh *et al*., 2007). Another of this type is the agar well diffusion which involves the puncturing of a well into solid agar, and the test sample is poured into the well.

The other common assay is the broth dilution method which, is commonly used for the establishment of the minimum inhibitory concentration (MICs). This parameter is important for assessing the antimicrobial potential of analytes. It involves the dilution with broth (in most cases Mueller-Hinton broth) of extract dissolved in a solvent. Then subcultured inoculums on agar slants is prepared in normal saline and compared with a 0.5 McFarland turbidity standard, and then diluted according to the desired inoculums density. In this thesis, we will look at *in vitro* antifungal and antibacterial activity assays using agar disc diffusion and broth dilution methods.

2.4.2 Antifungal activity

The method used to screen the crude methanol extracts was the agar disc diffusion method. Four test organisms were selected for this assay. Two clinical cultures of dermatophytes which included *Trichophyton tonsurans* Z6786 and *Trichophyton interdigitale* were selected on the basis that they are the most common *tinea* fungi isolated from patients of African origin who suffer from ringworms. Two additional fungi, the yeast; *Candida albicans* (NCPF 3153) and a mould; *Aspergillus fumigatus* were used as they cause common ailments.

Approximately 200 ml of distilled water was poured onto overgrown spores of fungi. The spores were lightly scrapped and 200 ml transferred into another bottle. The concentration and density of the suspension was determined and adjusted to 3 x 10^6 CFU MacFarland standard. Then 200 µl of the spore suspension was applied onto Sabouraud dextrose agar plate (Oxoid), spread evenly and dried; 6 mm diameter sterile discs loaded with 100 µg extract/disc and 50µg extract/disc were placed on the seeded agar. Ten microgram per milliliter miconazole (1-[2, 4-dichloro-β- [2, 4 dichlorobenzyl]-oxy) phenethyl] imidazole) nitrate salt) from Sigma and methanol (Fisher) were used as the positive and negative controls, respectively. The plates were incubated for 5-7 days for *T. tonsurans* and *T. interdigitale* and for 18 hrs for *C. albicans* and *A. fumigatus*. Disc diffusion assay was performed according to NCCLS guidelines. Each extract was tested in triplicates.

2.4.2.1 Antibacterial activity

2.4.2.1.1 Broth dilution method

Six clinically important test organisms were selected for the screening of crude methanol extracts from the 8 *Ficus* species under study. They were selected on the basis of the diseases they cause, in correlation with the traditional uses of the *Ficus* species in Zambia. Thus, three gram-positive bacteria; *Staphylococcus aureus* (NCTC 12695), *Enterococcus faecalis* 13379 (ISA) and *Streptococcus pneumoniae* (NCTC 12695) and three gram-negative bacteria included *Pseudomonas aeruginosa* (NCTC 10662), *Acinetobacter baumanii* (ATCC 19606) and *Escherichia coli* (NCTC 10418) were tested.

The stock concentration of the crude extracts was prepared (40 mg/ml) by dissolving 20 mg dry extract into 500 µl 100% dimethylsulfoxide DMSO (Sigma, D8418). This solution was further diluted by transferring 5 µl of the stock solution into 995 µl Muller-Hinton broth (MHB) (CM 0473, Iso-sensitest, Oxoid) inoculated with 1 x 10^5 CFU/ml bacterial suspension, to give a final concentration of 200 µg/ml of the extract. The prepared microtiter plates were then incubated for 18 hrs at 37^{o}C. The complete absence of bacterial growth was regarded as susceptible to the extracts. The results obtained were means of at least three independent assays.

2.4.2.1.2 The disc diffusion method

Four test organisms were selected for the screening of all the plant extracts of the 8 *Ficus* species under study. Three gram-positive bacteria; *S. aureus* (NCTC 12695), *E. faecalis* 13379 (ISA) and methicillin resistant *S. aureus* (MRSA 1199 B NorA) and one gram-negative bacteria; *E. coli* (NCTC 10418) were tested. Preparation of the agar (CM 0471, Iso-sensitest, Oxoid) and MHB broth was done according to the manufacturer's instructions. MRSA is a type of *S. aureus* that has become resistant to many antibiotics, including methicillin, penicillin, amoxicillin, and cephalosporins.

The method was performed according to BSAC (2005) standards. Approximately, 4-5 colonies of inoculum were touched with a sterile swab and suspended in 3 ml broth to give just visible turbidity. The suspension was standardized by measuring the wavelength (Helios α, Thermo Scientific spectrophotometer) of the bacterial suspension at 500 nm. Depending on the reading of the absorbance (Table 6) of different organisms, an appropriate amount of inoculum was transferred into 5 ml sterile broth to give a further dilution. The adjusted suspension was now used to inoculate the plates before applying the discs loaded with plant extract at a concentration of 100 µg extract/disc. The seeded plates were incubated (Genlab) for 18 hrs at 37^{o}C and inhibition was measured as the diameter in milimetres of the clear zone that had no bacterial growth. The assay was done in duplicates and the results recorded as means of the two experiments. The positive control was ciprofloxacin 1 µg/disc (CT 0623B, Oxoid) and 100 % methanol was the negative control.

Table 6: Absorbance and dilution for inoculum suspension.

Organisms	Absorbance at 500 nm	Volume (µl) to transfer to 5 ml sterile broth
E. faecalis	0.05	125
S. aureus	0.5	20
MRSA	0.496	25
E. coli	0.01	250

2.4.2.2 Anti-mycobacterial activity

The anti-mycobacterial activity was assessed by an MTT (3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide) assay based on the method detailed by Montoro *et al*. (2005). The assay assessed the ability of viable mycobacterial cells to reduce the MTT tetrazolium salt by actively growing cells to produce a blue formazan product. Thus, 100 µl of Middlebrook 7H9 media supplemented with 10% oleic acid-albumin-dextrose-catalase, 0.5% glycerol and 0.05% tween 80 were added to each sterile well of a 96-well flat bottom plate. In each well was also added 100 µl of approximately 2.5 x 10^7 *M. tuberculosis* strain H37Rv. Then serial dilutions of the extracts were prepared on the plates by adding 100 µl of the stock solution to the wells to achieve the final concentrations ranging from 256 µg/ml to 1 µg/ml. The plates were sealed then incubated with gentle rocking (30 rocks per min.) for 7 days at 37°C. MTT dye (10 µl filter-sterilized at 5 mg/ml in dH_2O) was added to each well and the plates were re-incubated for another 24 hrs. MICs were recorded as the lowest concentration at which a purple precipitate of formazan did not appear in the wells. Streptomycin (Sigma) was used as the positive control.

2.4.3 Antischistosomal activity

This assay was performed by using CD1 mice supplied by Charles River, UK which were maintained at St Mary's Hospital, Imperial College London. All experimentation was undertaken with the guidelines of the United Kingdom Animal's scientific procedures Act 1986 and approved by the London School of Hygiene and Tropical Medicine ethics committee.

2.4.3.1 Production of schistosomula

A Puerto Rican strain of *Schistosoma mansoni* was maintained by routine passage through *Biomphalaria glabrata* snails and CD1 mice. Schistosomula were prepared as described by Mansour and Bickle (2010). Here, cercariae of the Puerto Rican strain of *Schistosoma mansoni* (Doenhoff, *et al*., 1978) were shed in clean tap water from infected *Biomphalaria glabrata* snails exposed to direct illumination for 1 hr. The cercariae were then concentrated to 20 ml using an 8 µM filter (Sartorius CN, Scientific Laboratory Supplies, Ltd) on a concentration apparatus (Millipore) and cooled on ice for 1 hr. The

water was removed from the pelleted cercariae and replaced by cold serum free medium 169 (Basch, 1981) (M169) which was supplemented with 300 U/ml Penicillin (Gibco, UK), 300 µg/ml Streptomycin (Gibco, UK), and 160 µg/mL Gentamicin (Sigma-Aldrich, UK) (Incomplete M169). Under sterile conditions, cercariae were mechanically transformed into schistosomula using the 'Syringe Method' (James and Taylor, 1976). The cercarial head and tail suspension was layered onto a sterile gradient of 50% and 70% Percoll (Sigma-Aldrich, UK) in M169 in 15 ml polystyrene tubes (Lazdins, *et al*., 1982). These tubes were centrifuged for 10 min at 350 x *g* at 4 ºC, after which the supernatant layers were decanted and the cercarial heads (schistosomula) recovered from the 70% layer. They were washed 3 times in 10 ml of Incomplete M169 by centrifuging again at 400 x *g* and 4 ºC for 2 min. The schistosomula were then washed once more in M169 supplemented with 100 U/ml Penicillin, 100µg/ml Streptomycin and 5% foetal calf serum (Sigma-Aldrich, UK) (Complete M169 or cM169). The schistosomula were then transferred into 6-well plates (Nunc, UK) and incubated overnight at 37 ºC and 5 % CO_2.

2.4.3.2 Schistosomula Drug sensitivity assay

This is the primary larval assay in which 20 mg/ml stock solutions of extracts were prepared in 100% DMSO (Sigma-Aldrich, UK) immediately before use. Then 1 µl of test extracts were added to 100 µl of cM169 in each culture well of 96-well plates (Nunc, UK) and mixed well. The wells were topped up with another 100 µl of cM169 containing 100 schistosomula to give final concentrations of 100 µg/ml of extract in each well. Negative control wells contained larvae cultured in cM169 alone or cM169 with 0.5% DMSO. Positive control wells contained larvae cultured in Praziquantel (Sigma-Aldrich, UK) at 10 µg/ml (32 µM). After 3 days of culture the viability of schistosomula was assessed using an inverted microscope (Leitz Diavert Wetzlar, Germany) and any extracts which caused death (immotile, often showing a characteristic uniform shape and granular appearance) and/or morphological damage (showing a range of altered shapes, granularity and/or blebbing but still with some motility) of ≥ 70% was considered a hit. These hits were tested for IC_{50} in a secondary larval screen run at four serial 1 to 3 extract dilutions from 100 to 3.7 µg/ml or lower if necessary. The IC_{50} values (and IC_{70} values for certain extracts) were calculated using Microsoft XLfit version 5.1.0.0 (2006-2008 ID

Business Solutions Ltd). The average IC_{50} value for Praziquantel in the larval assay was 0.25 µg/ml (0.8µM). Larval hits in the primary larval screen were taken forward to be tested in the adult *ex-vivo* primary screen.

2.4.3.3 Adult worm culture conditions

Adult worm *ex-vivo* drug testing was performed as previously described by Ramirez, *et al*., (2007). The CD1 strain mice were infected by subcutaneous injection with 500 cercariae, freshly shed from infected *Biomphalaria glabrata* snails to obtain *Schistosoma mansoni* (Puerto Rican) adult worms. The worms were harvested, using sterile techniques, by portal perfusion 6 weeks post-infection using warm perfusion medium (Dulbecco's Modified Eagle's Medium [DMEM]), 2 mM l-glutamine, 100 U/ml penicillin, 100µg/ml streptomycin, 20 mM Hepes, 10 U/ml Heparin [Sigma-Aldrich, UK]). Under sterile conditions, the worms were washed 4 X, from host red blood cells by sedimentation in perfusion medium. The perfusion medium was then replaced with complete medium (cDMEM i.e., DMEM containing 10% foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin) and the worms were poured into 6-well plates, after which they were then distributed in test wells of 24-well plates (Nunc, UK) containing 1 ml complete medium to give at least five males and five females per well. The volume in the wells was then topped up to 2 ml of complete medium. Cultures and assays were incubated at 37 ºC and 5% CO_2. The effect of the extracts was recorded as evidenced by worm motility disturbances (e.g sluggishness or paralysis) and morphological changes like relaxation, shrinkage, curling, tegumental disruption and worm disintegration.

2.4.3.4 Adult wormd drug sensitivity assay

For the adult worm screening; 100 mg/ml stock solutions of extract were prepared in 100% DMSO immediately before use. 2 µl of test extracts were added to each culture well to give 100 µg/ml final concentrations. Negative control wells contained worms cultured in cDMEM alone or cDMEM with 0.1% DMSO. Positive control wells contained worms cultured in 10 µg/ml Praziquantel (Sigma-Aldrich, UK). The effect of the extracts was determined on the fifth day of culture using an inverted microscope (Leitz Diavert Wetzlar, Germany). Any extract that produced ≥ 70% worm motility

inhibition ± tegumental damage was recorded a hit in the primary screen. These active extracts were then tested for IC_{50} in a secondary screen run at four serial 1 to 3 dilutions of the extract starting with the concentration used in the primary screening i.e., 100 to 3.7 µg/ml or lower if necessary. The IC_{50} values were calculated using Microsoft XLfit version 5.1.0.0 (2006-2008 ID Business Solutions Ltd). The average IC_{50} value for Praziquantel, the positive reference was 0.36 µg/ml (1.15 µM).

2.4.4 Antiprotozoal activity

The plant extracts were screened for their *in-vitro* activity against four parasitic protozoa, which included; *Trypanosoma brucei rhodesiense*, *Trypanosoma cruzi*, *Leishmania donovani* and *Plasmodium falciparum*. These parasites cause diseases that are prevalent to tropical regions of the world, of which *P. falciparum* causes the deadly malaria.

2.4.4.1 Trypanocidal activity against Trypanosoma brucei rhodesiense

The stock of *Trypanosoma brucei rhodesiense*, STIB 900 strain was isolated in 1982 from a human patient in Tanzania (Witschel *et al*., 2012). After several mouse passages, the strain was cloned and adapted to axenic culture conditions (Baltz *et al*., 1985; Thuita *et al.*, 2008). Minimum Essential Medium (MEM) (50 µl) supplemented with 25 mM HEPES, 1 g/L additional glucose, 1% MEM non-essential amino acids (100x), 0.2 mM 2-mercaptoethanol, 1mM Na-pyruvate and 15% heat inactivated horse serum was added to each well of a 96-well microtiter plate. Serial drug dilutions of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml were prepared. Then 10^4 blood-stream forms of *T. brucei rhodesiense* STIB 900 in 50 µl were added to each well and the plate was incubated at 37°C under a 5% CO_2 atmosphere for 72 hrs. 10 µl of a resazurin (Sigma), solution (12.5 mg resazurin dissolved in 100 ml double-distilled water) was then added to each well and incubation continued for a further 3 hrs (Räz *et al*., 1997). The plates were read with a Spectramax Gemini XS microplate fluorometer (Molecular Devices Cooperation, Sunnyvale, CA, USA) using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Melarsoprol (Arsobal®, Sanofi-Aventis, provided by WHO) was used as a reference drug. Data was analyzed and IC_{50} values were determined using the microplate reader software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA).

2.4.4.2 Trypanocidal activity against Trypanosoma cruzi

For the *in-vitro* assay of *Trypanosoma cruzi*, rat skeletal myoblasts (L-6 cells) (ATCC) were used. These were seeded in 96-well microtitre plates at 2000 cells/well in 100 µL RPMI 1640 medium with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. After 24 hrs the medium was removed and replaced by 100 µl per well containing 5000

trypomastigote forms of *T. cruzi* Tulahuen strain C2C4 containing the β-galactosidase (lac Z) gene (Buckner *et al*., 1996). After 48 hrs, the medium was removed from the wells and replaced by 100 µl fresh medium with or without a serial drug dilution of seven 3-fold dilution steps covering a range from 90 to 0.123 µg/ml. After 96 hrs of incubation, the plates were inspected under an inverted microscope to assure growth of the controls and sterility. Then 50 µl of the substrate CPRG/Nonidet (CPRG: Roche Diagnostic; Nonidet: Calbiochem) was added to all the wells. A color reaction which could be read photometrically at 540 nm developed within 2-6. The data was then transferred into a graphic programme Softmax Pro (Molecular Devices), to calculate IC_{50} values. Benznidazole (Hoffmann-La Roche, provided by WHO) was the standard drug used.

2.4.4.3 Leishmanicidal activity against Leishmania donovani.

In this assay, amastigotes of *L. donovani* strain MHOM/ET/67/L82 (LSHTM) were grown in axenic culture at 37°C in SM medium at pH 5.4 supplemented with 10% heat-inactivated fetal bovine serum (FBS) under an atmosphere of 5% CO_2 in air. One hundred microlitres of culture medium with 10^5 amastigotes from axenic culture were seeded in 96-well microtitre plates. Serial dilutions of the plant extracts covering a concentration range from 90 to 0.123 µg/ml were prepared. Meanwhile, the seeded plates were inspected under an inverted microscope to assure growth of the controls and sterility of conditions after 72 hrs of incubation. Then 10 µl of resazurin solution (0.125 mg/ml resazurin in distilled water) (Mikus and Steverding, 2000) was added to each well and the plates incubated for another 2 hours. The plates were read with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analyzed using the software Softmax Pro (Molecular Devices Cooperation, Sunnyvale, CA, USA). Decrease of fluorescence (interpreted as inhibition) was expressed as percentage of the fluorescence of control cultures and plotted against the drug concentrations. From the sigmoidal inhibition curves the IC_{50} values were calculated. Miltefosine (Sigma) was used as a reference drug.

2.4.4.4 Antiplasmodial activity against blood stage Plasmodium falciparum.

The *in vitro* activity against erythrocytic stages of *P. falciparum* was determined by a modified [^{3}H]-hypoxanthine incorporation assay (Matile and Pink, 1990), using the

chloroquine- and pyrimethamine-resistant K1 strain and the standard drug chloroquine (Sigma). Since resistance development is the challenging factor hampering effective malaria treatment, the multidrug K1 strain was chosen for the assay. It was however, not possible to screen the extracts against the chloroquine sensitive strain as has been suggested by Wright (2010), because of time constraints.

In this method, [^{3}H]-hypoxanthine was used to determine the level of *in vitro* growth inhibition of *P. falciparum* in human erythrocytes (Calderon *et al.,* 2012). Thus, a serial dilution technique which measures the ability of the plant extracts to inhibit the incorporation of [^{3}H]-hypozanthine into the malaria parasite was performed. The parasite cultures were incubated in RPMI 1640 medium with 5% Albumax (without hypoxanthine) and they were then exposed to serial drug dilutions in 96-well microtiter plates. After 48 hrs of incubation at 37°C in a reduced oxygen atmosphere, 0.5 μCi [^{3}H]-hypoxanthine was added to each well. Cultures were incubated for a further 24 hrs before they were harvested onto glass-fiber filters and washed with distilled water. Radioactivity was measured using a Betaplate^{TM} liquid scintillation counter (Wallac, Zurich, Switzerland) and the results recorded as counts per minute (CPM) per well at each plant extract concentration and expressed as percentage of the untreated controls. IC_{50} values were calculated from graphically plotted dose-response curves.

2.4.5 Cytotoxic activity in the mammalian rat skeletal myoblast primary cell line L6

The cytotoxic activity assay was the same as that performed for *Trypanosoma brucei rhodesiense* above. The cytotoxity of the crude methanol extracts and subextracts was performed using L6 cells, which are a primary cell line derived from mammalian rat skeletal myoblast, in order to determine their selectivity. Briefly, the L6-cells (4×10^4) were maintained in 100 µl of RPMI 1640 medium supplemented with 1% L-glutamine (200 mM) and 10% FBS. Plant extract of seven 3-fold dilutions ranging from 90 to 0.123 µg/ml were added to the cultured L6 cells and incubated for 72 hrs. To the plates, 10 µl of 0.125 mg/ml resazurin solution was added as a viability indicator and they were incubated for another 2 hrs. Results were recorded by reading plates with a Spectramax Gemini XS microplate fluorometer using an excitation wavelength of 536 nm and an emission wavelength of 588 nm. Data were analysed using the microplate reader software Softmax Pro. Podophyllotoxin (Sigma) was the standard drug used.

2.4.6 *In vitro* FAS-II inhibition assay

There were three steps that characterized the FAS- II inhibition assay. Prior to performing the inhibition assay, the FAS enzymes were first expressed in *E. coli* BL21 (DE3)-CodonPlus-RIL cells (Stratagene) and then purified. Below are detailed outlines of the methods.

2.4.6.1 Protein Expression in E. coli BL21-CodonPlus (DE3)-RIL

BL21 and its derivatives are popular strains obtained from *E.coli* mammalian gut for recombinant protein expression. These systems offer the best characterized systems for overexpression of recombinant proteins as genes of interest can be introduced. Thus IPTG inducible protein expression can be performed because they contain T7 RNA polymerase under the control of the lacUV5 promoter. In this work the strain BL21-CodonPlus (DE3)-RIL (Stratagene) cells was used. This strain contains extra copies of the *argU, ileY,* and *leuW* tRNA genes which are rarely expressed in *E. coli*, as well as a gene for chlorophenicolacetyltranferase enzyme which mediates chloramphenicol resistance.

In this expression glycerol stocks of cloned FAS enzymes FabI (NcoI, BamHI (Perrozo *et al*, 2002)), FabG (NcoI, EcoRI (Tasdemir *et al*., 2006)) and FabZ (Nde, EcoRI (Kostrewa *et al*., 2005) were grown in separate batches. Thus, they were expressed in 1 L terrific broth medium (Fluka, T0918) made by weighing out 47.6g Terrific Broth medium into three 1litre flasks plus 8ml glycerol filled to 1 L with deionized water. The medium was autoclaved 121^{o}C for 45 minutes and then cooled, after which 1 mg chloramphenicol (Sigma, C0378) and 1 mg Kanamycin sulphate (Sigma, 60615) was added. The medium was then inoculated with frozen *E. coli* bacteria from a previously made glycerol stock stored at -80^{o}C and incubated over night at 37°C and 160 rpm. The enzyme expression was induced after about 18 hrs by adding 1 mM isopropyl-β-D-thiogalactpyranoside (IPTG) (Calbiochem, 420322) and further incubated for 5 hrs at 37°C and 160 rpm. The bacterial cells were harvested by centrifugation at 4°C and 5000 rpm (Avanti J-E centrifuge, rotor 16.25, Beckmann Coulter) for 20 mins and the pellets (containing the enzymes) were immediately frozen and stored at -20°C until needed.

2.4.6.2 Enzyme Purification

The enzymes were purified using the Nickel-NTA-affinity chromatography as all FAS-II enzymes were cloned with polyhistidine residues (His-tag). This method is well established (Porath *et al*., 1975) as it uses the ability of 6 histidine amino acids at either the N or C terminus of a protein to bind nickel (Ni^{2+}), which is also bound to an agarose bead by chelation using nitrilotriacetic acid (NTA) beads. When the lysed protein is poured onto the column, the His-tagged proteins bind to the NTA strongly while the low affinity proteins do not. Since imidazole has a greater affinity for Ni^{2+}, it is used to elute the protein. Low imidazole (20 mM) buffer is used to elute low affinity bound proteins while the high affinity bound protein is eluted from the NTA-beads with 500 mM imidazole. The collected protein is then processed by gel filtration using equilibrated PD-10 desalting column. This technique separates high molecular weight proteins from low molecular weight substances such as salts, free labels and impurities which maybe in the the protein.

The procedure was as follows: The frozen *E.coli* protein pellet was thawed and re-suspended in 35 ml cold lysis buffer which is made up of 20 mM TRIS (Aldrich,

154563), 500 mM Sodium chloride (NaCl; Acros organics, 2077900), 20 mM imidazole (Sigma-Aldrich, 56750), 1% glycerol (Sigma, 49767) at pH 8.0. To the suspension was added 1 mM PMSF (Sigma, 78830) in isopropanol and very minute amount of deoxyribonuclease I (DNase) from bovine pancrease (Sigma, DN25) for FabI and FabG only. The suspension was then lysed by passing it through the French Press (Thermo) twice under a pressure of 1500 *psi*. The lysate was centrifuged at 9000 rpm for 40 minutes at 4°C (Avanti J-E centrifuge, rotor 25.50, Beckmann Coulter) and the supernatant was filtered (Cellulose nitrate, Ø 0.45 µm).

The supernatant was loaded onto an Econo-Pac chromatography column (Bio-Rad, 732-1010), which was rinsed with distilled water and then washed with 20 ml cold lysis buffer and 10 ml cold 50 mM imidazole buffer B. This column, which is packed with 3 ml nickel-NTA agarose (Qiagen, 30210), and placed on top of the column, is a porous 30 µm polyethylene bed support. Before each use the column was washed with regeneration buffer containing 8 M (20 ml) Urea (Sigma U5378), 20 mM TRIS and pH 7.4, then followed by a wash with buffer containing 500 mM imidazole, 20 mM TRIS, 500 mM NaCl and 10% glycerol (V/V). It was finally equilibrated with ten times (20 ml) cold lysis buffer (20 mM imidazole).

From the column, the enzymes were eluted in 1 ml steps with 500 mM imidazole. The eluted fractions were tested for protein content by adding 10 µl of each fraction into tube containing 790 µl H_2O and 200 µl Bio-Rad protein assay dye reagent concentrate (Bio-Rad, 500-0006), noting the colour change of the dye from brown to blue as in

Figure 28 shows tubes 3, 4, 5 and 6 as containing the protein. Fractions containing protein were combined and then concentrated to 2.5 ml in an Amicon ultra-15 centrifugal filter unit with Ultracel-10 membrane (Millipore, UFC901024) by centrifugation at 4000 g (Megafuge 1.0R, Heraeus) at room temperature. The enzyme was desalted using a PD-10 desalting column (GE Healthcare, 17-0851-01) with a cold desalting buffer containing 20 mM 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) (Sigma, 54457), 150 mM NaCl all at pH 7.4 to elute the protein.

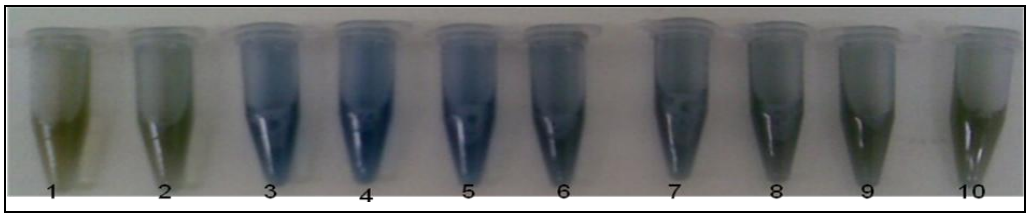


Figure 28: Presence of protein in Bio-Rad protein assay dye reagent.
(The blue colour change from brown indicates the presence of protein in vials 3, 4, 5 and 6).

The enzyme concentration was determined spectrophometrically by measuring the absorption of 10 µl protein in 990 µl 20 mM HEPES buffer C using a semi-micro cuvette of quartz glass (45 mm H x 12.5 mm D x 12.5 mm W, column1400 µl, light path 10 mm) (Hellma, 104-QS) at 280 nm on a Lambda 25 UV/VIS spectrometer (Perkin Elmer). The following formula was used to determine the concentration:

$[c] = \text{mean } A_{protein} \times 100 / A_1^{0,1\%}$

Where $A_1^{0,1\%}$ with His-Tag values for the enzymes are 0.951 for FabI, 0.586 for FabG and 0.606 for FabZ. Enzyme concentrations below 4 mg/ml were futher concentrated in an Amicon ultra-4 centrifugal filter unit with Ultracel-10 membrane (Millipore, UFC801024). The protein was stored in 50% glycerol (Sigma-Aldrich, 49767) at -20°C to be used within 3 months under these conditions.

2.4.6.3 Enzyme documentation by SDS-PAGE

The enymes were documented by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE), which is the most widely used method to determine the complexity and molecular mass of constituent polypeptides in a protein sample. It is simple and quick to use, and also polypeptide compositions of small amounts of proteins can be analyzed. The proteins are readily solubilized by sodium dodecyl sulphate (SDS), an ionic detergent, designed to dissociate all proteins into individual polypeptide subunits. It accumulates stoichiometrically at the protein (1 molecule SDS per 2-3 amino acids) so that the amount of amino acids and negative charge is proportional. The electrophoretical mobility of a protein is therefore proportional to the logarithm of its molecular weight. The tertiary and secondary structure of the protein is dissolved by breaking down

hydrogen bonds and straightening of the molecule. Dithiothreitol (DTT) or C-mercaptoethanol in the SDS Sample buffer reduce intermolecular disulfide bonds between cysteins and disrupt the quaternary structure.

Samples were collected from the total lysate (T_c) before centrifugation, the supernatant (S) and pellet (P) after centrifugation, the flow through after loading the column (FT), the flow through (W) after washing the column with 50 mM imidazole, from each fraction containing enzyme before combination (vial numbers 3,4 and 5 as in

Figure 28) and from enzyme fractions after desalting (vial numbers 1, 2 and 3). All the samples were added up to 20 µl with sample buffer containing 166 mM TRIS pH 6.8, 10% glycerol, 2% (W/V) Sodium Dodecyl Sulphate (SDS) (Sigma-Aldrich, L6026), 3.5% 2-mercaptoethanol (Sigma, M7522), 17% bromphenol blue xylene cyanole dye solution (Sigma, B3269) and heated for 5 minutes at 95°C. The purpose was to denature the protein so that most of the polypeptides could bind to SDS in a constant weight ratio allowing the SDS-polyacrylamide complexes have an identical charge density and migrate in the polyacrylamide gels of the correct porosity strictly according to polypeptide size. Because of an alkaline pH-value used during electrophoresis and the interaction with SDS the proteins move towards the anode, a low range SDS-PAGE standard (Bio-Rad, 161-0304) was used as a marker.

The samples were thus loaded into the 15% polyacrylamide stacking gel which was prepared with tetramethylethylenediamine (TEMED) (Sigma, T9281) and 10% (W/V) ammonium persulphate (APS) (Sigma-Aldrich, 248614), 0.5 M TRIS pH 6.8, 30% acrylamide, 10% SDS and deionized water. The separating gel was composed of TEMED, 10% APS, 30% acrylamide, 1.5 M TRIS pH 8.8, 10% SDS and deionized water. Electrophoresis was then run on 200 V (constant) and a maximum of 400 mA for 50 minutes using a running buffer containing 25 mM TRIS, 192 mM glycine (Sigma, 50046) and 0.1% (W/V) SDS. After electrophoresis, the gel was transferred into a staining solution composed of 0.1% (W/V) brilliant blue G (Sigma, 27815), 50% methanol and 6% glacial acetic acid (VWR, analytical grade) for 30 seconds in a microwave set at the maximum power setting (Cookworks, 650 W). It was then destained for at least 12 hrs in 5% methanol and 7.5% glacial acetic acid.

2.4.6.4 Enzyme inhibition assay

The activities of extracts against FabI, FabG and FabZ were determined spectrophometrically by determining IC_{50} values, in which the changes in the light absorbed by the sample reaction mixture was recorded. The enzymes under study were two reductases (FabI and FabG) and a dehydratase (FabZ). In the enzyme inhibition assay as illustrated in Figure 29, Fab G (β-oxoacyl-ACP reductase) reduces the substrate acetoacetyl-CoA to β-hydroxyacyl-CoA in the presence of NADPH, in which NADPH gets oxidized to $NADP^+$. The changes in the absorption of the cofactors at 340 nm in the absence of specific inhibitors (compound or extract) are measured and compared to the reduction measured with the inhibitor. In the same manner, FabI (Enoyl-ACP reductase) reduces the double bond of the substrate crotonyl-CoA to yield butyryl-CoA in the presence of cofactor NADH. The oxidation of NADH to NAD^+ in the reaction is measured by observing the decrease in absorption at 340 nm. For the third enzyme, FabZ (β-hydroxyacyl-ACP dehydratase) the same principle applies, however, it oxidizes the reverse reaction of crotonoyl-CoA to β-hydroxyacyl-CoA and the change in absorbance is measured at 263 nm. At this wavelength the reduction measured is due to the elimination of the double bond in crotonoyl-CoA. If a compound or extract inhibits an enzyme, the decrease in the absorption will be prevented. By increasing and decreasing the compound or extract concentration, complete inhibition and no inhibition can be measured. DMSO was used as a positive control as it was the solvent in which the extracts were dissolved and it provided full enzyme activity at 0.1% in its final concentration in the assay.

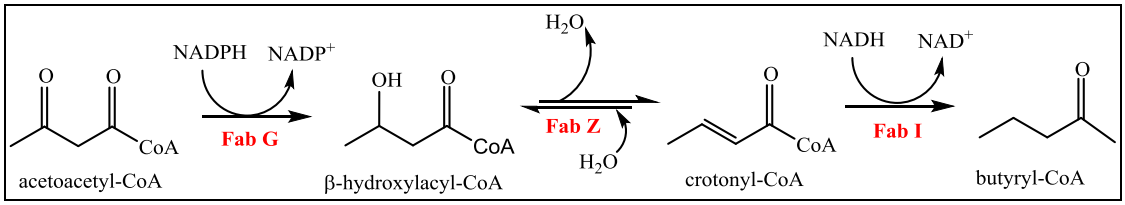


Figure 29: Illustration of FAS enzymes catalyzing the oxidation of NADH/NADPH to NAD^+/ $NADP^+$.

Before the assay, one milligram of the standards and extracts were dissolved in 100% DMSO (Sigma, D8418) with stock concentration of 10 mg/ml (to achieve 0.1% final

concentration in reaction mixture). Depending on how many samples had to be tested, a specific amount of the enzyme was taken from the stock and diluted with the HEPES buffer C (20 mM HEPES, 150 mM NaCl, pH 7.4). The cofactor and the substrates were dissolved in deionized water at a concentration of 1 mg/ml. During the assay, they were ten-fold diluted to lower concentrations with 100% DMSO until full enzyme activity was detected. The highest concentration measured for the extracts was 50 µg/ml. For FabG inhibition, 100 µM of the cofactor NADPH (Fluka, 93220) was added to 1 µg enzyme and sample mixed well. The reaction was started by addition of acetoacetyl coenzyme A sodium salt hydrate (acetoacetyl-CoA) (Sigma, A1625) substrate. The mixture was then read for 1 min at 340 nm. The same was performed for FabI, however, using NADH (Sigma, 43420) as co-factor and 50 µM 2-butenoyl coenzyme A lithium salt (Crotonyl-CoA) (Sigma, C6146, UK) as a substrate. Meanwhile, FabZ was measured at 263 nm for 2 min in the presence of 25 µM crotonyl-CoA and 1 µg enzyme. The negative control was the measurement of the 0.1% DMSO activity in the absence of the enzyme. The components for the enzyme assay were added into a quartz cuvette (Hellma, 104-10-40) and then HEPES buffer C was added up to 1 ml in the order as listed below;

Volume	Reagent
996 µl	Measuring buffer (20 mM HEPES and 150 mM HCl)
1 µl	Cofactor
1 µl	Enzyme/s
1 µl	DMSO
1 µl	Substrate/s

Determination of activity was done by calculating the mean slope of at least three measurements (absorption against time) of the negative control. To determine the activity of the enzyme in the presence of the compound or extract, the following formula was used:

$$Enzyme\ activity\ in\ \%\ (X) = \frac{change\ in\ absorbance\ for\ sample}{average\ change\ in\ absorbance\ for\ negative\ control} X\ 100$$

The activities of different extracts (У-values) were plotted against the extract concentrations (X-values) (Figure 30). If the extract inhibited the enzyme, a decrease of the activity was seen descending as the extract concentrations increased. The IC_{50} value could then be read on the plot. Sample concentrations were decreased until 100% activity was achieved. The limitations of these measurements were either the absorption of the spectrophotometer or the solubility of the sample. When the extracts were more concentrated, they had high concentration of chromophores (colour intensity), hence all the light was absorbed leading to a positive slope. In our experiments, the highest concentration was pegged at 50 µg/ml. Hence to find out the enzyme percentage inhibition, the change in absorbance (in this caes change in activity) was calculated divided by the average change in absorbance for the negative controls. The sample concentration to start the assay was 10 µg/ml and all the samples that recorded enzyme activity less than 50 % were next measured at 50 µg/ml, while those that gave enzyme activity more than 50 % were measured next at 1 µg/ml. However, prior to the measurement of samples, pure standard compounds were measured to ascertain the viability of the enzymes. Plots for change in absorbance for the positive controls is illustrated in Figure 30A. The standard substrates used for as posistive controls for FabI was triclosan (Sigma, 72779) and that of FabG and FabZ was e*pigallocatechin gallate* (EGCG) (Fluka 50299). The concentration of the standards (triclosan and EGCG) which inhibited 50% of the enzyme activity (IC_{50}) was calculated and a graph of activity against concentration was plotted as illustrated in Figure 30B.

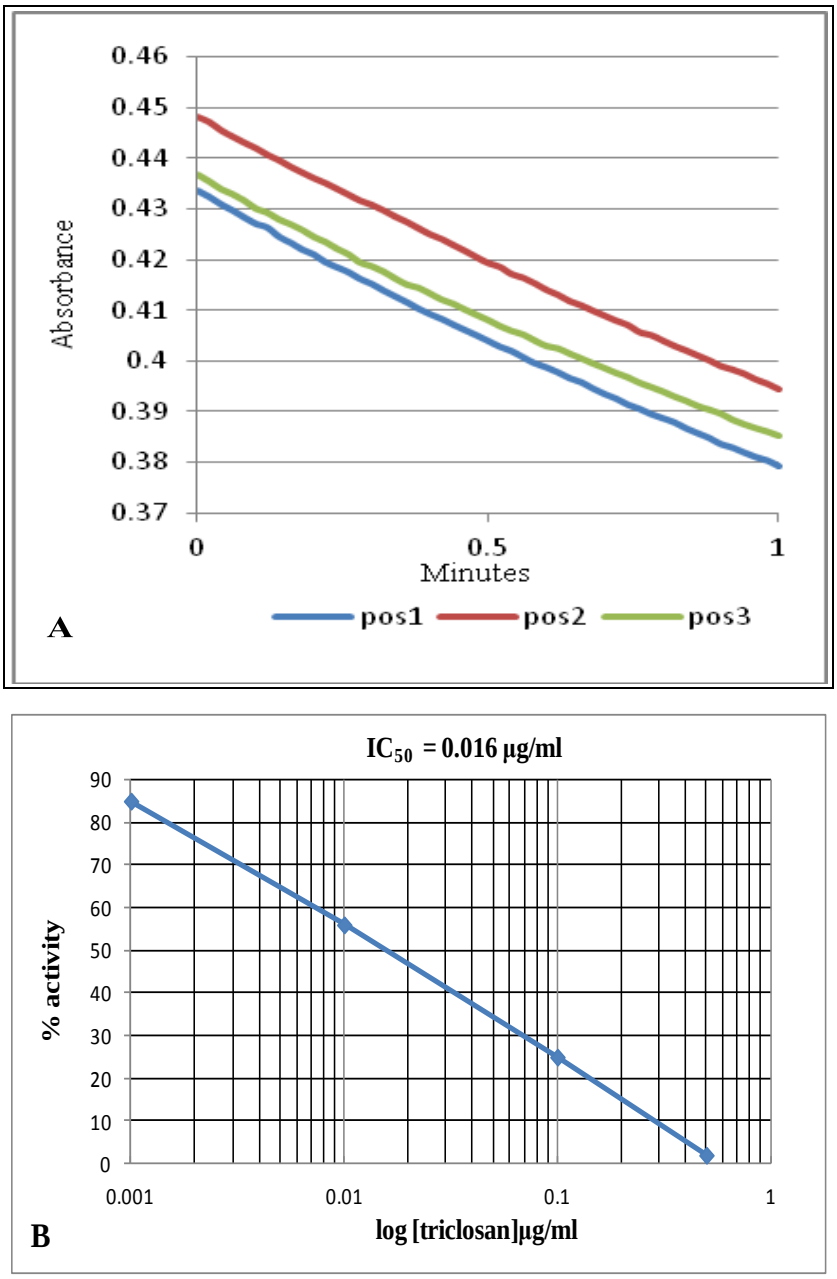


Figure 30: A) Plots for change in absorbance for negative controls. B) Activity against concentration for triclosan, a standard substrate used for FabI.

3. RESULTS AND DISCUSSION

3.1 PLANT EXTRACTION

Finely ground plant materials of the eight *Ficus* species were extracted with 100% methanol by continuous maceration on a magnetic stirrer for over 24 hrs. Table 7 (below) shows the percentage yields from dry mass of the obtained crude extracts (CR-MeOH) which range from 3.96% (root extract of *F. ingens*) to 16.95% (leaf extract of *F. lutea*). However the % yield of the CR-MeOH of the large scale extraction of *F. sansibarica* was higher with 23% yield. There were only nine out of the 24 extracts that yielded over 10% of the dry mass.

The CR-MeOH extracts were then subjected to liquid-liquid partitioning. The subextracts were partitioned according to polarity and the % yield was calculated from the yield of the CR-MeOH extract. The aq. MeOH subextracts had the highest yields which can be attributed to the presence of large amounts of sugars. The partitioning provided a coarse separation of the the metabolites into nonpolar (hexane), medium polar ($CHCl_3$) and polar (aq.MeOH) compounds.

Table 7: The percentage (%) yield of the plant extractions

Species	Plant part	% yield from dry mass	% yield from CR-MeOH extract		
		CR-MeOH	Hexane	Chloroform	Aq-MeOH
F. ingens	L	8.45	9.26	10.62	34.8
	S	9.15	16.5	14.6	34.0
	R	3.96	14.9	15.0	33.5
F. lutea	L	16.95	5.2	6.6	33.7
	S	13.45	11.0	7.7	44.3
	R	11.80	10.4	23.1	49.5
F. natalensis	L	15.20	8.9	8.3	46.1
	S	8.80	8.8	8.5	63.4
	R	11.50	5.7	9.9	47.1
F. ovata	L	11.0	11.8	12.1	48.4
	S	9.60	14.2	11.9	35.9
	R	13.15	13.7	11.6	45.6
F. sansibarica	L	13.55	12.2	24.05	36.5
	S	9.75	5.12	3.14	36.2
	R	8.08	13.9	12.4	45.5
F. sycomorus subsp. *gnaphalocarpa*	L	9.10	16.7	12.7	39.7
	S	8.25	8.4	8.4	66.4
	R	12.45	6.4	11.2	60.3
F. sycomorus subsp. *sycomorus*	L	7.15	16.7	7.2	27.4
	S	5.41	12.8	6.0	50.5
	R	7.32	21.9	18.4	20.2
F. wakefieldii	L	9.55	14.1	21.1	45.8
	S	7.74	21.6	9.3	56.2
	R	9.22	12.4	12.0	36.8
F. sansibarica (large scale extraction)	L	23.5	7.35	16	53.66

L- leaves, S- stem bark and R- root bark.

3.2 PHYTOCHEMICAL PROFILING

The use of herbal medicines has been in existence for centuries and has never stopped since. Their worldwide use has even increased in the last decade and developing countries like Zambia is not an exception. As infectious diseases are predominant and other debilitating non-communicable illnesses are on the increase, the use of herbal medicines by consulting traditional healers is part of most households' primary care system (Ndubani and Höjer, 1999). Regardless of the use of these traditional herbal medicines over many centuries, only a very small percentage of plant species have been scientifically validated in Zambia. Even worse, the safety and efficacy of the extracts and active ingredients of these medicines are not documented.

However, the World Health Organization (WHO) as the custodian of global health matters has encouraged the consistent use of traditional plant based medicines because of their accessibility and affordability to many communities burdened with diseases. As such, it has developed technical guidelines for quality control assessments (Springfield *et al*., 2005). To this effect, TLC and HPLC are the separation techniques in pharmacopoeial monographs most commonly reported.

TLC has the advantage of requiring minimal infrastructure so it would be economical to use for phytochemical screenings. However, HPLC provides enhanced resolutions and separation (Rewald *et al*., 1994). HPLC has been a technique of choice as a reference tool for the identification and quality assessment of herbal medicines (Liu *et al*., 2007; Ma *et al*., 2007; Springfield *et al*., 2005). Even more the coupling of HPLC to the Diode array detector (DAD) has increasing use for the screening of drugs, and also for natural products (Springfield *et al*., 2005). With the tool of analysis in metabolomics, on top of HPLC analysis, NMR is also increasingly being used.

3.2.1 Thin Layer Chromatography (TLC)

The crude extracts being a mixture of many different chemical constituents with different functional groups, of different polarities and in variable concentrations, an appropriate separation solvent system was selected after several trials. The selection was based on the solvent system's ability to separate the chemical constituents distinctively with spots

appearing with R_f values that were between 0.1 and 0.9. Based on the results, the solvent system that separated the crude methanol extracts and aq. MeOH subextracts quite reasonably constituted ethyl acetate: methanol: water: acetic acid (41:5:4: 0.5). The best solvent system found for hexane subextracts was hexane: ethyl acetate: acetic acid (8:2:0.5), and that of chloroform subextracts contained chloroform: ethyl acetate: acetic acid (6:4:0.5).

The developed TLC plates were visualized under UV light at 254 and 365 nm to detect UV active or absorbing plant constituents and were then sprayed with 4% vanillin in sulphuric acid reagent spray and heated to optimize colour development. Two other visualizing reagents were used. These are natural product spray and Dragendorff's reagent used to visualize polyphenolic compounds and alkaloids, respectively.

3.2.1.1 Crude MeOH extracts and aq. MeOH subextracts

The chemical composition of the CR-MeOH extracts of the leaves was dominated by pink and yellow spots which, appeared at the bottom and middle of the plates (Figure 31A). This implies that the extracts constituted medium polar to polar compounds. However, less prominent violet/purple spots also appear at the top of the plate indicating the presence of nonpolar components. The TLC of the aq. MeOH subextracts (Figure 31B) of the leaves resembled that of the CR-MeOH profile, except for the violet/purple spots which were missing. Track 9 denoting the leaf extract of *F. lutea* leaves exhibited a different profile from the rest as it did not have distinct chemical components highlighted. As for the stem bark and the root bark, the CR-MeOH extract and the aq. MeOH subextracts were run on the same plate because of the similarities that were observed during the process of mobile phase selection. The chemical composition of the CR-MeOH extracts of the stem bark and root bark extracts resembles that of the leaves (Figure 31A and B). The CR-MeOH extract was then subjected to liquid-liquid partitioning to yield the hexane, $CHCl_3$ and aq. MeOH subextracts.

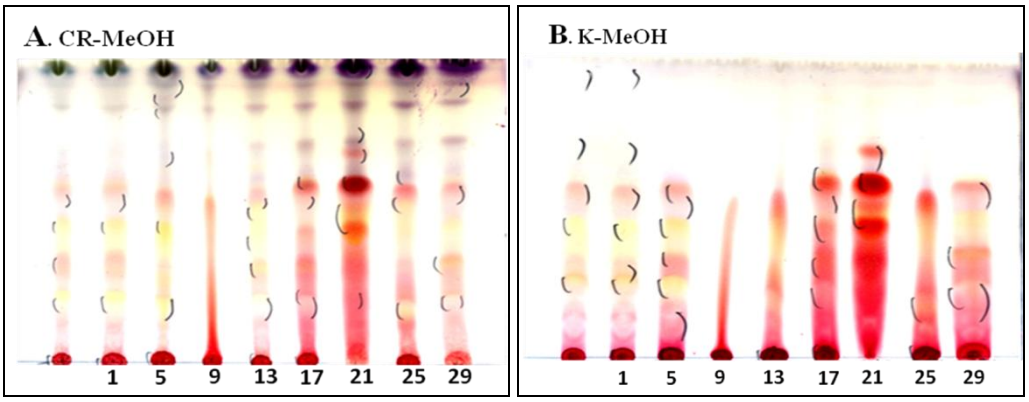


Figure 31: TLC profiles of leaf CR-MeOH extracts (A) and the aq. MeOH subextracts (B).

1 - *F. ingens*; 5 - *F. sycomorus* subsp. *gnaphalocarpa*; 9 - *F. Lutea*; 13 - *F. natalensis*; 17 - *F. ovata*; 21 - *F. sansibarica*; 25 - *F. sycomorus* subsp. *sycomorus*; 29 - *F. wakefieldii*

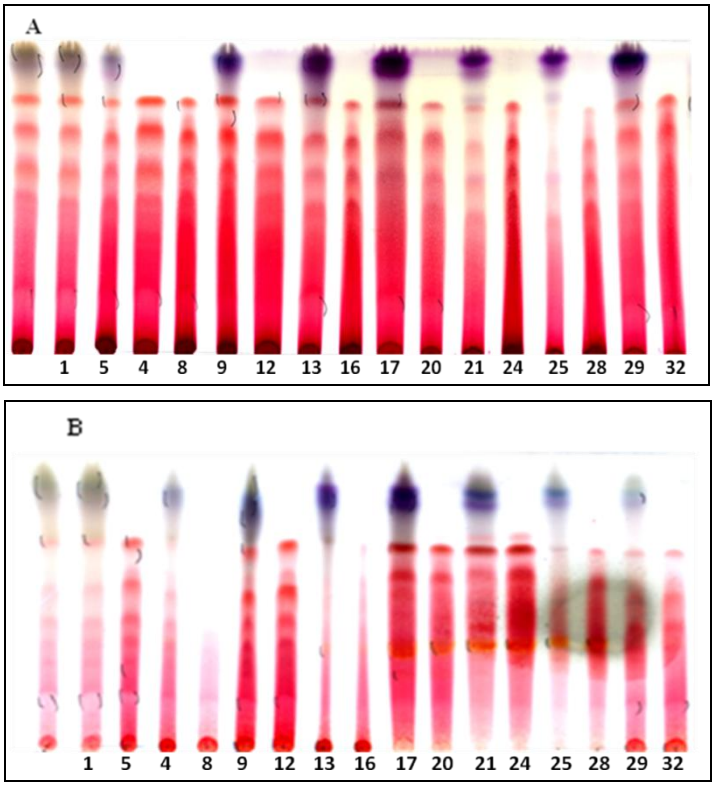


Figure 32: TLC plates of the CR-MeOH extracts and aq. MeOH subextracts of the stem barks (Plate A) and the root bark (Plate B).

Plate A – ethyl acetate: methanol: water (41:5:4) Plate B – ethyl acetate: methanol :water (40.5:5.5:4)

5, 8 - *F. sycomorus* subsp. *gnaphalocarpa*; 1, 4 - *F. ingens*; 9, 12 - *F. Lutea*; 13, 16 - *F. natalensis* ; 17, 20 - *F. ovata*; 21, 24 - *F. sansibarica*; 25, 28 - *F. sycomorus* subsp. *sycomorus*; 29, 32 - *F. wakefieldii*

3.2.1.2 Hexane subextracts

The hexane substracts were the first to be obtained dominated by the least polar constituents. The TLC analysis was run with a predominantly nonpolar mobile solvent to obtain a separation that was dominated by purple spots at the top of the plate (Figure 33). On the TLC plate (Figure 33A), tracks 2 (*F. ingens*) and 6 (*F. lutea*) did not exhibit an abundance of these metabolites. However, subextracts of the leaves (Plate A) show great similarity to the root extracts (Plate C), which are dominated with purple and blue spots around the middle of the plate. Plate D of the roots was sprayed with Dragendorff's reagent to highlight the possible presence of alkaloids which appear as brown spots. Tracks 2, 6, 10 (*F. ingens, F. sycomorus* subsp. *sycomorus, F.lutea* and *F.natalensis*) exhibited a strong presence of brown spots which in this case could represent alkaloids. The stem barks exhibited quite a rich profile with orange, pink, purple and blue spots on plate B (Figure 33).

Comparison between the plant species revealed that track 2 for *F. ingens* leaves and barks were as rich in different types of compounds as was tracks 6, 14 and 18 representing *F. sycomorus* subsp. *gnaphalocarpa, F. natalensis* and *F. ovata*. Meanwhile, tracks 22 for *F. sansibarica*, 26 (*F. sycomorus* subsp. *sycomorus*) and 30 (*F. wakefieldii*) of the leaves were very similar to the profile of the stems, while track 22 for *F. sansibarica* of the root extract was very different, with no nonpolar spots shown.

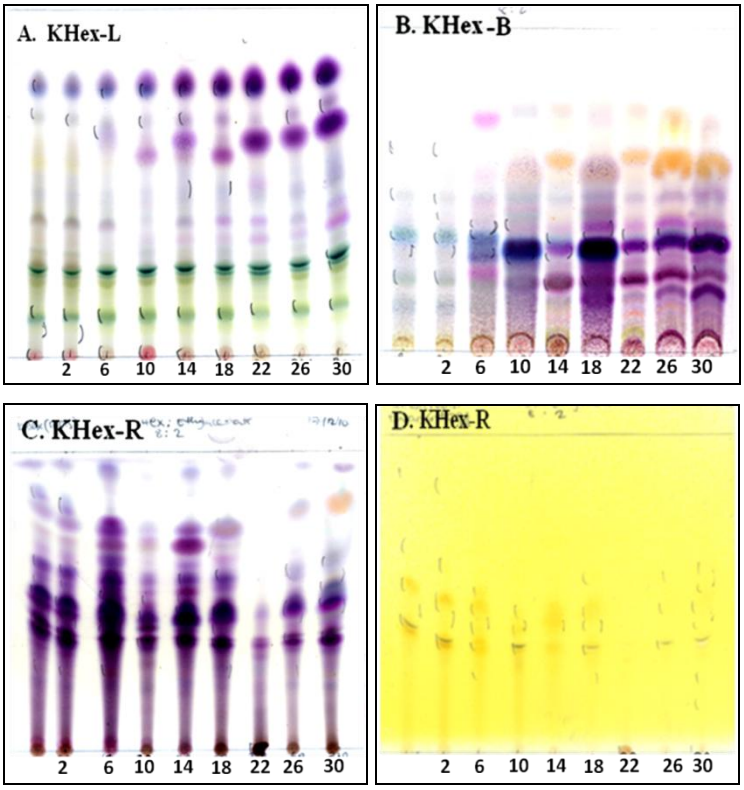


Figure 33: TLC for the hexane subextracts of the leaves (Plate A), stem barks (Plate B) and the root barks (Plates C and D).

Plates A-C were sprayed with VS. Plate D was sprayed with Dragendorff's reagent. The mobile phase was hexane: ethyl acetate (8 : 2)

2 - *F. ingens* ; 6 - *F. sycomorus* subsp. *gnaphalocarpa*; 10 - *F. lutea*; 14 - *F. Natalensis*; 18 - *F. ovata*; 22 - *F. sansibarica*; 26 - *F. sycomorus* subsp. *sycomorus***;** 30 - *F. wakefieldii*

3.2.1.3 Chloroform subextract

The $CHCl_3$ subextracts were dominated by the compounds of medium to high polarity and as such a medium to high polar mobile solvent was used in the analysis. Thus chloroform/ethyl acetate (6:4) was used as mobile phase. The TLC Plates A-C (Figure 34) were sprayed with NP/PEG and observed under UV 366 nm to detect flavonoids and related polyphenolic compounds which are abundant across the genus *Ficus*. Figure 34 (leaf extract) was dominated by red spots at the top end of the plate depicting the presence of chlorophyll. Nonetheless, track 11 (*F. lutea*) did not show chlorophyll spots and any other abundant flavonoids. This can be observed as well on plate D which was sprayed with VS. Different classes of flavonoids have been highlighted by different colours of spots; for instance orange spots could depict the presence of flavonol or flavone glycosides. The dark and light blue spots at the bottom of the plate could represent phenol carboxylic acids and/or coumarins. Apart from *F. lutea*, tracks 3 (*F. ingens*) and 23 (*F. sansibarica*) have a slightly different chemical profile. Most flavonoids also exhibit yellow to red colour because of conjugated chromophores present (Qiao *et al.*, 2011).

In Figure 34, Plates A - C were sprayed with NP/PEG, plate D with VS and Plate E with Dragendorff's reagent. The plate for the $CHCl_3$ of root extracts was characterized by deep orange, bright blue and fluorescent spots. Track 15 (*F. natalensis*) showed a unique composition of just dark blue spots. Track 3 (*F. ingens*) on the stem bark plate B has a pronounced bright blue fluorescent spot unlike other species, which on the leaf extract plate A appears as purple spots. Dragendorff's reagent didn't visualize the alkaloids present as evidenced by (Figure 34**Error! Reference source not found.**E). When comparing the leaf to the stem bark extracts, a significant difference is the absence of the spots presumably of chlorophyll which appear in red in the leaf extracts. In addition, the comparison of leaf extracts to root extracts reveals appearance of bright orange and green fluorescent spots under UV 366 nm in the roots. The apparent red spots in the roots are thus not an indication of chlorophyll, but compounds that have conjugated chromophores.

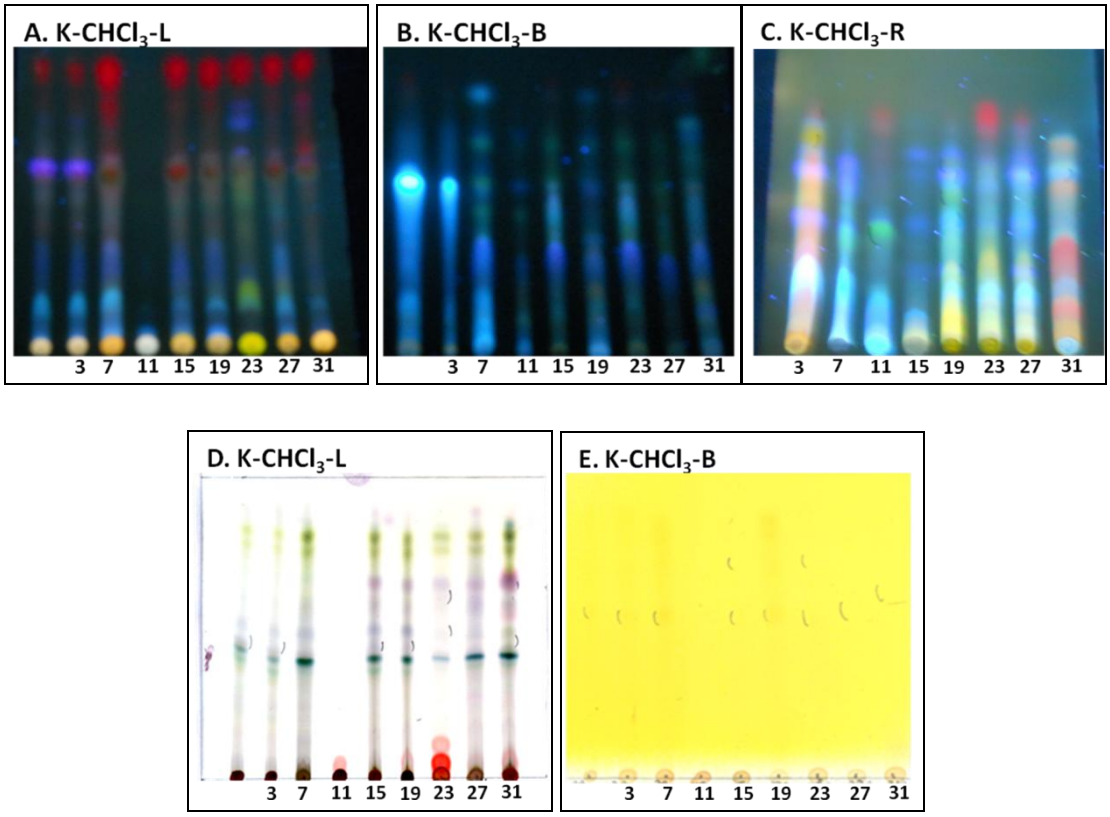


Figure 34: TLC plates of the $CHCl_3$ subextracts of the leaves (Plates A and D), stem barks (B and E) and the root barks (Plate C).

Plates A-C were sprayed with NP/PEG and viewed under wavelength 366 nm. Plate D was sprayed with VS and plate E was sprayed with Dragendorff's reagent.
The mobile phase used was Chloroform: ethyl acetate (6:4)

3 - *F. ingens*; 7- *F. sycomorus* subsp. *gnaphalocarpa*; 11 - *F. lutea*; 15 - *F. natalensis*; 19 - *F. ovata*; 23 - *F. sansibarica*; 27 - *F. sycomorus* subsp. *sycomorus*; 31 - *F. wakefieldii*

The TLC screenings were done for rapid detection of the different plant constituents; however, for complex crude extracts it offers lower accuracy and precision.

Overall, the TLC plates for the crude, hexane, chloroform and aqueous methanol extracts and subextracts were dominated with purple, orange, red, yellow and blue spots. The TLC plates were viewed under 254 nm and 366 nm UV light to identify the type of chemical compounds that were constituted in the extracts. One group of compounds that were evident in the *Ficus* extracts are flavonoids which cause fluorescence quenching

under the UV light at 254 nm. Meanwhile, under the 366 nm UV light, flavonoids show dark yellow, green, or blue fluorescence (Waksmundzka-Hajnos *et al*., 2008). The red colour of spots which were also prominent could denote flavonoids because of conjugated chromophores present in the molecules (Effendy *et al*., 2011; Qiao *et al*., 2011). The resolution of the chemical constituents was not precise, as was observed from the broad spots (tailing) which is one of the signs indicating the presence of charged functional groups. However, an attempt was made to reduce the tailing by acidifying the mobile phases with 0.1% acetic acid which is sufficient to reduce the dissociation of ions. With VS visualizing spray, red or purplish-red spots are normally produced by catechins and proanthocyanidins immediately after spraying and warming the plates, while flavanones and dihydroflavonols develop more slowly (Waksmundzka-Hajnos *et al*., 2008). Much as the spots could not be quantified, in this thesis, we can only assume that the intensity and size of the spots indicate the abundance of the group of compound in the extract, or it maybe that it is the most dissolved group of compounds in the solvent used both for extraction and for the chromatographic analysis. To sum up these results, the CR-MeOH extracts of the leaves, stem barks and root barks of *F. lutea* and *F. sansibarica* displayed different TLC profiles. Notably as well, are the $CHCl_3$ subextracts of the stem and roots of *F. ingens*.

3.2.2 Proton Nuclear Magnetic Resonance (^{1}H NMR)

The CR-MeOH extracts and liquid-liquid partitioned subextracts of the *Ficus* species were subjected to ^{1}H NMR analysis. The ^{1}H NMR profiles of all the species were dominated by signals between δ_H0 - δ_H2, δ_H3 - δ_H5 and δ_H6 - $\delta_H7.5$ indicating the presence of aliphatic groups, sugars and some aromatic compounds. The chemical shifts (δ) given in ppm for the solvents used to dissolve the samples appeared at $\delta_H7.27$ for $CDCl_3$ and $\delta_H3.31$ for CD_3OD. The crude methanol (CR-MeOH) extracts were measured in CD_3OD, while the hexane subextracts containing nonpolar compounds were measured in $CDCl_3$. The $CHCl_3$ subextracts have medium and high polar compounds and were thus measured in both $CDCl_3$ and CD_3OD. The aq. MeOH subextract contained hydrophilic compounds and were measured in CD_3OD.

3.2.2.1 CR-MeOH extracts

The ^{1}H NMR profiles for the CR-MeOH extracts did not show definite functional groupings because it is composed of all kinds of compounds with different chemical class, polarity and solubility. However, the spectra in all the species were dominated by signals between δ_H0 - δ_H2 and δ_H3 - δ_H5 indicating the presence of aliphatic groups, probably arising from alkyl chains of fatty acids and sugars respectively. However, the CR-MeOH extract of the leaves of *F. sansibarica* (Figure 35a) exhibited an exceptionally different profile which showed prominent peaks in the low-field aromatic region.

3.2.2.2 The Hexane subextracts

The hexane subextracts clearly showed aliphatic peaks upfield ranging between δ_H0 - δ_H2 in all the extracts (Figure 35B-36B). Peaks also appeared between δ_H3 - $\delta_H5.5$ to indicate presence of functional groups with oxygenation and double bond protons. To compare the different plant parts, further down field, peaks were observed from δ_H7 - $\delta_H9.5$ in all the leaf extracts and only in *F. ingens* (both stem bark and root bark) which may indicate the presence of aldehydes and carboxylic acids.

3.2.2.3 The Chloroform subextract

The $CHCl_3$ subextracts were measured in both $CDCl_3$ and CD_3OD. Both spectra of the subextracts of all the species indicated to be qualitatively similar as in the hexane subextracts; except for the subextract of the leaves of *F. sansibarica* (Figure 35). The subextracts measured in CD_3OD had the same peaks appearing in the CR-MeOH extracts (Figure 35D). Meanwhile, when the subextract was measured in $CDCl_3$, only aliphatic groups with signals between δ_H0 - δ_H2 were predominant, even though the same peaks also appeared in the CD_3OD samples although with less intensity. This denotes that the peaks in the aromatic region arise from water soluble polyphenolic compounds and their glycosides abundant in this species. Notable aromatic peaks were observed in the $CDCl_3$ samples between $\delta_H7.5$ - δ_H8 only in the extracts of *F. sansibarica* (Figure 35E), *F. natalensis* (as well as root bark), *F. ingens* and *F. lutea* stem barks.

3.2.2.4 The aq. MeOH subextract

The aq. MeOH subextracts had predominant peaks in the δ3 - δ5 region in all the species (Figure 35E). These signals are likely to arise from anomeric protons of sugars, which also give peaks between δ_H4 - δ_H5. The subextract of *F. sansibarica* was dominated by the same compounds found in the CR-MeOH and the CD_3OD soluble compounds of the $CHCl_3$ subextracts. The peaks that appear between δ_H6.5 - δ_H7 could be as a result of water soluble polyphenolic compounds and their glycosides.

From these results, we can conclude that *F. sansibarica* is chemically different predominantly possessing aromatic compounds with glycosidic bonds.

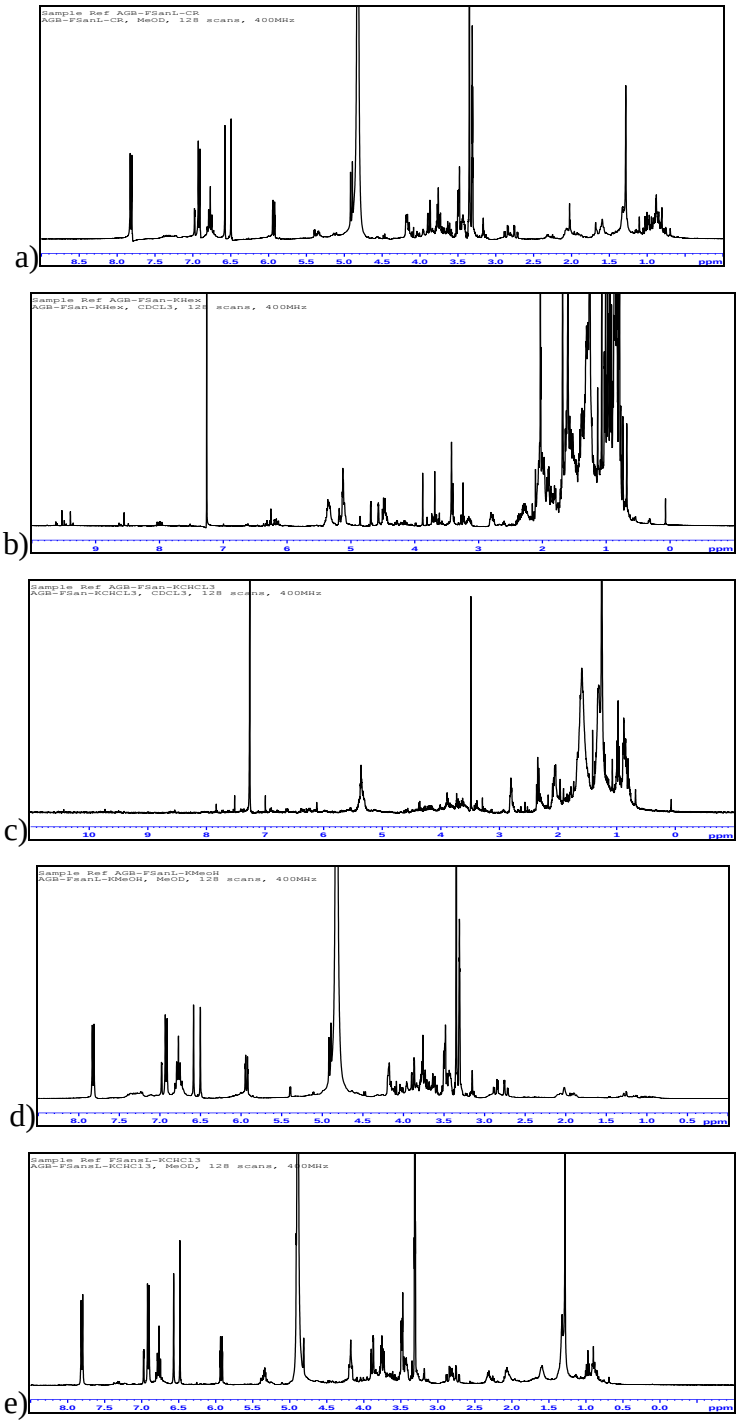


Figure 35: ^{1}H NMR profiles of the CR-MeOH extracts and the liquid-liquid partitioned subextracts obtained from the leaves of *F. sansibarica.* a) CR-MeOH extract (in MeOD). b) hexane subextract (in $CDCl_3$). c) $CHCl_3$ subextract (in $CDCl_3$), d) $CHCl_3$ subextract (in MeOD) and e) aq. MeOH subextract (in MeOD)

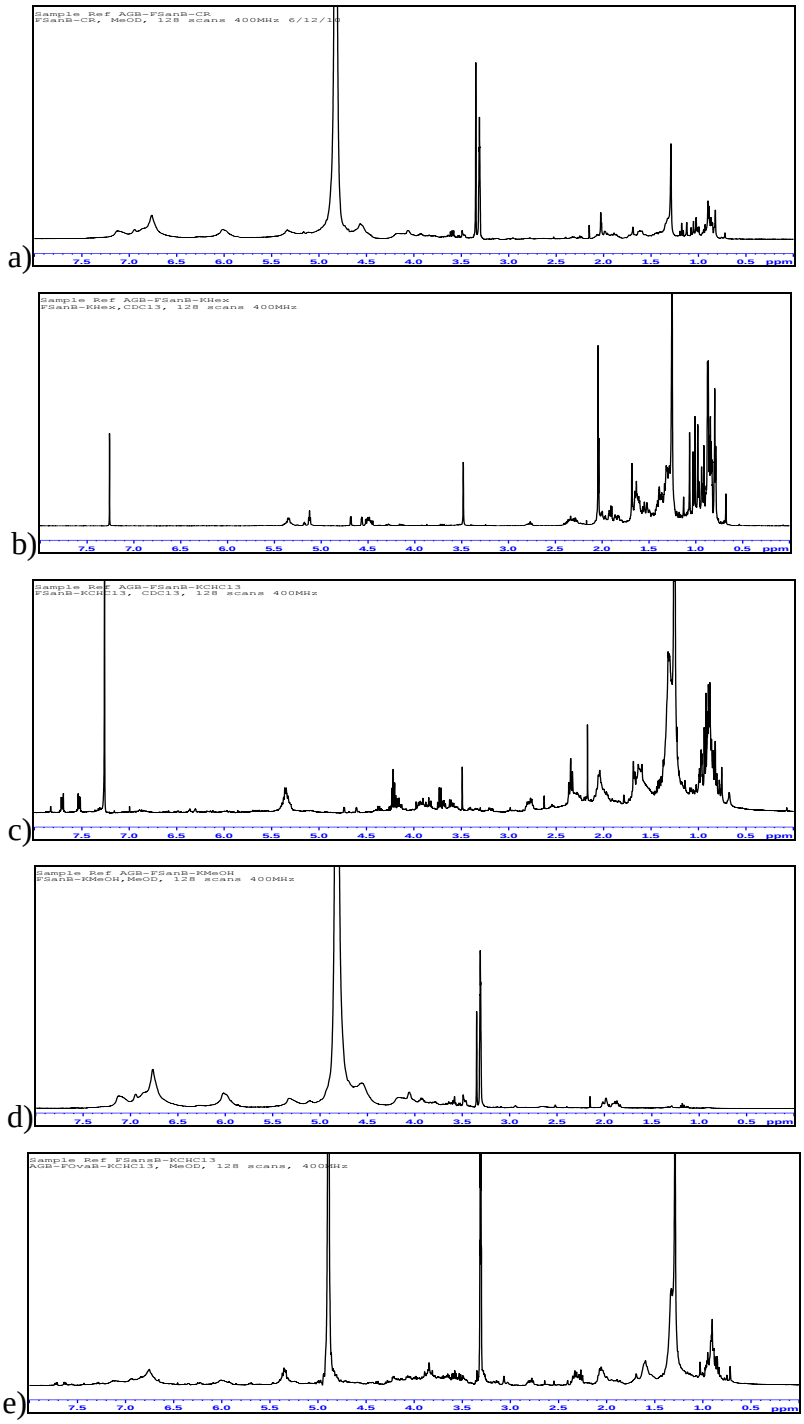


Figure 36: ^{1}H NMR profiles of the CR-MeOH extracts and partitioning subextracts obtained from the stem bark of *F. sansibarica.* a) CR-MeOH (in MeOD). b) hexane subextract (in $CDCl_3$). c) $CHCl_3$ subextract (in $CDCl_3$), d) $CHCl_3$ subextract (in MeOD) and e) aq. MeOH subextract (in MeOD).

3.3 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC) FINGERPRINTING

In HPLC, qualitative extract identification can be achieved through comparing retention time and UV spectra, such that an HPLC-UV profile of an unknown active principle can be useful towards its identification (Springfield *et al*., 2005). Hence HPLC fingerprinting was performed for the eight *Ficus* species to target phenolic compounds. The separation gradient was modified from the established conditions by Giner *et al.* (1993). The gradient was slightly acidified with 0.2% acetic acid because the more acidic the mobile phase, the more precise the separation would be (Plazonić *et al*., 2009; Andersen and Markham, 2006). However, this phenomenon did not apply with our extracts. As observed in TLC analysis, because of high ionization, the resolution of the chemical constituents in the *Ficus* species was not precise due as was shown by the tailing. However, the further investigation of the optimum pH for which the phenolic compounds constituted in the crude extracts would be ideally separated was not done, as we did not really know the phytochemical profile of the crude extracts. Crude extracts are a consortium of many compounds with different functional groups nonetheless, these constituents exhibited to be very closely related as was observed in the phytochemical investigation of *F. sansibarica* which will be discussed in Section 1.3.

The analysis was first performed on twelve standard phenolic compounds that have been reportedly identified and isolated within the genus *Ficus*. This was done in order to establish their retention times (RTs) and their UV spectra. The standards included; Gallic acid, Catechin hydrate (unhydrous), Caffeic acid, (-) Epicatechin 90%, EGCG, Rutin, Quercitrin (Glucoside + rhamnoside), Apigenin-7-glucoside, Quercetin, Luteolin, Genistein and Kaempferol. The cocktail of the standards was also run during the sequence analyses of the methanol crude extracts of *Ficus* species. Since the RTs of the standards in the cocktail were known, a correction factor was applied to the unknown crude extract chromatograms to monitor any major shifts in the retention time. From the results obtained, phenolic compounds appear to be the major chemical constituents of the *Ficus* species, as it has been reported before (Aref *et al*., 2010). While there is no single wavelength ideal for the detection of the peaks of all classes of compounds, given that

they display absorbance maxima at different wavelengths (Andersen and Markham, 2006), two wavelengths were used. The chromatograms were viewed at 254 nm and 360 nm but were however, extracted using the short wavelength 254 nm because all the standards used showed considerable absorbance at this wavelength.

Phenolic compounds are ubiquitous in the plant kingdom and are thus the most studied phytochemicals in plant research (Mradu *et al*., 2012), however identifying and isolating them in plant species that are used for medicinal purposes is imperative. Besides, newer ones are yet to be discovered as more than half the plants in the plant kingdom have not yet being explored. These compounds are chemically diverse secondary metabolites, which are characterized by aromatic rings with one or more hydroxyl functional groups (Harbone *et al*., 1999).

3.3.1 Peak identification by Retention time and UV spectra

UV/Vis spectra have long been used for structural analysis of flavonoids (Atoui *et al*., 2005). A flavonoid spectrum normally consists of two maxima ranging between 240–285 nm for band II and 300 –550 nm for band I. The position and relative intensities of these maxima can give information on the nature of the flavonoids including their oxygenation, hydroxylation and the degree of substitution of the hydroxyl groups (Švehlíková *et al*., 2004). The HPLC profile of the cocktail containing 12 standards exhibited closely related retention times and elution sequence as was for standards when they were analyzed individually. Thus the peaks in the cocktail were identified and labeled based on the retention times (Table 8) and UV spectra of the standards (Figure 37). This data was now used to support the identification of the assumed phenolic compounds constituting the crude extracts and subextracts of the *Ficus* species.

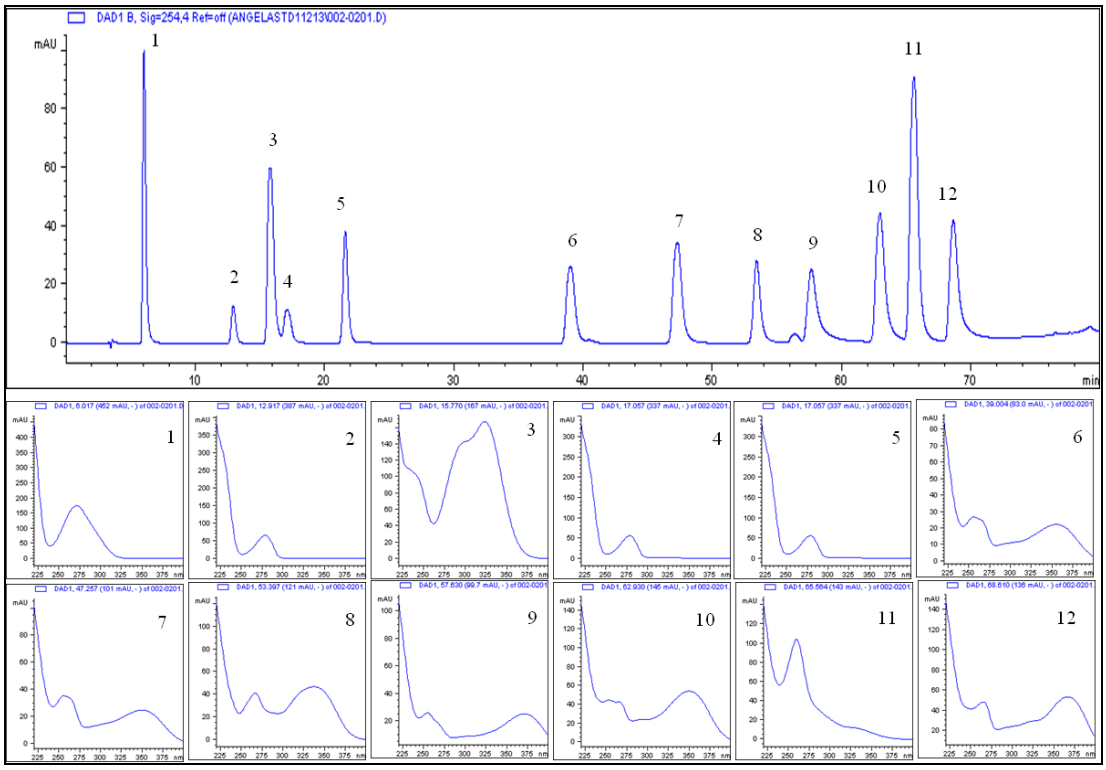


Figure 37: HPLC chromatograms and UV spectra of standards

1.Gallic acid; 2. Catechin hydrate (unhydrous); 3. Caffeic acid; 4. (-) Epicatechin 90%; 5. EGCG; 6. Rutin; 7. Quercitrin (Glucoside + rhamnoside); 8. Apigenin-7-glucoside; 9. Quercetin; 10. Luteolin; 11. Genistein; 12. Kaempferol.

Table 8: HPLC retention times (RT) of phenolic standards

Position on chromatogram	Standards	Retention time (minutes)
1	Gallic acid	6.087 ± 0.127
2	Catechin hydrate	13.091 ± 0.163
3	Caffeic acid	15.760 ± 0.004
4	*Epi*catechin	17.368 ± 0.248
5	EGCG	21.859 ± 0.210
6	Rutin	39.433 ± 0.427
7	Quercitrin	47.592 ± 0.286
8	Apigenin-7-glucoside	53.736 ± 0.319
9	Quercetin	57.954 ± 0.444
10	Luteolin	63.263 ± 0.466
11	Genistein	65.874 ± 0.368
12	Kaempferol	68.906± 0.388

3.3.2 Chemical variation among the *Ficus* species

Plants usually contain a series of similar polyphenolics, with slight change in substitution patterns or functional groups. These compounds may show very similar properties causing their separation be very challenging (Qiao *et al*., 2011). Much as there is no HPLC method that can detect all chemical constituents in particular crude extract using a single analysis (Wolfender, 2009), a degree of qualitative chemical variation of the predominant and UV detectable compounds was noted among the eight *Ficus* species that were studied and between the plant parts. The authentication of the presence and absence of similar compounds is however, tentative as the concentrations of these compounds maybe varied among different extracts, a factor that is relevant to the activity of medicinal plants but not to their correct identification (Springfield *et al*., 2005).

Thus, from the fingerprint results, only the prominent peaks (about 10 peaks in each chromatogram) were recorded. The peaks were classified according to their RT and UV spectra at 254 nm wavelength to evaluate chemical variations and markers between the species. A limitation to this process however, is that some compounds that have weak chromophores were not detected and at this wavelength, soluble compounds like tannins were also excluded since they do not absorb at this wavelength (Peschel *et al*., 2012).

For the leaf CR-MeOH extracts, the most predominant compound eluted was at about RT 14.5 for all the species except for *F. sansibarica* and *F. wakefieldii* (Table 9). The UV spectra for this compound in these species are related to that of the standard caffeic acid, and the retention time too is very close. The compounds that eluted at about RT 9.46 occurred in seven species except for *F. sansibarica*, and their UV spectra were also similar to caffeic acid. The compound eluting at about RT 39.433 ± 0.427 was the most predominant in *F. sansibarica* and *F. wakefieldii* (with lesser intensity), with their UV spectra were resembling those of the standards Apigenin-7-glucoside and quercitrin respectively. This echoes the results obtained in the TLC and ^{1}H NMR analyses in which *F. sansibarica* extracts exhibited quite an outstandingly different chemical profile.

Another group of compounds that were common in all the extracts were associated with catechin-type derivatives. These were eluted at RT 17.34, and their spectra matched that of the standards epicatechin and *epi* gallocatechin gallate. Three species (*F. ingens, F. natalensis* and *F. sycomorus* subsp. *gnaphalocarpa*) did not have any metabolite elute at this RT. The compounds that eluted at RT 41.1 had their UV spectra relating to that of rutin. This metabolite occurred in all the species. While most of the extracts contained at least compounds eluted between RT 45.138 – RT 51.87 with similar UV spectra associated with quercetin and kaempferol, *F. sansibarica* did not have any detectable compounds eluted within this RT.

The CR-MeOH extracts of the leaves were thus grouped into four compound categories identified according to the UV spectra. These include; caffeic acid, catechin, rutin and apigenin-7-glucoside associates. Beyond 50 minutes RT, the resolution was not very clear to categorize the extracted UV spectra.

Table 9: HPLC retention times (RT) of the leaf CR-MeOH extracts

RT (minutes) ***Ficus***	**9.46±**	**11.947**	**13.013**	**14.973**	**16.561**	**17.341**	**32.548**	**35.519**	**38.271**	**39.559**	**41.119**	**45.138**	**46.614**	**47.649**	**48.576**	**51.873**	**53.120**	**54.066**	**61.094**	**64.361**	**66.23**	**78.896**
ingens	+	+	+	+					+	+	+	+		+	+	+		+		+		
lutea	+	+	+	+	+	+	+	+		+	+		+		+				+	+		
natalensis	+	+	+	+	+				+	+	+	+		+			+					
ovata	+	+	+	+	+	+	+	+	+	+	+		+			+			+			
sansibarica						+		+		+	+											
sycomorus subsp.*gnaphalocarpa*	+		+	+	+				+		+	+	+				+					
sycomorus sbsp. *sycomorus*	+	+	+	+	+	+		+	+		+	+		+							+	+
wakefieldii	+	+	+	+	+	+	+	+	+	+	+	+		+	+			+				

+ = denotes presence of metabolite eluting at the indicated RT

The HPLC profiles of the CR-MeOH of the stem barks were not very different from those obtained in the leaf extracts (Table 9). Similar to the leaf extracts, the compound that eluted at RT 14.534 was the highest in concentration in six species except for *F. sansibarica* and *F.sycomorus* subsp. *sycomorus*. The UV spectra were similar to that of standard caffeic acid. The predominant compound in *F. sansibarica* and *F. sycomorus* subsp. *sycomorus* eluted at RT 9.090 and the UV spectra were equivalent to that of the standard caffeic acid as well. The UV spectra of the compounds that eluted at RT 8.8 are related to that of gallic acid. They occurred in all the species. There are some metabolites that were eluted at times exclusively to particular species. For instance, *F. ingens* extract showed a peak at RT 47.8 which was similar to quercitrin both in RT and spectrum (Figure 43 and Table 8). From the actual chromatograms (not shown in thesis), it was observed that the chemical constituents that elute after RT 40 min were not precisely resolved, hence poorly generated peaks in turn made it difficult to identify which standard they could be associated with. The RTs 15.8, 16.48, 17.383 and 21.26 based on their UV spectra were associated to catechin and epicatechin- type derivatives. This category occurred in all the species, except in *F. ingens*. The compound that eluted at RT 53.8 was found in *F. sansibarica* and *F. wakefieldii*, however the UV spectra were not similar. The UV spectrum for *F. sansibarica* was similar to that of apigenin-7-glucoside while that of *F. wakefieldii* was related to the UV spectrum of quercetin.

The extracts of the stem bark were thus grouped into five compound categories that could be identified according to UV spectra. These included; gallic acid, caffeic acid, catechins/epicatechin, apigenin-7-glucoside and quercetin.

Table 10: HPLC retention times (RT) of the stem bark CR-MeOH extracts

RT(minutes) ***Ficus***	**8.859**	**9.090**	**9.940**	**11.894**	**12.057**	**13.051**	**14.534**	**15.824**	**16.483**	**17.383**	**19.098**	**21.261**	**23.968**	**29.458**	**31.210**	**37.680**	**39.559**	**47.8**	**48.458**	**53.888**	**54.043**	**77.101**	**78.896**
ingens	+	+	+				+							+				+					
lutea	+		+	+	+		+	+		+			+		+	+					+		
natalensis	+		+		+	+	+	+	+		+	+		+									+
ovata	+		+	+			+	+		+	+		+		+	+							
sansibarica	+	+	+		+		+	+		+	+	+							+	+		+	
sycomorus subsp.*gnaphalocarpa*	+				+		+	+	+				+	+									+
sycomorus sbsp. *sycomorus*		+			+		+	+		+	+	+					+						
wakefieldii	+						+			+			+		+	+	+			+			+

+ = denotes presence of metabolite eluting at the indicated RT

Table 11 shows the results for CR-MeOH extracts of the root barks. There were some relative qualitative differences in the chemical constituents of the root bark extracts. Most constituents did not absorb well at 254 nm, and like in the stem bark extracts, the compound that eluted at RT 8.84 with the UV spectrum similar to that of gallic, was common in all the species. On the other hand, compounds that eluted between RT 12.07 and RT 17. 01 did not occur in *F. sansibarica*, and in *F. sycomorus* subsp. *sycomorus*, for which their UV spectra were similar to caffeic acid. Then all the extracts eluted compounds at RT 39. 5 however, they had different UV spectra. The UV spectra of *F. sansibarica* and *F. ovata* were similar to that apigenin-7-glucoside, while the rest of the species showed UV spectra identical to quercetin derivatives. Since catechin-type derivatives were not detectable, it can be assumed that they were in very low concentration.

The extracts of the root barks were thus grouped into five compound categories identified according to UV spectra. These included; Gallic acid, caffeic acid, catechins/epicatechin, apigenin-7-glucoside and quercetin groups.

Table 11: HPLC retention times (RT) of the root bark CR-MeOH extracts

RT (minutes) ***Ficus***	**8.843**	**9.377**	**9.807**	**11.827**	**12.073**	**12.680**	**14.973**	**16.561**	**17.090**	**17.341**	**19.132**	**23.292**	**25.681**	**27.478**	**29.396**	**35.070**	**37.667**	**39.559**	**44.899**	**46.454**	**47.681**	**53.886**	**75.508**	**78.896**
ingens	+						+		+			+	+	+	+		+	+	+	+	+	+		
lutea	+		+			+	+	+								+		+					+	
natalensis	+	+			+		+	+							+									+
ovata	+						+											+	+					
sansibarica	+		+	+						+								+						
sycomorus subsp.*gnaphalocarpa*	+				+		+	+			+	+						+						
sycomorus subsp. *sycomorus*	+																	+						
wakefieldii	+		+				+		+									+					+	+

+ =denotes presence of metabolite eluting at the indicated RT

In view of the prominent peaks that were analyzed based on the RT and UV spectra, the CR-MeOH extracts of the stem barks and the root barks are qualitatively similar in polyphenolic constitution. Meanwhile, the CR-MeOH extracts of the leaves present a slightly different chemical profile, with no peaks associated to gallic acid.

Other than the intrinsic differences between the *Ficus* species, there are extrinsic factors that collectively and significantly determine the presence and concentration of the secondary metabolites of interest in a plant extract. These factors include the location and geographical conditions like rainfall, temperature and soil type. In addition, the season, time of harvesting and extraction method used also contribute to the chemical compounds profile. The *Ficus* species used in this thesis were collected from the same environmental structure, except for *F. sycomorus* subsp. *gnapharlocapa* which was collected in an area with much less rainfall and termite mound-type of soil. To this effect, it can be assumed that the qualitative and more so quantitative variation observed in the fingerprints are more of intrinsic in nature.

On the other hand, plant secondary metabolites such as flavonoids contribute to the plant-environment interactions including plant defense against insects or microbes and abiotic stress reactions (Ong *et al.*, 2011; Newton *et al*., 2002; Stobiecki, 2000). Thus, when the plant is exposed to unfamiliar harsh environmental conditions such as excessive UV light, wounding or infection, it defends itself or quickly adapts by inducing the biosynthesis of more secondary metabolites (Waksmundzka-Hajnos *et al*., 2008; Bednarek *et al*., 2001). This could explain why the stem back extracts were found to be the most active in most of the biological activities that were screened. It is usually the stem barks that are easily chiseled for the latex and/or for the bark. These injured plants then develop a defense mechanism by igniting the biosynthesis of more metabolites, which become useful with medicinal properties.

While identification of the peaks in a LC–UV chromatogram is possible by comparing retention times and UV spectra with authentic samples or from a databank, we did not manage to identify the exact phenolic compounds constituted in the *Ficus* species studied. Hence, to avoid drawing wrong conclusions about the phenolic compounds present, we only categorized them into groups similar to the standards based on their retention time and UV spectra. These categories include; gallic acid, caffeic acid, catehin/epicatechin, rutin, apigenin-7-glucoside and quercetin similarities. We can thus make conclusions that qualitative similarity in HPLC profiles was noted intraspecifically albeit infraspecific variation in the chemistry of plant secondary metabolites is the one that is mainly quantitative (Springfield *et al*., 2005), which is the reason why the plant species are also used indiscriminately.

Overall, *F. sanisibarica* is chemically different from the other seven species, as this fingerprinting has revealed, confirming the results obtained in the TLC and NMR profilling. Our results also demonstrate that HPLC-DAD fingerprints of crude extracts can be generated for different medicinal plants by using retention time and UV spectra provided a library of specific spectra of standard compounds already exist. Some information about the chemical constituents present in a herbal plant extract that is needed for validation in its use can be acquired through this method.

3.4 BIOLOGICAL ACTIVITY

This thesis reports for the first time on an array of bioactivities of eight selected *Ficus* species collected in Zambia. The dried crude extracts and subextracts were screened *in vitro* for antimicrobial, antiparasitic and enzyme inhibition activities. The antimicrobial activities included antifungal, antibacterial and antimycobacterial assays, while antiparasitic activites included antischistosomal, antileishmanial, antitrypanosomal and antiplasmodial.

3.4.1 Antifungal activity

The CR-MeOH extracts at concentrations of 100 µg extract/disc and 50 µg extract/disc were screened using the disc diffusion assay. The extracts of the leaf, stem bark and root bark of all the eight *Ficus* species did not exhibit any antifungal activity against *Trichophyton interdigitale*, *Trichophyton tonsurans*, *Aspergillus fumigatus* and *Candida albicans* at both concentrations, hence I did not display the table of results. However, the growth of the *Trichophytons* around the dics was thinner and inconsistent compared to the growth away from the discs (Figure 38, plates A and B), suggesting that the extracts had some effect on the fungi. There was confluent growth of the *A. fumigatus* and *C. albicans* and no zones of inhibition were observed in comparison to the positive control which was miconazole with the concentration of 10 µg/disc. (Figure 38, plates C and D). Miconazole showed zones of inhibition in the ranges between 35 mm against *C. albicans* to 45 mm against *T. interdigitale.*

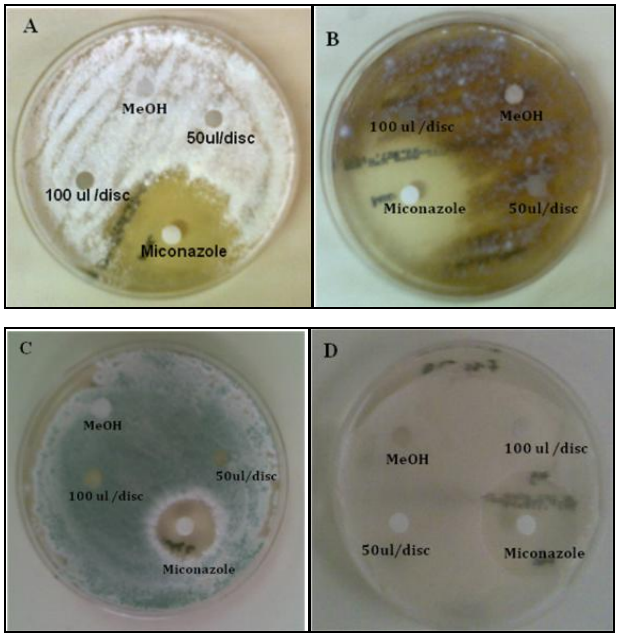


Figure 38: Antifungal activity of the *F. sansibarica* CR-MeOH extracts

A-*T. interdigitale*; B- *T. tonsurans*; C - *A. fumigatus* and D - *C. albicans*

The results exhibited are in agreement with some earlier studies on *F. congensis* Engl. syn *Ficus trichopoda* Bak (van Noort *et al*., 2007). The hexane fraction was found inactive against fungi like *A. fumigatus* (Alaribe *et al*., 2011). However, in this same study (Alaribe and collegues), using the broth dilution method the hexane extract was active against *C. albicans* with a MIC value of 1 mg/ml. Kuete *et al*. (2009) showed how the CR-MeOH extract of the stem bark of *F. ovata* collected in Cameroon had activity against clinical isolates of *C. albicans* in the broth microdilution method and obtained a MIC value of 156 µg/ml. The hexane extract of the stem bark of *F. sycomorus* has also been reported to have weak activity against *C. albicans* with MIC of 1000 µg/ml (Maregesi *et al*., 2008). In all these instances of recording activity, the method used was broth dilution method, which appears to be the effective method to use for the screening of fungi.

In broth microdilution bioassays the concentration of the test compound in the medium is defined and available to exhibit its potential in the broth, while the concentration of the test compound applied onto a disc may or may not all diffuse through the agar in order to

be available to exhibit its potential (Hadacek and Greger, 2000). Disc diffusion method is highly depended on water solubility of the test components to diffuse through agar (Shane *et al*., 1999). It would therefore, be assumed that the method used against fungi herein was not appropriate and also the concentration might have been insufficient for the secondary metabolites constituted in the *Ficus* species to exert activity. Therefore, the compounds of lower solubility in polar solvents would have no activity using this method. Our results shown by the nonpolar compounds are clearly an indication of the limitation arising in the method used.

In addition, CR-MeOH extracts are very complex mixtures of compounds of varying polarities, and antifungal properties of *Ficus* species have been attributed to the presence of pathogenesis-related (PR) proteins, which are found in the milky latex (Taira *et al*., 2005; Ricardo *et al*., 2004). However, metabolite classes such as flavonoids and isoflavonoids have also been reported to have antifungal properties. Cushnie and Lamb (2005) reviewed how polar flavonoids possessed activity against *C. albicans* and some *Aspergillus* species, and how the activity of *propolis* against dermatophytes and *candida* spp. have been attributed in part to its high flavonoid content. Flavonoids have been reported in some of the *Ficus* species that were screened (Hubert *et al*., 2011; El-Sayed *et al*., 2010; Kuete *et al*., 2009). The possible antifungal constituents in the *Ficus* species screened may have been in very minute concentrations to exert noticeable activity. As evidenced in the particular flavonoids that have antifungal properties include catechins and epiafzelechins which have been isolated from *F. ovata* (Kuete *et al*., 2008).

3.4.2 Antibacterial activity

The antibacterial activity of the CR-MeOH extracts was assessed by both broth dilution and agar disc diffusion methods. These methods both depend on the effective solubility of the extracts in the test medium in order for them to exert maximum activity against the organism. In the broth dilution method, activity denoted the total inhibition of bacterial growth by the observation of clear broth in the plates. Activity of the extracts using disc diffusion method was measured (mm) by the size of clear zones observed around the disc.

3.4.2.1 Broth dilution method

There were six organisms that were tested. These were three gram-negative bacteria which included *Escherichia coli, Acinetobacter baumanii* and *Pseudomonas aeruginosa*, and three gram-positive bacteria which included *Staphylococcus aureus, Streptococcus pneumoniae* and *Enteroccocus faecalis*.

The only gram-negative organism that was susceptible to some of the leaf extracts at 200 µg/ml of *F. ingens, F. sansibarica* and *F. wakefieldii* (Table 12) was *E. coli.* Using the breakpoint testing, extract concentrations lower than 200 µg/ml did not show activity. Other researchers have however, reported activity of different kinds of extracts (dichloro methane, petroleum ether, ethanol and water extracts) obtained from the leaves of *Ficus craterostoma* with MIC of 6.25 µg/ml (Madikizela *et al*., 2012). Alaribe and colleagues (2011) also report the hexane extract of *F. congensis* against *E. coli* to exhibit an MIC of 8 mg/ml against *E. coli.*

As for the gram-positive organisms, *Streptococcus pneumoniae* was the most sensitive to the extracts. It was susceptible to five of the stem bark and two of the leaf crude extracts. *S. aureus* and *E. faecalis* were only susceptible to four stem bark extracts. Other extracts that showed activity were *F. lutea* and *F. wakefieldii* leaves. Overall, the stem barks were the most active compared to the leaf and root bark extracts.

Table 12: Antibacterial activity of the CR-MeOH extracts (200 µg/ml) using the broth dilution method.

Test Organisms	Leaves				Stem bark				Root bark			
	Gram -ve	Gram +ve			Gram -ve	Gram +ve			Gram -ve	Gram +ve		
	Ec	Sa	Ef	Sp	Ec	Sa	Ef	Sp	Ec	Sa	Ef	Sp
F. ingens	+	-	-	-	-	-	-	-	-	-	-	-
F. lutea	-	-	-	+	-	-	-	+	-	-	-	+
F. ovata	-	-	-	-	-	-	-	+	-	-	-	-
F. natalensis	-	-	-	-	-	+	-	+	-	-	-	+
F. sansibarica	+	-	-	-	-	-	-	+	-	-	-	-
F. syc. gnaphalocarpa	-	-	-	-	-	+	-	-	-	-	-	-
F. sycs. sycomorus	-	-	-	+	-	-	+	+	-	-	-	-
F. wakefieldii	+	-	-	+	-	+	-	-	-	-	-	+
Clotrimoxazole	+				+				+			

- = bacterial growth; + = inhibited bacterial growth; Ec-*Escherichia coli;* Sa- *Staphyloccocus aureus;* Ef-*Enteroccocus faecalis;* Sp- *Streptococcus pneumoniae.*

3.4.2.2 Agar disc diffusion method

None of the extracts showed activity against the gram-negative bacteria screened. The results for gram-negative screening are not shown in Table 13. However, selective activity was observed against gram-positive bacteria; *S. aureus*, MRSA and *E. faecalis* (Table 13, Figure 39). The CR-MeOH of the stem barks was more active against the three gram-positive bacteria, while only the leaf $CHCl_3$ subextracts of *F. lutea* and *F. ovata* were active to only against *S. aureus*, MRSA and *E. faecalis* (was generally resistant to all except for the $CHCl_3$ subextract of *F. lutea*). Of the subextracts, antibacterial activity was observed mostly in the aq. MeOH subextracts of all the plant parts with inhibition zones ranging from 6 mm to 11 mm against the three test organisms. It is interesting to note that all, except two (leaf subextracts from *F. ingens* and *F. sycomorus* subsp. *gnaphalocarpa*), aq. MeOH subextracts showed activity against *E. faecalis*, an organism that is most susceptible to non polar metabolites.

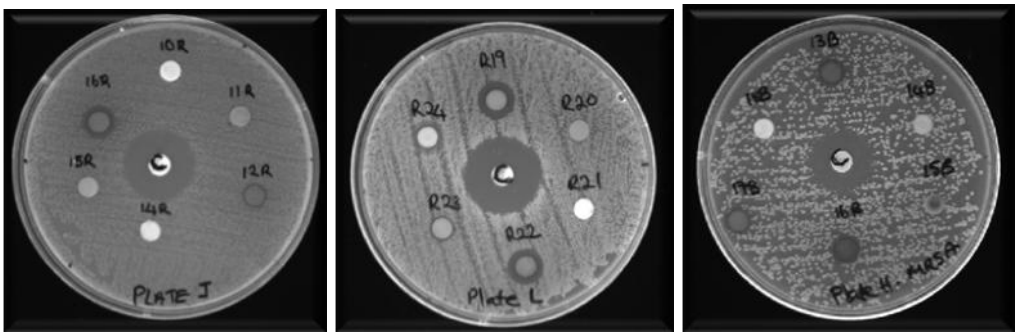


Figure 39: Plates of antibacterial activities of the *Ficus* species CR-MeOH extracts and subextracts

A- *E. faecalis*; B- *S. aureus*; C - MRSA

Table 13: Antibacterial activity of the crude extracts and subextracts (100 µg/disc) using the disc diffusion method.

Plant extracts		ZONES OF INHIBITION (mm)								
	Type of Extract	LEAF			STEM BARK			ROOT BARK		
Test organisms		*Sa*	*MSa*	*Ef*	*Sa*	*MSa*	*Ef*	*Sa*	*MSa*	*Ef*
F. ingens	CR-MeOH	-	6	-	6	9	6	7	6	-
	Hexane	-	-	-	-	-	-	10	-	-
	$CHCl_3$	-	-	-	-	-	-	-	-	-
	aq. MeOH	8	6	-	9	11	8	-	8	6
F. lutea	CR-MeOH	10	9	8	8	8	6	-	8	6
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	10	12	7	-	-	-	-	-	-
	aq. MeOH	10	9	8	10	8	8	8	10	7
F. natalensis	CR-MeOH	-	-	-	7	8	-	8	-	-
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	7	-	-	6	6	-	-	-	-
	aq. MeOH	9	8	7	10	10	7	10	6	10
F. ovata	CR-MeOH	7	9	-	7	8	-	7	7	-
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	7	8	-	-	-	-	-	-	-
	aq. MeOH	11	9	8	10	10	7	11	9	9
F. sansibarica	CR-MeOH	-	-	-	8	7	6		-	-
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	-	-	-	7	6	-	-	-	-
	aq. MeOH	8	-	8	10	10	7	9	8	6
F. sycomorus subsp. *gnaphalocarpa*	CR-MeOH	-	-	-	7	9	6	7	8	6
	Hexane	-	-	-	-	6	-	-	-	-
	$CHCl_3$	-	-	-	-	-	-	7	6	-
	aq. MeOH	8	8	-	9	10	8	9	7	6
F. sycomorus subsp. *sycomorus*	CR-MeOH	7	8	-	6	8	6	-	-	-
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	-	-	-	7	6	-	-	-	-
	aq. MeOH	10	10	8	7	8	7	9	9	9
F. wakefieldii	CR-MeOH	7	7	-	8	8	6	7	-	6
	Hexane	-	-	-	-	-	-	-	-	-
	$CHCl_3$	-	-	-	-	-	-	-	-	-
	aq. MeOH	10	10	7	10	10	7.5	10	8	7.5
Ciprofloxacin 1µg		22	20	22	22	20	22	22	20	22

(-) implies no inhibition observed. Ec-*E. coli;* Sa- *S. aureus;* Ef- *E. faecalis;* Sp- *S. pneumoniae*

The extracts and subextracts showed selectivity in activity against the gram-positive bacteria as compared to the gram-negative bacteria. This is consistent with the pattern of *in vitro* inhibition assays emerging from other studies (Anago *et al*., 2011; Maregesi *et al*., 2008; Zhang and Rock, 2004; Izzo *et al*., 1995). It is often observed that gram-negative bacteria such as *E. coli* and *P. aeruginosa* have low sensitivity towards plant extracts. It has been proposed that the inactivity could be due to the presence of the extra outer membrane in the cell wall which acts as a barrier to prevent the influx of foreign substances and larger molecules in and out of the cell (Maregesi *et al*., 2008). In particular, *E. coli* are generally impermeable to ionized or charged molecules, with the exception of those for which it possesses specific transport systems (Osman 2012; Leive, 1965). Certain charged or ionized substances can however, enter *E. coli* despite the general permeability barrier since it possesses a number of active transport systems, and the treatment of the *E. coli* with EDTA before susceptibility testing enhances its permeability (Leive, 1965).

The crude extracts and subextracts of the *Ficus* species were composed of ionized metabolites as was exhibited in the TLC analysis in which there was tailing of some components in the extracts. This could be one of the reasons they could not exhibit any activity against *E. coli.* Another possibility is that the active constituents were not made available to the microbes due to insolubility and or insufficient concentration of the active metabolites. There are several studies that have reported the inactivity of some *Ficus* species extracts against *E. coli* and other gram-negative bacteria. For instance, chloroform extracts of the aerial parts of *F. lutea* was found inactive against two gram-negative bacteria *E. coli* and *P. aeruginosa* and a gram-positive *S. aureus* (Marwah *et al*. 2006). Yet in another study Aref and colleagues (2010) report that the alcohol extracts of the aerial parts of *F. carica* did not exhibit any activity against *E. coli*, and a gram positive bacteria *S. aureus* and a fungi *C. albicans*. In this experiment however, the concentration of the extract on the disc was much lower (25 μg/ml) than in our experiment (100 μg/ml). In another study, the water extracts of all the plant parts of *Ficus platyphylla* were also reported inactive against *E. coli* and *P. aeruginosa* but the ethanol extracts exhibited activity with zones of inhibition ranging between 12.3 mm -16 mm (Kubmarawa *et al*., 2009).

On the contrary, there are other studies that have shown non-volatile extracts of *Ficus* species to possess antibacterial activity against gram-negative bacteria (Vital *et al*., 2010; Kuete *et al.*, 2008). For example, crude ethanol extracts of *F. septica* Burm exhibited antibacterial activity against *E. coli* and *S. aureus* with zones of inhibition of 19.5 ± 1.8 mm for both bacteria (Vital *et al*., 2010).

The disc diffusion results are in line with the broth dilution results for CR-MeOH extracts of *F. sycomorus* subsp. *gnaphalocarpa*, *F. natalensis* and *F. wakefieldii* stem barks, against *S. aureus* and also for *F. sycomorus* subsp. *sycomorus* stem bark against *E. faecalis.* Overall, the antibacterial activity was not very significant with the inhibition diameters distributed over a narrow range due to the low concentration of the active metabolites being availed to exert activity. The crude methanol extracts being a mixture of numerous chemical compounds with different functional groups and properties, the active metabolites would be in small concentration to exert activity against the organisms' compared to the aq. MeOH subextracts that has more polar metabolites which are even easily soluble to be availed to exert activity. In this screening, the aq-MeOH subextracts were thus the most active. These extracts are rich in polar components which in solution easily diffuse through the agar and inhibit bacterial growth given that the agar disc diffusion is depended on water solubility. Among the chemical constituents presumably contained in the aq. MeOH subextracts are phenolic compounds, of which flavonoids and isoflavonoids are some of the chemical classes reported to exhibit antibacterial activity (Kuete *et al.*, 2008). Alkaloids, tannic acid and saponins isolated from *F. sycomorus* have also been reported to have strong antibacterial activity, however, highly toxic to the host liver (Garba *et al*., 2006). These results are quite encouraging in that, plant extracts used in traditional herbal preparations are extracted with water in which case only the more polar metabolites are extracted. Hence, the moderate antimicrobial activity observed in the aq. MeOH fractions indicate that the decoctions used traditionally may or may not be effective in the infections they are used for. It has to be borne in mind that higher concentrations of the extracts maybe used in traditional preparations.

3.4.3 Antimycobacterial activity

As discussed in the introduction, tuberculosis is a prevalent condition in Africa. In Zambia, a combination of various parts of *F. sycomorus* and *F. sur* are recorded as popular remedies against this condition (Fowler, 2007). According to my knowledge, this the first time *Ficus* species collected in Zambia has been screened for antimycobacterial activity. Previously only two *Ficus* species namely *Ficus chlamydocarpa and Ficus cordata* -which are used traditionally in Cameroon in the treatment of tuberculosis- have been studied for their antimycobacterial activity (Kuete *et al*., 2008).

The antimycobacterial activity was assessed by the ability of actively growing mycobacteria to reduce the tetrazolium salt MTT into a blue formazan product within the mitochondria. Of the eight *Ficus* species screened for antimycobacterial activity, five were active with MICs ranging between 128 µg/ml and 256 µg/ml (Table 14). Interestingly, there is a high variability as to which part of the plant is responsible for this biological activity. Overall, the activity can be attributed to nonpolar principles present in hexane or chloroform extracts. Only *F. lutea* may contain more polar active principles such as flavonoids (Marwah *et al*., 2007). Because m*ycobacterium* species are quite resistant to pharmacological treatment, some i*n vitro* screenings for antimycobacterial activity have generally considered concentrations of up to 500 µg/ml as a positive result (Newton *et al*., 2002). Moreover, many screenings are run with less pathogenic *Mycobacterium* species such as *Mycobacterium bovis, M. aurum* and *M. smegmatis* which cannot always predict the activity in *M. tuberculosis* (Kuete *et al.,* 2008; Newton *et al*., 2002). In our case, the genuine human pathogen *M. tuberculosis* was used, so the activities of our samples can be considered quite promising and the stem bark extract from *F. ovata* is the best candidate for a future bioactivity-guided isolation of antimycobacterial compounds. Yet *F. cordata* -with a reported MIC of 39.1 µg/ml against *M. tuberculosis*- is still more potent than the Zambian *Ficus* species here screened. The flavonoids genistein, alpinum, laburnetin and luteolin - isolated from *Ficus chlamydocarpa*- are reported to be active against *M. tuberculosis* (Kuete *et al*., 2008). In our HPLC analysis we could not detect any genistein in the active samples (see section 2.4), but the other flavonoids may be present seeing the numerous baseline peaks that

were not accounted for. Besides, we did not have these HPLC standards to co-run with the samples.

Our results therefore, do support the reported antimycobacterial ethnopharmacological uses of *F. sycomorus*. In Zambia, the most common subspecies is *sycomorus,* which did not exhibit activity at the highest initial concentration in our tests. However, the root bark of the subspecies *gnaphalocarpa* did show activity. Ethnopharmacological reports are to be taken with caution in this case seeing that there is poor distinguishing between the species and subspecies, hence different species have the same names, usually *mutaba or mukunyu*, as mentioned in Section 1.6. To further complicate matters all the plant parts are reportedly used, echoeing the report by Hubert *et al*. (2011), and here we show that only a very specific part of the plant is active. This correlates with the case of *Ficus chlamydocarpa* and *Ficus cordata* which are indiscriminately used in Cameroon, in the treatment of tuberculosis, but only *F. cordata* is active upon *M. tuberculosis*. Further, the same authors were able to isolate active metabolites from the inactive extract only, whilst they did not do so from the active one (Kuete *et al*., 2008).

In terms of convenience, the leaves of *F. lutea* may provide a more accessible and sustainable antimycobacterial herbal remedy although they were less active *in vitro* than *F. ovata* stem bark, because all the (*F. lutea* leaves) extracts showed the same level of activity. Perhaps further studies could demonstrate if its *in vivo* antimycobacterial activity could mirror the *in vitro* one. This brings to the discussion the possible differences in bioavailability of chemicals from one or another species and this maybe a major factor in the ethnopharmacological uses, although not a consideration in traditional use of the plant extracts.

Table 14: Anti-mycobacterial activity of extracts from Zambian *Ficus* species against *Mycobacterium tuberculosis.* Only plant extracts that showed some activity are tabulated.

Plant species	**Type of extract**	**Activity (MIC µg/ml)**		
		Leaves	**Stem Barks**	**Root Barks**
F. ingens	CR-MeOH	>256	>256	>256
	Hexane	>256	256	256
	$CHCl_3$	>256	>256	>256
	aq. MeOH	>256	>256	>256
F. lutea	CR-MeOH	256	>256	>256
	Hexane	256	>256	>256
	$CHCl_3$	256	>256	>256
	aq. MeOH	256	>256	>256
F. natalensis	CR-MeOH	>256	>256	>256
	Hexane	>256	>256	256
	$CHCl_3$	>256	>256	>256
	aq. MeOH	>256	>256	>256
F. ovata	CR-MeOH	>256	>256	>256
	Hexane	>256	256	>256
	$CHCl_3$	>256	128	256
	aq. MeOH	>256	>256	>256
F. sansibarica	CR-MeOH	>256	>256	>256
	Hexane	>256	>256	>256
	$CHCl_3$	>256	>256	>256
	aq. MeOH	>256	>256	>256
F. sycomorus subsp. *sycomorus*	CR-MeOH	>256	>256	>256
	Hexane	>256	>256	>256
	$CHCl_3$	>256	>256	>256
	aq. MeOH	>256	>256	>256
F. sycomorus subsp. *gnaphalocarpa*	CR-MeOH	>256	>256	>256
	Hexane	>256	>256	>256
	$CHCl_3$	>256	>256	256
	aq. MeOH	>256	>256	>256
F. wakefieldii	CR-MeOH	>256	>256	>256
	Hexane	>256	>256	>256
	$CHCl_3$	>256	>256	>256
	aq. MeOH	>256	>256	>256
Streptomycin		0.125		
Isoniazid		0.0625		

3.4.4 Antischistosomal activity

The antischistosomal activity was assessed in different stages of the parasite's growth. Firstly, the assay was perfomed on schistosomula (the migratory larvae stage), and any extracts which caused death (evidenced by immotile larvae, often showing a characteristic uniform shape and granular appearance) and/or morphological damage was considered a hit, and thus tested for IC_{50} in an *ex vivo* secondary larval screening. The results are presented according to the plant part (leaves, stem barks and root barks) and discussed according to the type of subextracts most active. The root barks generally come out as the most active with activity being attributed to the less polar fractions.

For the leaves, Table 15 reveals that only chloroform subextracts were active on the larvae stage of the worms with an exception of the subextract of *F. lutea*. Thus the seven lethal subextracts that caused more than 70% morphological larval damage or deaths were then tested in the adult assay. In this assay, none of the extracts were lethal to the adult worms.

The results of the stem bark extracts show that the hexane and the $CHCl_3$ subextracts were more active with eight crude and aqueous methanol subextracts, and one hexane subextract of *F. wakefieldii* inducing marked morphological changes (damage or death) to the larvae (Table 16). These active extracts and subextracts were then tested in the adult assay, in which only three subextracts showed this level of activity. These include hexane (IC_{50} values of 35 μg/ml) and $CHCl_3$ (IC_{50} of 17.8 μg/ml) subextracts of *F. ovata* and the hexane subextract of *F. sycomorus* subsp. *gnaphalocarpa (*IC_{50} of 20.3 μg/ml).

For the root extracts presented in Table 17, all the extracts and subextracts were deleterious to the larvae, except for seven aqueous methanol subextracts. Of these, only five were lethal in the adult assay: the $CHCl_3$ subextracts of *F. ovata, F. sycomorus* subsp. *sycomorus* and *F. sycomorus* subsp. *gnaphalocarpa;* the hexane subextracts of *F. sycomorus* subsp. *gnaphalocarpa* and *F. natalensis.* The later gave the lowest IC_{50} value of 3.8 µg/ml for the larvae stage and IC_{50} of 71.2 µg/ml for the adult stage. Notably, $CHCl_3$ subextract of the stem bark of *F. ovata* was most active against the adult worms 17.8 µg/ml, followed by its hexane subextract and that of *F. sycomorus* subsp. *gnaphalocarpa*.

To compare the results between the plant parts, most of the stem and root bark extracts demonstrated strong antischistosomal activity. The aq-MeOH subextracts were the least active with only three species causing damage to over 70% larvae. As for the subextract types, the most active were the $CHCl_3$ subextracts, which are generally composed of compounds that have medium polarity. These results agree with already published data reported by Abdel-Hameed *et al.* (2008), in which the $CHCl_3$ fraction had the highest activity, and the EC_{84} values were 42.6 and 47.1 µg/ml against male and female adult worms respectively.

Table 15: Antischistosomal activity of the leaf extracts

Plant species	Type of Extract	Larval assay		Adult assay	
		% dead larvae	IC_{50} (100 to 1.23 µg/ml)	% Motility reduction (at100 µg/ml)	IC_{50} (100 to 3.7 µg/ml)
F. ingens	CR-MeOH	0	-	-	>100
	Hexane	39.1	-	0	>100
	$CHCl_3$	100(dead)*	64.4	-	>100
	Aq. MeOH	30.8	-	-	>100
F. lutea	CR-MeOH	39.9	-	-	>100
	Hexane	100(dead)	62.1	0	>100
	$CHCl_3$	46.7	-	-	>100
	Aq. MeOH	52	-	-	>100
F. natalensis	CR-MeOH	9.4	-	-	>100
	Hexane	0	-	-	>100
	$CHCl_3$	34.5	-	-	>100
	Aq. MeOH	41.3	-	-	>100
F. ovata	CR-MeOH	34.3	-	-	>100
	Hexane	0	-	-	>100
	$CHCl_3$	100(dead)	36.3	50	100
	Aq. MeOH	36.9	-	-	>100
F. sansibarica	CR-MeOH	10.1	-	-	>100
	Hexane	0		-	>100
	$CHCl_3$	100(dead)	37.8	0	>100
	Aq. MeOH	6.4	-	-	>100
F. sycomorus subsp. *gnaphalocarpa*	CR-MeOH	19.5	-	-	>100
	Hexane	0	-	-	>100
	$CHCl_3$	100(dead)	>100	50	100
	Aq. MeOH	0	-	-	>100
F. sycomorus subsp. *sycomorus*	CR-MeOH	26.2	-	-	>100
	Hexane	29.7	-	-	>100
	$CHCl_3$	100(dead)	33	0	>100
	Aq. MeOH	30.1	>100	43.1	>100
F. wakefieldii	CR-MeOH	27	-	-	>100
	Hexane	14	64.4	25	>100
	$CHCl_3$	100(dead)	-	-	>100
	Aq. MeOH	59.5	-	-	>100

*Larvae dead within the 3 days of the assay; (-) implies not active; (0) implies no mortality observed

Table 16: Antischistosomal activity of the stem bark extracts

Plant species (Stem barks)	Type of Extract	**Larval assay**		**Adult assay**	
		% dead larvae	IC_{50} (100 to 1.23 µg/ml)	% Motility reduction (100 µg/ml)	IC_{50} (100 to 3.7 µg/ml)
F. ingens	CR-MeOH	**100**(dead)*	>100	33.3	>100
	Hexane	100(dead)	34.9	0	>100
	$CHCl_3$	100(dead since day 1)	32.4	41.7	>100
	Aq. MeOH	63.1	-	-	>100
F. lutea	CR-MeOH	100(dead)	36.4	48.2	>100
	Hexane	100(dead since day 1)	12	37.5	>100
	$CHCl_3$	100(dead since day 1)	33.4	37.5	>100
	Aq. MeOH	64.4	-	-	>100
F. natalensis	CR-MeOH	100(dead since day 1)	33.1	20	>100
	Hexane	100(dead since day 1)	64.4	0	>100
	$CHCl_3$	100(dead since day 1)	35.8	43.8	>100
	Aq. MeOH	71.9	>100	51.3	100
F. ovata	CR-MeOH	100(dead since day 1)	36.2	37.5	>100
	Hexane	100(dead since day 1)	11.4	80	35
	$CHCl_3$	100(dead since day 1)	11.1	100	17.8
	Aq. MeOH	64.2	-	-	>100
F. sansibarica	CR-MeOH	54.7	79.1	33.8	>100
	Hexane	100(dead)	64.4	0	>100
	$CHCl_3$	100(dead)	36.1	56.3	100
	Aq. MeOH	71	-	-	>100
F. sycomorus subsp. *gnaphalocarpa*	CR-MeOH	100(damaged)	61.2	65.6	100
	Hexane	100(dead since day 1)	10.3	100	20.3
	$CHCl_3$	100(dead since day 1)	12.1	50	100
	Aq. MeOH	57.3	-	-	>100
F. sycomorus subsp. *sycomorus*	CR-MeOH	61.3	-	-	>100
	Hexane	100(damaged)	>100	12.5	>100
	$CHCl_3$	100(dead)	64.4	61.6	97.7
	Aq. MeOH	76	>100	66.7	100
F. wakefieldii	CR-MeOH	70.9	>100	46.3	>100
	Hexane	35.00	-	-	>100
	$CHCl_3$	100(dead)	34.8	35.8	>100
	Aq. MeOH	53.9	-	-	>100

*Larvae dead within the 3 days of the assay; (-) implies not active; (0) implies no mortality observed

Table 17: Antischistosomal activity of the root bark extracts

Plant species (Root barks)	Type of Extract	**Larval assay**		**Adult assay**	
		% dead larvae	IC_{50} (100 to 1.23 µg/ml)	% Motility reduction (100 µg/ml)	IC_{50} (100 to 3.7 µg/ml)
F. ingens	CR-MeOH	100(dead)*	34.7	13.3	>100
	Hexane	100(dead since day 1)	21.5	0	>100
	$CHCl_3$	100(dead)	12.2	41.7	>100
	Aq. MeOH	66.2	-	-	>100
F. lutea	CR-MeOH	90.3	75.8	25	>100
	Hexane	100(dead)	14.6	32.5	>100
	$CHCl_3$	100(dead)	31.8	25	>100
	Aq. MeOH	59.3	-	-	>100
F. natalensis	CR-MeOH	100(dead since day 1)	31.9	46.9	>100
	Hexane	100(dead since day 1)	3.8	87.5	71.2
	$CHCl_3$	100(dead since day 1)	8.2	100	32.6
	Aq. MeOH	70.4	65.6	55.6	100
F. ovata	CR-MeOH	100(damaged)	37.2	58.1	100
	Hexane	100(dead since day 1)	36.2	46.3	>100
	$CHCl_3$	100(dead since day 1)	21.5	79.2	98.6
	Aq. MeOH	38.6	-	-	>100
F. sansibarica	CR-MeOH	100(dead)	64.4	51.7	>100
	Hexane	100(dead)	34.3	29.2	>100
	$CHCl_3$	100(dead since day 1)	21.5	75	>100
	Aq. MeOH	0	-	-	>100
F. sycomorus subsp. *gnaphalocarpa*	CR-MeOH	100(dead)	36	50	100
	Hexane	100(dead since day 1)	19.7	100	31.7
	$CHCl_3$	100(dead)	11.7	80	60.8
	Aq. MeOH	60.8	-	-	>100
F. sycomorus subsp. *sycomorus*	CR-MeOH	100(dead)	64.4	50	100
	Hexane	100(dead in 3 days)	35.8	0	>100
	$CHCl_3$	100(dead since day 1)	12	77.1	58.8
	Aq. MeOH	16.5	-	-	>100
F. wakefieldii	CR-MeOH	95.7	64.4	70	71.2
	Hexane	86.2	78.3	25	>100
	$CHCl_3$	100(dead since day 1)	21.5	50	100
	Aq. MeOH	62.7	-	-	>100

*Larvae dead within the 3 days of the assay; (-) implies not active; (0) implies no mortality observed

Members of the genus *Ficus* have been reported to possess antihelminitic activities, especially by the milky latex (Lansky and Paavilainen, 2011; de Amorin, 1999) and there are some pharmacological studies that have been performed to confirm their use. For example the crude methanol extract of the bark of *F. trijuja* was the only species with activity amongst others (*F. bengalensis, F. benjamina, F. racemosa, F. microcarpa, F. oblique, F. pyriformis*) that were screened against schistosoma. The activity was reported with LC_{50} and LC_{90} values of 14.4 µg/ml and 39.5 µg/ml (Yousif *et al*., 2012). In another study by Abdel-Hameed *et al*. (2008), the $CHCl_3$ fractions exhibited some activity against the worms. It was further shown that male worms are more susceptible to the extracts than female worms. However, in our screening, we did not record the results according to the sex of the worms, to further make a comparison. The milky latex of *F. insipida* and *F. carica* have also been found to exhibit mild activity against the adult worms, and exhibited very high toxicity in mice (de Amorin, 1999).

Since Praziquantel (PZQ) has been the only available treatment for schistosomiasis for many years, there have been efforts to develop alternative therapies such as vaccines and novel drugs (Coultas and Zhang, 2012). Praziquantel however, has little or no effect on larvae and immature forms, but eliminates mature worms. It could therefore be assumed that the hexane and $CHCl_3$ subextracts that exhibited high activity against the larvae and not against the adult worms have a different mode of action to that of praziquantel. Also, the subextracts that demonstrated being lethal against both the larvae and the adult worms may have another mode of action.

As suggested by Coultas and Zhang (2012), developmental stages of the schistosome life cycle, such as schistosomules and adult worms should be targeted. From the screening results, we have observed that the schistosomules are very susceptible, as already reported in earlier studies (Coultas and Zhang, 2012). It is thus the ideal target for drug development. There are some trioxane-containing drugs that are being tested against schistosomes and these have shown to be potent (Portela *et al*., 2012), and depending on what maybe obtaining in our extracts, they may offer a different class of active metabolites and hence broaden the choice of drugs to use against schistosoma.

3.4.5 Antiprotozoal activity

There are no data about the antiprotozoal activity of the selected *Ficus* species collected in Zambia or abroad, apart from one report on *F. ingens* of Saudi Arabian origin (Al-Musayeib *et al*., 2012). The CR-MeOH extracts and subextracts were screened for antiprotozoal activity against the amastigote stages of *Leishmania donovani* MHOM-ET-67/L82 and *Trypanosoma cruzi* Tulahuen C4. Also for screening was the trypomastigotes stage of *Trypanosoma brucei rhodesiense* STIB 900 and the *P. falciparum* K1 blood stage (IEF) parasites. The cytotoxicity activity was determined against rat skeletal myoblast cell line-L6 cells, for which the selective index (SI) against *P. falciparum* parasites was calculated. The results of the cytotoxicity data of all the extracts and subextracts revealed that most of the extracts were not entirely safe towards mammalian cells as the selective indices (SI) were less than 10 for almost all the parasites. There are a few exceptions which have been highlighted.

The results are categorized in terms of the most susceptible protozoan, the most active plant parts used and thirdly the type of extraction which was more active. *P. falciparum* was the most sensitive parasite towards all the extracts (Table 20). Of the 32 extracts and subextracts, there were nine aerial subextracts and 18 stem bark extracts and subextracts that exhibited IC_{50} values lower than 10 µg/ml. The most active subextract was the lipophilic hexane subextract of the leaves of *F. ingens* with IC_{50} of 5.76 µg/ml. The next sensitive parasites were *T. brucei rhodesiense* and *L. donovani* with hexane and $CHCl_3$ subextracts of *F. sycomorus* also showing IC_{50} values lower than 10 µg/ml (IC_{50} values of 7.46 µg/ml and 6.54 µg/ml for hexane and $CHCl_3$ respectively against *L. donovani*). Meanwhile, *T. cruzi* was the least sensitive protozoan.

In terms of the extracts, those obtained from the stem barks were the most active. Eighteen crude methanol extracts had IC_{50} values below 10 µg/ml. The most active species was *F. ovata* with IC_{50} 4.76 µg/ml followed by *F. ingens* (IC_{50} 7.24 µg/ml), while *F. sansibarica* was the least active with IC_{50} value of 23.6 µg/ml (Table 19).

The $CHCl_3$ subextracts were the most active type of extract with three having IC_{50} values less than 2 µg/ml. The highest activity was observed in *F. ovata* stem bark (IC_{50} 1.14

µg/ml), followed by the same plant part of *F. sycomorus* subsp. *gnaphalocarpa* (IC_{50} 1.49 µg/ml) and *F. wakefieldii* (IC_{50} 1.92 µg/ml). The aq. MeOH subextracts showed moderate activity in comparison to the hexane and $CHCl_3$ subextracts.

As for cytotoxity, the extracts and subextracts showed some degree of toxicity, as all showed cytotoxic IC_{50} values higher than 10 µg/ml except for $CHCl_3$ subextract of the root bark of *F. wakefieldii* which was very toxic with IC_{50} value of 7.57 µg/ml. The impact of the cytotoxicity was further evaluated in terms of the selectivity index (SI) which is the selective activity of the extracts against the parasite compared to its toxicity for the L6 cells. In this case the SI values were calculated against *P. falciparum* blood stage. The SI corresponds to the ratio between cytotoxic IC_{50} values and the *Pf*K1 parasitic IC_{50} values. A value greater than 1 implies more selective against the L6 cells, while a value lower than 1 is considered to be more selective to the parasite. The selectivity indices for *T. brucei rhodiense*, *T. cruzi* and *L. donovani* were all below the value of 10, suggesting a level of toxicity against the L6 cells. This indicates the *Ficus* extracts would not be safe to use against these protozoans. However, the leaf extracts did not show selectivity except for the hexane extracts of *F. ingens* and *F. ovata* which exhibited mild selectivity with SI index ratios of 10.5 and 10.6 respectively. The rest had SI index ratios lower than 10. The $CHCl_3$ subextracts of the root barks of *F. ingens* and *F. ovata* showed a bit more selectivity with SI index ratios of 14.2 and 16.3 respectively. The $CHCl_3$ subextract of *F. wakefieldii* was found to be the most selective against *P. falciparum* with the selecitivity index ratio of 22.8. Another six subextracts with SI ratio above 10 included the $CHCl_3$ subextracts of *F. ingens* (14.6), *F. lutea* (18.5), *F. ovata* (13.9), *F. sycomorus* subsp. *gnaphalocarpa* (15.6) and *F. sycomorus* subsp. *sycomorus* (11.7).

Table 18: Antiprotozoal activity and cytotoxic activity on (IC_{50} in µg/ml) of the leaf extracts

Plant species	**Type of extract**	***T.b. rhod*$^{\alpha}$**	***T. cruz*$^{\beta}$**	***L. dono*$^{\gamma}$**	***P.falc*$^{\delta}$**	**Cytot$^{\varepsilon}$**	**SI**
F. ingens	CR-MeOH	31.1	79.9	45.6	23.8	70.5	3.0
	Hexane	21.3	41.8	11.7	5.76	60.5	10.5
	$CHCl_3$	27.3	62.5	37.4	14.1	67.5	4.8
	Aq. MeOH	27.5	66.4	86.2	29.6	76.9	2.6
F. lutea	CR-MeOH	35.5	73.3	96.7	21	67.9	3.2
	Hexane	24.3	52.6	18.9	8.29	54.4	6.6
	$CHCl_3$	33.3	76.2	43.3	21	65.7	3.1
	Aq. MeOH	37.2	64.7	>100	17.9	51.9	2.9
F. natalensis	CR-MeOH	30.7	79.5	72.4	27.3	60.1	2.2
	Hexane	25.1	47.5	16.7	6.65	59.9	9.0
	$CHCl_3$	30.5	54.5	21.2	8.93	59.2	6.6
	Aq. MeOH	37.8	73.7	>100	23.3	70.4	3.0
F. ovata	CR-MeOH	31.4	70.9	70.1	19.4	52.2	2.7
	Hexane	15.3	50.6	15.8	6.04	64.3	10.6
	$CHCl_3$	22.2	67.2	10.5	18.1	69.8	3.8
	Aq. MeOH	38.7	72.1	>100	27	70.4	2.6
F. sansibarica	CR-MeOH	34.5	78.8	68.6	28.6	72.6	2.5
	Hexane	32.2	42.2	20.5	10.4	45.1	4.3
	$CHCl_3$	30.1	58.6	55.3	15.2	73.1	4.8
	Aq. MeOH	34.8	79.7	>100	43.4	>100	2.3
F. sycomorus gnaphalocarpa	CR-MeOH	31.1	72.6	48.7	26.2	70.1	2.7
	Hexane	27.6	47.9	15.9	8.54	71.8	8.4
	$CHCl_3$	27.1	64.2	19.1	9.41	73.2	7.8
	Aq. MeOH	36.9	66.8	>100	32.7	75.8	2.3
F. sycomorus. sycomorus	CR-MeOH	25.9	63.9	59.1	18.8	48.4	2.6
	Hexane	11.3	51.9	7.46	6.78	58.9	8.7
	$CHCl_3$	9.63	21.3	6.54	5.88	57.8	9.8
	Aq. MeOH	27.6	57.1	>100	20	47.5	2.4
F. wakefieldii	CR-MeOH	28.3	70.4	56.3	22.1	49.7	2.2
	Hexane	14.5	58.7	10.3	11	70.9	6.4
	$CHCl_3$	16.7	54.8	14.8	10.8	61.8	5.7
	Aq. MeOH	38.4	75.9	99.9	21.1	70.1	3.3
Reference drugs		**0.005[1]**	**0.464[2]**	**0.171[3]**	**0.073[4]**	**0.007[5]**	

Note: $^{\alpha}$*Trypanosoma brucei rhodesiense*; $^{\beta}$*Trypanosoma cruzi*; $^{\gamma}$*Leishmania donovani*; $^{\delta}$*plasmodium falciparum*; $^{\varepsilon}$cytotoxic L-6 cells**;** [1]Melarsopro; [2]Benznidazole; [3]Miltefosine; [4]Chloroquine; [5]Podophyllotoxin

Table 19: : Antiprotozoal activity and cytotoxic activity (IC_{50} in µg/ml) of the stem bark extracts

Plant species	**Type of extract**	***T.b. rhod*$^{\alpha}$**	***T. cruz*$^{\beta}$**	***L. dono*$^{\gamma}$**	***Pf*** K1$^{\delta}$	**Cyt** L6$^{\varepsilon}$	**SI**
F. ingens	CR-MeOH	32.1	45.5	23.4	7.24	59.2	8.2
	Hexane	12.3	16.9	14.3	3.3	36.9	11.2
	$CHCl_3$	15.9	18.3	7.95	2.27	33.1	14.6
	Aq. MeOH	37.1	60.3	>100	20.7	63.2	3.1
F. lutea	CR-MeOH	33.9	32.8	57.3	8.9	67.9	7.6
	Hexane	5.99	10.6	8.79	3.36	19.5	5.8
	$CHCl_3$	11.4	17.8	10	2.5	46.3	18.5
	Aq. MeOH	40.4	61.7	>100	17.9	45.7	2.6
F. natalensis	CR-MeOH	20.6	37.8	28.2	7.52	48.4	6.4
	Hexane	39.1	11.3	44	19.9	75.5	3.8
	$CHCl_3$	31.4	52.9	36.2	7.93	68.8	8.7
	Aq. MeOH	38.2	61.5	>100	18.8	45.9	2.4
F. ovata	CR-MeOH	14.9	18.5	30.1	5.89	44.8	7.6
	Hexane	4.14	6.33	6.12	2.85	19.8	6.9
	$CHCl_3$	4.48	5.59	4.12	1.14	15.9	13.9
	Aq. MeOH	31.4	55.7	>100	17.3	43.4	2.5
F. sansibarica	CR-MeOH	11.9	85.3	83.8	24	43.1	1.8
	Hexane	18.6	26.9	37.9	19.7	45.1	2.3
	$CHCl_3$	16.3	66.1	36	7.5	46.9	6.3
	Aq. MeOH	14.6	69.1	>100	21.9	37.2	1.7
F. sycomorus gnaphalocarpa	CR-MeOH	36.9	53.7	53.7	9.79	53.8	5.5
	Hexane	10.2	8.61	6.84	2.76	16.7	6.1
	$CHCl_3$	8.48	11.1	6.57	1.49	23.3	15.6
	Aq. MeOH	39.5	55.5	>100	18.6	44.7	2.4
F. sycomorus. sycomorus	CR-MeOH	33	70.3	>100	23.2	46.4	2.0
	Hexane	24.7	35.3	15.7	18.8	55.9	3.0
	$CHCl_3$	15.7	>100	>100	8.53	>100	11.7
	Aq. MeOH	16.6	63.2	>100	22.5	39.5	1.8
F. wakefieldii	CR-MeOH	37.2	65.3	69.2	12.7	69.5	5.5
	Hexane	12.9	46.8	14.2	8.89	19.9	1.5
	$CHCl_3$	12.4	23.3	7.34	1.92	43.7	22.8
	Aq. MeOH	37.6	55.9	>100	16.5	44.2	2.7
Reference drugs		**0.005**[1]	**0.464**[2]	**0.171**[3]	**0.073**[4]	**0.007**[5]	

[1]Melarsopro, [2]Benznidazole, [3]Miltefosine, [4]Chloroquine, [5]Podophyllotoxin

Table 20: Antiprotozoal activity and cytotoxic activity (IC_{50} in µg/ml) of the root bark extracts

Plant species	**Type of extract**	***T.b. rhod*$^{\alpha}$**	***T. cruz*$^{\beta}$**	***L. dono*$^{\gamma}$**	***Pf* K1$^{\delta}$**	**Cyt** L6$^{\varepsilon}$	**SI**
F. ingens	CR-MeOH	10.6	53.8	32.3	8.35	46.5	5.5
	Hexane	5.35	20.6	11.9	6.15	33.2	5.4
	$CHCl_3$	6.89	18.9	6.37	2.24	31.8	14.2
	Aq. MeOH	16.3	75.9	>100	22.5	70.4	3.1
F. lutea	CR-MeOH	17.6	53.9	43.3	15.1	71.3	4.7
	Hexane	16.5	52.2	48.4	16.5	53.6	3.2
	$CHCl_3$	15.6	30.9	14.6	6.04	40.8	6.7
	Aq. MeOH	18.4	63.6	>100	33.7	41.9	1.2
F. natalensis	CR-MeOH	8.95	49.7	34.8	13.4	42.7	3.2
	Hexane	2.07	11.8	11.1	5.99	15.4	2.6
	$CHCl_3$	2.04	13.5	8.61	2.62	14.2	5.4
	Aq. MeOH	18.1	62.9	>100	23.8	35.2	1.5
F. ovata	CR-MeOH	18.6	58.1	43.7	13.9	73.8	5.3
	Hexane	6.3	24.0	28.3	7.78	40.2	5.2
	$CHCl_3$	13.9	36.7	9.97	2.66	43.3	16.3
	Aq. MeOH	16.5	78.7	>100	31.8	74.3	2.3
F. sansibarica	CR-MeOH	16.9	55.5	32.2	14.6	44.5	3.0
	Hexane	6.29	9.75	10	6.09	13.2	2.2
	$CHCl_3$	17.7	34.1	10.8	2.8	17.1	6.1
	Aq. MeOH	17.9	75.4	>100	37.7	74.3	2.0
F. sycomorus subsp *gnaphalocarpa*	CR-MeOH	16.7	46.5	28.7	11.2	44	4.0
	Hexane	6.01	17.9	12.1	6.46	43.1	6.7
	$CHCl_3$	3.14	13.3	5.85	2.92	17.4	6.0
	Aq. MeOH	18.2	70.1	86.7	23.7	37.1	1.6
F. sycomorus. sycomorus	CR-MeOH	16.2	43.6	20.6	6.42	24	3.7
	Hexane	13.4	40.5	22.7	7.94	47.2	6.0
	$CHCl_3$	6.83	20.1	7.39	2.01	4.62	2.3
	Aq. MeOH	17.1	88.8	>100	46.8	72.1	1.5
F. wakefieldii	CR-MeOH	14.2	61.4	47.8	12.4	46.9	3.8
	Hexane	36.6	56.9	30.1	17.9	23.2	1.3
	$CHCl_3$	9.49	47.4	10.4	2.32	7.57	3.3
	Aq. MeOH	15.1	70.7	>100	20.3	40.9	2.0
Reference drugs		**0.005**[1]	**0.464**[2]	**0.171**[3]	**0.073**[4]	**0.007**[5]	

[1]Melarsopro, [2]Benznidazole, [3]Miltefosine, [4]Chloroquine, [5]Podophyllotoxin

The results in the antiprotozoal assays reveal that *P. falciparum* was the most susceptible parasite towards all the extracts. The chloroform subextracts were the most active, while it is the stem barks which exhibited more activity than the other plant parts. The IC_{50} data of the extracts in the cell line L6 helps to determine whether an activity is specific or just general in toxic effect ("non specific activity"). When the selectivity index is calculated, we observed that the active extracts against *T. cruzi* showed nonspecific affects.

The CR-MeOH extract of the three plant parts of *F. ingens* (leaves, stem bark and root bark) showed activity against *P. falciparum* with IC_{50} values of 23.8 (SI 3.0), 7.24 (SI 8.2) and 8.35 (SI 5.5) µg/ml respectively, with low SI values. These findings are in agreement with literature data published recently by Al-Musayeib *et al*. (2012) who reported the antiprotozoal activity against *P. falciparum* of the methanol extract of *F. ingens* growing in Saudi Arabia to be IC_{50} 8.4 ± 2.3 with SI 3.87. While the variation in the area of collection and ecological factors has an effect on the quality and quantity of chemical constituent of the plants, it seems not to have been the case with *F. ingens*. The probable active metabolites could be inherent and can be identified as markers for the species. The activity of CR-MeOH extracts of *F. ingens* for other protozoans ranged from, *T. cruzi* 31.2 ± 4.3, SI 1.04 to *T. brucei* 8.0 ± 2.2, SI 4.08 (Al-Musayeib *et al*., 2012). The IC_{50} values of *T. brucei rhodiense* and *T. cruzi* also tally with those obtained by Al-Musayeib *et al*. (2012). *T. cruzi* has been generally reported less sensitive than T. *brucei rhodiense* because it has an intracellular test system (Jensen *et al*., 2012; Mokoka *et al*., 2011). This echoes already published data (Mokoka *et al*., 2011), in which the dicloromethane/methanol [1:1] extract of *Agathosma apiculata* was 140 times more active against *P. falciparum* than against *T. cruz* (with IC_{50} value of 17 µg/ml), 53 times more active than against *L. donovani* and 37 times active than against *T. brucei rhodiense*.

3.4.6 Enzymology- Enzyme purification and SDS-PAGE

The recombinant enzymes FabI, FabG, and FabZ were purified by Nickel-NTA-Affinity-chromatography method (Porath *et al*., 1975) after being disrupted by a French press. The purification was documented by 15% SDS-PAGE, which was performed to verify the successful purification of the protein. The disrupted protein samples were collected from the cell lysate (T) to confirm the numerous types of proteins contained in the sample. After centrifuging the cell lysate, the supernatant (S) which contained all soluble protein was documented. This is the portion in which the proteins of interest were also captured. The pellet (P) which had insoluble proteins and other cell debris was next, followed by the flow through (F) which was collected after passing through the nickel-NTA column. The protein in the portion does not have the His-tag. The column was washed (W) with 50 mM imidazole to remove all protein in volumes of 1 ml , 3 times (E3, E4, E5) with 500 mM imidazole. The protein was then loaded onto a PD-10 column for desalting and collected 3 times (E1*, E2* E3*). All these collected samples were then ran on an SDS-PAGE.

The purified enzymes gave homogenous bands, and the molecular weights of the proteins were estimated by comparing with the low range SDS marker protein standard which was the first and last bands (m) on the gels. The contents of the marker (m) are indicated in kDa and were (top to bottom) BSA, ovalbumin, carbonic anhydrous, trypsin inhibitor and lysozyme. The size of rhe protein subunit of FabG was estimated to be about 32 kDa (Figure 40), while that of FabI was 45 kDa (Figure 41) which is typical of the molecular weight of FabI (average of 45.3 kDa). The protein band of FabZ was estimated to be 21 kDA as shown in Figure 42.

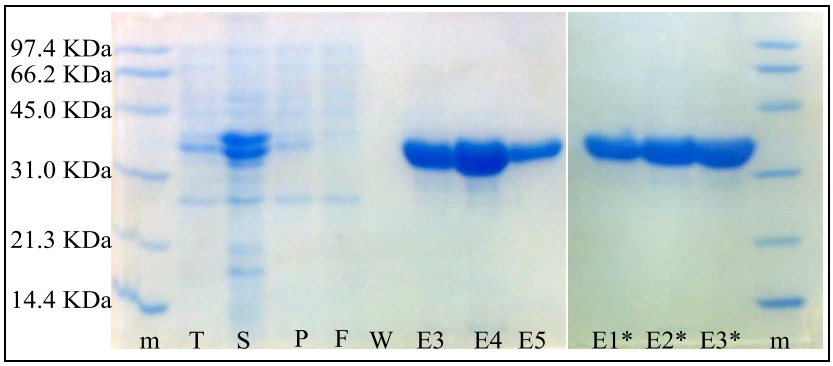


Figure 40: SDS-PAGE for FabG purification.

m: Low range SDS-Standard, T: cell lysate, P: insoluble protein fraction, S: soluble protein fraction, F: flow-through, W: combined washing fractions, E: elution fractions from Nickel-NTA column, E*: elution fractions from PD-10 column

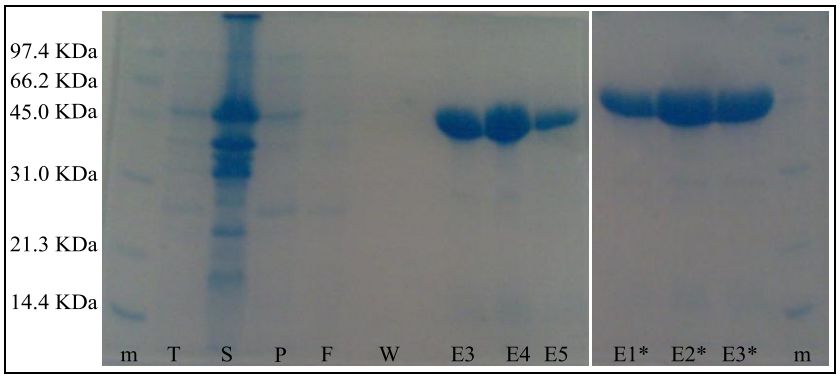


Figure 41: SDS-PAGE for FabI purification.

m: Low range SDS-Standard, T: cell lysate, P: insoluble protein fraction, S: soluble protein fraction, F: flow-through, W: combined washing fractions, E: elution fractions from Nickel-NTA column, E*: elution fractions from PD-10 column

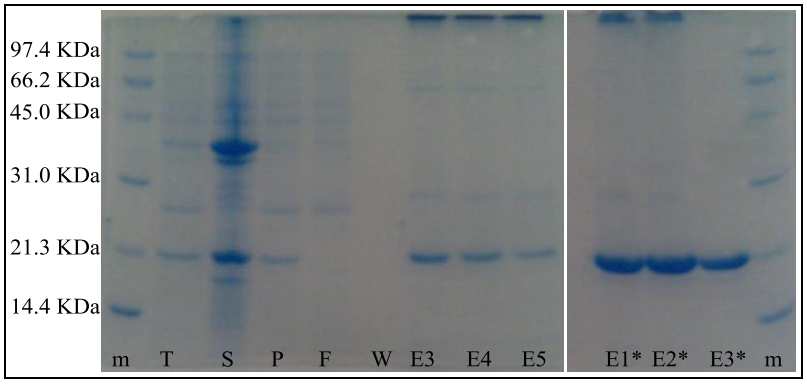


Figure 42: SDS-PAGE for FabZ purification.

m: Low range SDS-Standard, T: cell lysate, P: insoluble protein fraction, S: soluble protein fraction, F: flow-through, W: combined washing fractions, E: enzyme fractions from Nickel-NTA column, E*: enzyme fractions from PD-10 column after desalting

3.4.7 Plasmodial FAS-II enzyme inhibition

The malaria liver stage is asymptomatic, and yet it is the best stage for prophylaxis because it avoids the development of the symptomatic blood stage. This makes it a good target for intervention against malaria through liver stage *Pf*FAS-II enzyme inhibition. The assay was thus performed spectrophometrically and a total of 96 extracts were assayed for the activity of three recombinant enzymes (FabG, FabI and FabZ) that are part of the enzyme cascade involved in the type II fatty acid synthesis elongation process. The plant extracts were assayed at two concentrations, firstly at 10 µg/ml as the initial concentration, then at 1 µg/ml. At higher concentrations the colour intensity of the plant extract most often interfered with the measurement and it thus led to a positive slope, in which case measurements were done at a lower concentration (in this case 1 µg/ml).

The CR-MeOH extracts of the root barks at 1 µg/ml recorded the least activity with FabZ being the most susceptible (Table 21). The inhibition was up to 96.6% for *F. sycomorus* subsp. *sycomorus*. The leaf and stem bark extracts were inactive against all three enzymes, except for *F. wakefieldii* stem bark with FabZ inhibition of 79.6%. The only CR-MeOH to inhibit FabI was *F. sansibarica* leaf extract (62%).

Enzyme activity was also measured for the liquid-liquid paritioned subextracts. The hexane subextracts showed inhibition less than 50% except for four notable inhibition results recorded for *F. natalensis* root extract (97.8%) and *F. sycomorus* subsp. *sycomorus* (97.3%) against FabI, while *F. ingen*s (94.7%) and *F. sansibarica* (92.5%) inhibited FabG. The inhibition activity of the $CHCl_3$ subextract was just about 50% on average as well, with the roots showing the least activity.

Overall, the aq. MeOH subextracts were the most active, among them the leaves exhibited more significant activity against FabI. The aq. MeOH subextracts recorded the highest inhibition of up to 98.6% for *F. ingens*, so was that of the stem bark of *F. sycomorus* subsp. *sycomorus* (over 90 % against all FAS-enzymes; FabG, FabI and FabZ). FabZ was more sensitive than FabG which had an inhibition of over 90% for leaf and root subextracts of *F.ingens, F. sansibarica* and *F. sycomorus* subsp. *sycomorus*.

Table 21: Plasmodial FAS-II enzyme (FabG, FabI and FabZ at 1µg/ml) inhibition in Percentage (%).

Plant species	Type of Extract (µg/ml)	LEAF			STEM BARK			ROOT BARK		
		FabG	FabI	Fab Z	FabG	FabI	FabZ	FabG	FabI	FabZ
F. ingens	CR-MeOH	-	-	-	-	-	-	-	-	40.7
	Hexane	0	7.9	11.6	9	0	9.4	94.7	2	0
	$CHCl_3$	39	64.9	65.2	44.3	6	41.6	83.5	0	0
	Aq. MeOH	0	98.6	91	66.1	0	89.3	47.5	52.5	80.3
F. lutea	CR-MeOH	0	0	0	-	-	-	0	-	90.6
	Hexane	5	29.3	33.8	31.5	0	0	88.6	7	33.8
	$CHCl_3$	59	89	75	70.7	57.9	66.2	4.9	27.1	7.9
	Aq. MeOH	61.8	92.2	84.4	75.7	73.9	83.9	9.5	92.7	91.8
F. natalensis	CR-MeOH	0	0	0	-	-	0	0	-	-
	Hexane	0	35.2	14.2	33.4	16.1	18.5	25	97.8	0
	$CHCl_3$	22.5	57.6	48.4	84	78.4	84.1	25.3	49.9	12.6
	Aq. MeOH	76.2	95.8	88.3	89.9	19.1	88.5	30.1	79.6	85.3
F. ovata	CR-MeOH	0	0	0	0	0	0	0	0	-
	Hexane	9.3	59	21.9	29.6	0	7.4	2.3	29.2	0
	$CHCl_3$	0	77.6	66.9	30.8	0	27	65.3	0	0
	Aq. MeOH	46.4	97.6	88.4	84.9	70.4	92.4	2.3	49.9	28.5
F. sansibarica	CR-MeOH	-	62	-	-	-	-	-	-	-
	Hexane	0	45.2	0	44.4	0	0	92.1	59.9	0
	$CHCl_3$	1.3	34.9	0	71.1	0	62.8	1	22.4	0
	Aq. MeOH	-	50	14.1	92.6	90.9	0	90	82.9	0
F. sycomorus. gnaphalocarpa	CR-MeOH	0	-	0	0	-	0	0	-	96.6
	Hexane	0	41.4	11.9	33	8.5	22.2	64.9	0	0
	$CHCl_3$	48	81	65.7	49.5	0	57.8	62.2	65.3	41.3
	Aq. MeOH	63	95.6	83.2	92.2	85.7	0	80.4	85.9	90.4
F. sycomorus. sycomorus	CR-MeOH	-	-	-	-	-	-	0	-	65.8
	Hexane	13.6	39.7	5.1	40.1	0	0	4.1	97.3	0
	$CHCl_3$	0	44.4	23.2	76.8	62.9	70.2	0	0	0
	Aq. MeOH	3.7	98.8	85.3	91.2	90.3	96.2	39.9	95.8	43.2
F. wakefieldii	CR-MeOH	-	-	-	-	-	79.6	-	-	87.1
	Hexane	0	45.1	0	45.8	12.4	1.6	3.1	0	0
	$CHCl_3$	0	52.7	28	57.3	0	48.5	39.9	0.6	29.6
	Aq. MeOH	52.9	86	73.1	77.8	66.7	93	47.6	97.5	72.9
IC_{50} values of Standard drugs (µg/ml)		0.7[1]	0.017[2]	0.2[1]	0.6[1]	0.03[2]	0.3[1]		0.03[2]	0.34[1]

[1]*Epi*gallocatechin gallate (EGCG), [2]Triclosan, - implies no activity, in most cases self absorbance. 0 – implies no activity at 1µg/ml.

The results show that the extracts were most active against the FabZ enzyme, while the least activity was observed against FabG. The most extracts being the aq. MeOH extracts. These extracts were mainly composed of very polar metabolites to which the FAS-II enzyme activity could be attributed to. Since the *Ficus* species screened herein have clearly indicated constituting an array on flavonoids in the phytochemical profiling (Section 3.3), enzyme inhibition could therefore be attributed to the presence of these flavonoids. Compounds such as luteolin, catechins and gallocatechins have been isolated from the genus *Ficus* and are reported to exhibit significant enzyme inhibition (Tasdemir *et al*., 2006). Catechin-type metabolites have been identified in our extracts. In particular, it is reported that isoflavonoids show moderate activity against FabZ, while epicatechin gallates and gallocatechin gallates are potent inhibitors of all three enzymes (Tasdemir *et al*., 2006; Zhang and Rock, 2004). In addition highly hydroxylated and planar polyphenols have shown good enzyme inhibition with minimal selectivity. As Cushnie and Lamb (2005) suggests, flavonoids with homogenous structures inhibit a variety of biochemical enzymes while Tasdemir *et al*. (2006) argues that flavonoids (such as catechins and epicatechins) that carry a free hydroxyl group at C-3, neither have enzyme nor antiplasmodial activity.

The mechanisms by which the enzymes are inhibited by polyphenols are not the same for all the enzymes. Enzyme activity is due to the interaction of enzymes with the different parts of the substrates and/or the inhibitors or enhancers (Cushnie and Lamb, 2005). Inhibition against FabG is noncompetitive with regard to the substrate and cofactor (Tasdemir *et al*., 2006). For FabI, the mechanism depends on whether it is extracted from *E. coli* or *P. falciparum* because there are differences in the NADH-protein interactions in the enzymes of the two organisms. Bacterial FabI is competitively inhibited with respect to NADH (Zhang and Rock, 2004), while *Pf*FabI is noncompetitively inhibited. Inhibitors against FabZ are competitive, in that they compete with the substrate for the same binding site. Other researchers have used FabZ models to show that larger molecule inhibitors such as flavonoids bind differently to FabZ in comparison to smaller ones (Jensen *et al*., 2012). In this case, the overall activity by our extracts against the enzymes culminates from factors including the differences of the FAS-II enzyme inhibition

mechanisms and the chemical constituents of the extracts. *F. ovata* has emerged as the most active in the inhibition of the three FAS-II elongation enzymes.

The FAS-II activity did not correlate well with the antiplasmodial activity. For instance, the $CHCl_3$ subextracts exhibited the highest antiplasmodial activity with some having IC_{50} lower than 2 μg/ml. The stem bark of *F. ovata* exhibited an IC_{50} of 1.14 μg/ml against chloroquine resistant strains of *P. falciparum* K1 and then the percentage of inhibition against FabG, FabI and FabZ was 30% and below. However, the aq. MeOH subextract of this particular species showed significant FAS-II enzyme inhibition of 70% and above. The lack of correlation between enzymatic and antiparasitic *in vitro* activities was already observed in several studies. For instance, in a study by Tasdemir *et al*. (2006), a number of polyphenols with *in vitro* activity against chloroquine-sensitive and chloroquine-resistant *Plasmodium* parasite strains NF54 and K1 respectively, did not inhibit any of the FAS-II target enzymes. Zhang and Rock (2004) as well reported how antibacterial activity did not correlate with the inhibition of FAS-II enzymes in *E. coli* fatty acid biosynthesis. This suggests that the extracts may have other targets in the *Plasmodium* other than the FAS-II enzymes. Also the FAS-II pathway in the enzyme assay is an isolated entity which is ideal in its operation, while in the antiplasmodial assay, there ae many metabolisms or pathways which are taking place in the parasite that have enhance or inhibit the viability of the parasites.

3.5 PHYTOCHEMICAL INVESTIGATION OF *FICUS SANSIBARICA*

As discussed in section 3.2; TLC, NMR and HPLC analyses have shown that *F. sanisbarica* has a different chemical profile from the other seven species, even though its biological activities were moderate. Despite this activity profile, it was selected for detailed phytochemical investigation coupled with FAS-II enzyme inhibition testing because of its different phytochemical profile.

3.5.1 Large scale extraction and liquid-liquid partitioning

The crude methanol extraction and liquid-liquid partitioning of the dried and ground aerial parts of *Ficus sansibarica* (321g) was performed as described in Chapter 2.2.2 to yield crude MeOH extract (75g), hexane subextract (3.4g), $CHCl_3$ subextract (10g) and aq. MeOH subextract (40g) (Figure 43).

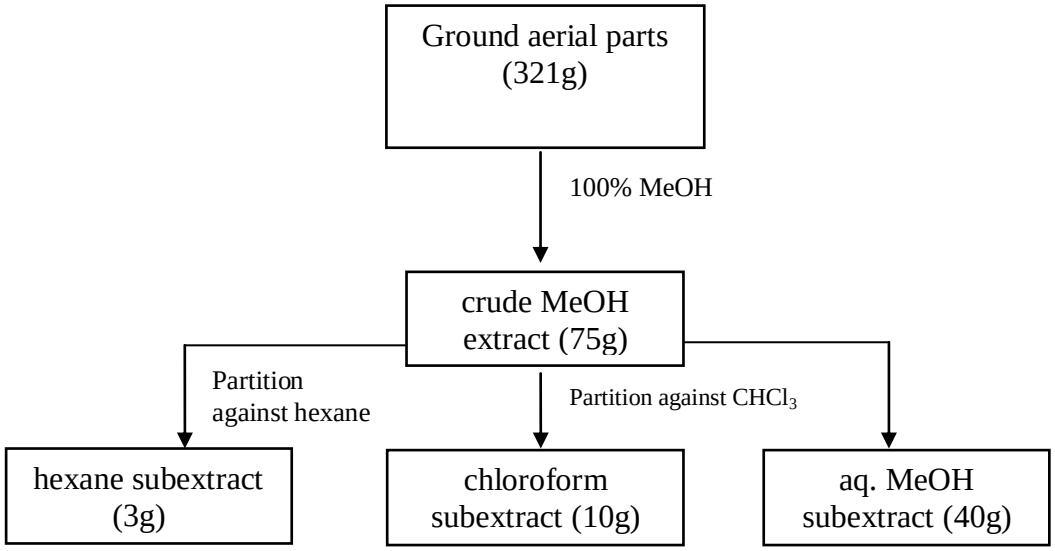


Figure 43: Extraction scheme for *F. sansibarica* for phytochemical investigation.

3.5.2 FAS-II inhibition assay

The *F. sansibarica* extracts and subextracts were tested for inhibition activity against FAS –II enzymes and the results are presented in Table 22. The aq-MeOH subextract was the most active with IC_{50} values close to the standards at 0.13 µg/ml for FabG, 1µg/ml for FabI and 0.25 µg/ml for FabZ. The hexane and $CHCl_3$ subextracts both showed inhibition over IC_{50} of 50 µg/ml for FabG and FabI, while IC_{50} of 2 µg/ml for FabZ. Meanwhile, FabZ was found to be the most susceptible with IC_{50} values of 0.25 -2 µg/ml for the hexane and aq. MeOH subextracts respectively.

Table 22: FAS-II inhibition assay (IC_{50} in µg/ml) of the crude extract and subextracts of *F. sansibarica*

Extract	**FabG**	**FabI**	**FabZ**
Crude MeOH	-	> 50	-
Hexane	> 50	> 50	2.0
Chloroform	> 50	> 50	2.0
Aq. methanol	0.13	1.0	0.25
Standards	0.2[1]	0.01[2]	0.3[1]

EGCG[1] and Triclosan[2]

3.5.3 Phytochemical profiling of *F. sansibarica* sub-extracts obtained by large scale extration

The extracts and subextracts were then analyzed by TLC (Figure 44) and ^1H NMR (Figure 45). The mobile solvent systems used for the TLC analysis was hexane/ethylacetate (8:2) for n-hexane subextract and chloroform/ethylacetate (6:4) for chloroform subextract. Both analyses showed the same chemical profile as obtained in the screening (Section 3.3.1 above), revealing a strong presence of aromatic compounds. From the ^1H NMR profiles of the CR-MeOH, $CHCl_3$ and aq. MeOH extracts, there appear to be similar metabolites in high concentration. Thus, further evaluation of the $CHCl_3$ and aq-MeOH subextracts was performed to isolate the most predominant metabolites. The TLC analysis was performed on silica gel 60 F_{254} plates and sprayed with 4% vanilin /sulphuric acid for visualization, while the NMR analysis was performed as has been outlined in Section 2.4.2.1.

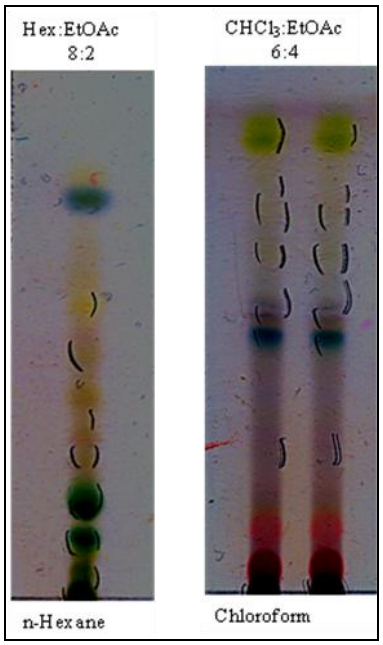


Figure 44: TLC analysis of the crude and sub-extracts of *F. sansibarica* leaves

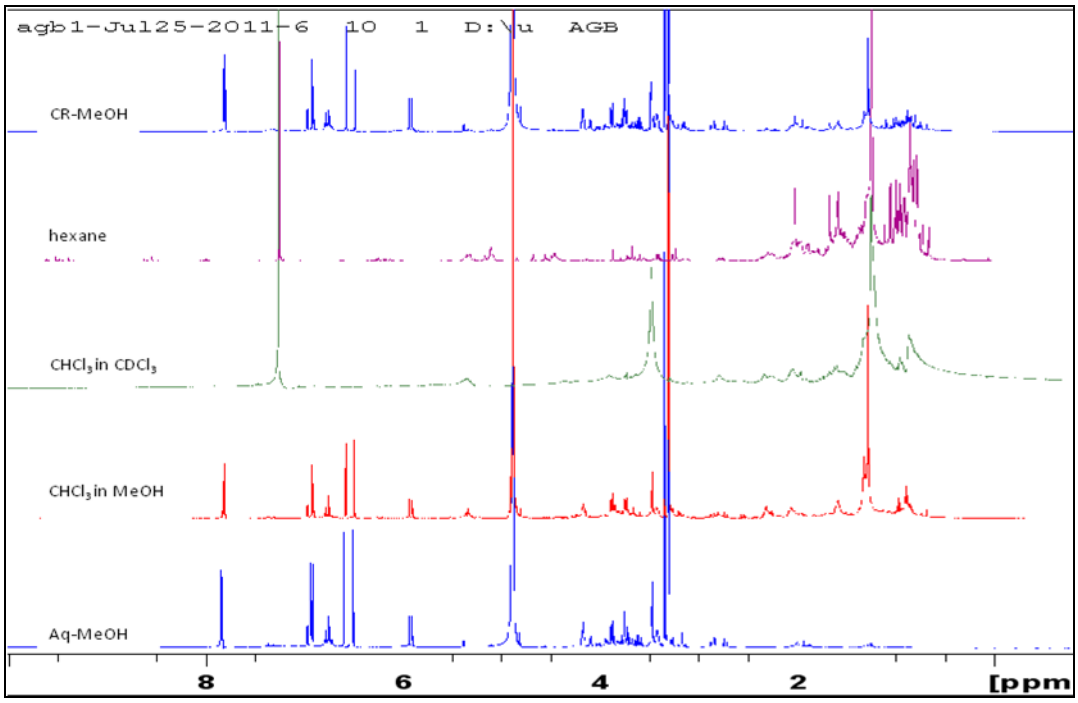


Figure 45: [1]HNMR spectra (500MHz) of *F. sansibarica* leaf crude and sub-extractions.

3.5.4 Investigation of the chloroform subextract

The $CHCl_3$ subextract (about 10 g) was fractionated by open column chromatography (OCC) with sephadex L-20. One hundred and fifty grams sephadex filling a 6 cm diameter column was used for every 1g of the $CHCl_3$ subextract to the separated. An isocratic mobile flow of 100% MeOH was applied resulting in fourteen fractions as the TLC plate in Figure 46 shows after being pooled following TLC (normal silica gel) and ^1H NMR analyses. The TLC showed fraction 10 to be well separated with fairly distinct components and fraction 9 which was very different from the other fractions in its response to the mobile phase and solubility in methanol. Fraction 4 had a distinct dark blue spot.

Further attempt to separate and isolate components in fraction 4 was launched with a two step silica VLC run. The first run was performed with the initial mobile phase of 100% methanol and then the second run was with a step-gradient mobile phase from $CHCl_3$ to methanol (in 10% increments). Unfortunately, the isolation process did not yield any pure metabolites as further isolations were not possible due to diminished sample size. The ^1H NMR however, revealed that compounds in this fraction were rich in nonpolar principles as the chemical shifts were concentrated in the regions $\delta_H 0$ - $\delta_H 2$ and $\delta_H 3$ - $\delta_H 4$.

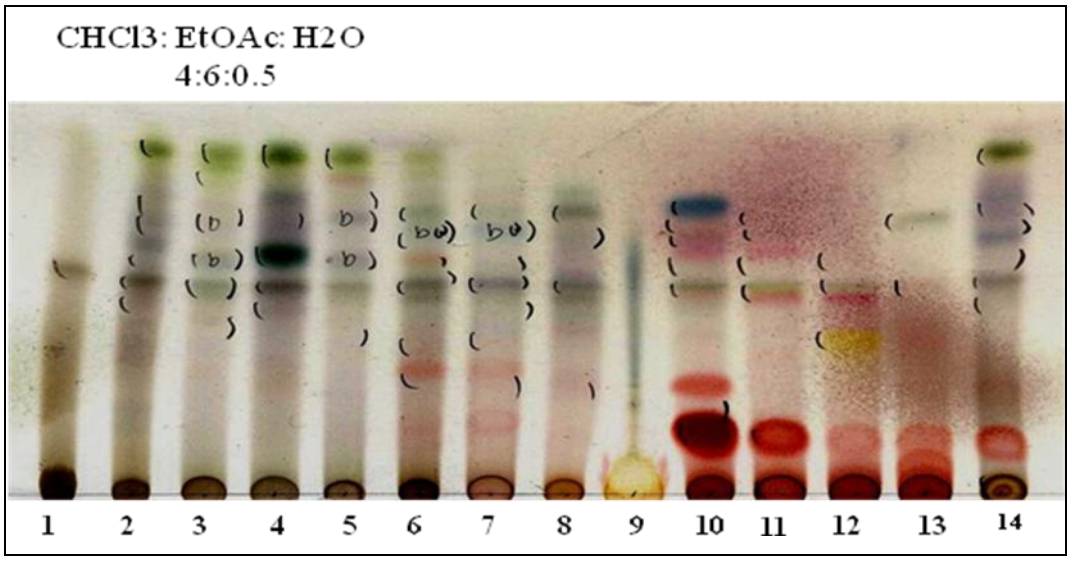


Figure 46: TLC (sprayed with VS) for the sephadex fractions of *F. sansibarica* leaves.

All the fractions showed some enzyme inhibition activity, with FabZ being the most inhibited (Table 23). The first six fractions showed higher rate of inhibition against FabZ such that IC_{50} values were calculated in the ranges 0.5 - 4 µg/ml. The rest exhibited moderate inhibition. In particular, fraction 4 did not show considerable activity against the FabG and FabI, while it exhibited inhibition of FabZ with IC_{50} of 4 µg/ml. Fractions 9 and 10 exhibited IC_{50} values of 3.5 µg/ml and 8 µg/ml respectively, against FabI. Fraction 11 also showed significant inhibition of IC_{50} of 2.3 µg/ml against FabI.

Table 23: FAS-II inhibition assay (IC_{50} in µg/ml) of the sephadex fractions of chloroform subextracts of *F. sansibarica*

Fractions	FabG	FabI	FabI	FabI	FabZ	FabZ
	Inhibition % at 10 µg/ml	Inhibition % at 10 µg/ml	Inhibition % at 1 µg/ml	IC_{50}	Inhibition % at 10 µg/ml	IC_{50}
1	74.1	69.1	35.6	nd		0.5
2	66.9	na	Na	nd		1
3	90.3	88.3	49.8	nd		1
4	42.1	na	61.6	nd		4
6	98	88.4	0	4.6		0.9
7	90.3	99.4	86.4	nd	96.5	1
8	66.8	92.7	57.3	nd	92	0.4
9	35.9	69.6	25.6	3.5	58	na
10	25.7	51.7	27.2	8	34.7	7
11	65.5	85.35	35.6	2.3	88.7	0.6
12	95.4	99	78	nd	98.4	0.01
13	92.7	93.7	81.4	nd	0	
IC_{50} of standards	0.27[1]			0.015[2]	0.19[1]	

[1] EGCG; [2] Triclosan; na- not active; + slope; nd – not determined

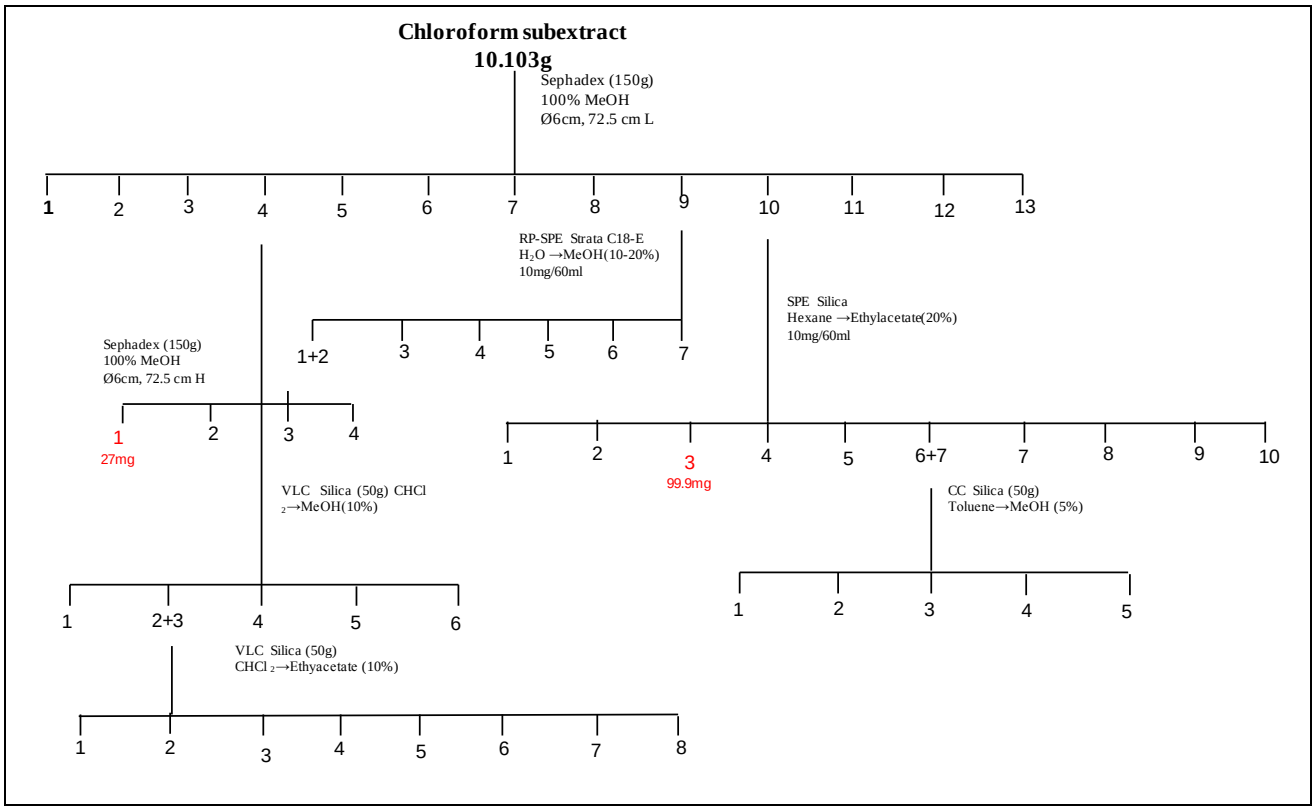


Figure 47: Fractionation scheme of the chloroform subextract.

3.5.4.1 Further separation of Fraction 10

Fraction 10 (460 mg) was also subjected to further separation by silica SPE (for every 150 g plant extract, a 10g/60 ml SPE SI-1 silica column was used) with an increase in polarity of the mobile phase by step-gradient from hexane to ethyl acetate (20% increment) to yield 10 fractions, with a pure component of Fraction 3 (99 mg). Figure 48 below shows the TLC analysis that led to arrive at F10-3. This compound yielded as F10-3 appeared as whitish solid. Further separation of the combined fractions 6 and 7, however it did not yield any purer chemical components. The isolation procedure of fraction 10 is outlined in Figure 47 above.

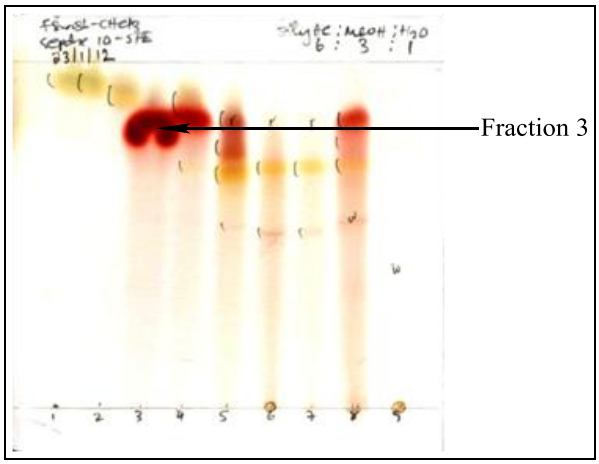


Figure 48: The TLC plate of F10-3, sprayed with 4% vanillin/sulphuric acid spray
(Mobile phase was ethylacetate: MeOH: H_2O, 6:3:1)

Since the crude $CHCl_3$ subextract did exhibit some FAS-II enzyme inhibition, the subfractions of fraction 10 were tested for FabG, FabI and FabZ inhibition. The first three fractions 1, 2 and 3 showed inhibition with IC_{50} values ranging between 0.35 µg/ml to 11.6 µg/ml (Table 24). FabI and FabZ were the most susceptible, while FabG was not. F10-3 exhibited appreciable activity against FabI and FabZ with IC_{50} values of 4.2 µg/ml and 3.5 µg/ml respectively, while it did not exhibit activity against FabG.

Table 24: FAS-II inhibition assay (IC_{50} in μg/ml) of the $CHCl_3$ subextract fraction 10 of *F. sansibarica*

Fractions	FAB G	FAB I	FAB Z
Fr. 1	n.a*	11.6	0.35
Fr. 2	15	5.4	0.4
Fr. 3	n.a	4.2	3.5
Fr.4	24	3	4.0
Fr.5	6.4	n.a	6.0
Fr. 6	10.6	n.a	n.d
Fr. 7	n.a	n.a	4.3
Fr. 8	2.8	n.a	8.7
Fr. 10	10.8	n.a	9
EGCG	0.4	-	0.04
Triclosan	-	0.02	-

n.a = not active; n.d = not determined.

There seems to be particular attention attached to the compounds in high concentration as the active agents against microorganisms. This, however, may not be the case because their action could be a result of the combined effect of more than one compound which, with another contributes to the increased or maybe reduced pharmacological activity. Thus, the minute compounds which maybe active or inactive may tend to influence bioavailabilty of the active metabolite to the pharmacokinetic reactions that will be undergone (Svoboda and Hampson, 1999). Thus the chemical constituents may have synergistic effects, which agree with Chalchat *et al*. (1997) who pointed out that correlating biological activity to the extracts' chemical composition should be tentative, because the involvement of less abundant compounds should be considered. It is worth noting that biological activities could be due to single compounds in a very complicated concert of synergistic or antagonistic activities (Svoboda and Hampson, 1999). With regard to the above statement, the isolated compound F10-3 as epicatechin exhibited the inhibitory potential against FabZ and FabI enzymes with IC_{50} of 3.5 μg/ml and 4.2 μg/ml

respectively (shown as Fr. 3 in Figure 48). The isolated epicatechin was however, not 100%pure, hence the activity can be attributed to the principle compound of the isolate, the epicatechin or to the minute 'impurities' that were providing synergistic or added effect on the main compound. In literature, it has been reported by Tasdemir *et al*. (2006) that pure epicatechin (Buchs, Switzerland) standard does not exhibit any inhibition against any of the three Fas-II enzymes. Tasdemir *et al*. (2006) alludes to the fact that methylation of the hydroxyl groups on the flavones or flavonol moiety removes almost all activity against all FAS-II enzymes. Thus catechins and epicatechins, carrying a free hydroxyl group at C-3, neither inhibit the enzymes nor have antiplasmodial activity. Meanwhile, epicatechin gallates and gallocatechin gallates are potent inhibitors of all three enzymes (Tasdemir *et al*., 2006). Thus, it is essential to co-test the isolated pure compound with the relevant standard to ascertain the source of activity that can be claimed.

Most often than not it has been proven in many studies that isolated compounds that are not pure are active while the purified forms lose activity (Lansky and Paavilainen 2011; Weathers *et al*., 2011). This explains why many traditional remedies are potent in many instances even though cytotoxicity is not taken into consideration; it is the 'chemical impurities' that may help reduce any toxic effects (Lansky and Paavilainen, 2011). Hence a huge challenge comes in when the active metabolites cannot be identified during the process of bioactivity-guided isolations because they may lose efficacy along the process or the sample be lost in the process. The natural products that are known to inhibit FAS-II enzymes are cerulenin and thiolactomycin which target FabB, FabF and FabH (Kırmızıbekmez *et al*., 2004).

3.5.4.1.1 Structure elucidation of compound F10-3

The compound F10-3 (Figure 49) was isolated from the chloroform extract of the leaves of *F. sansibarica.* It was obtained as a whitish amorphous powder with the ESI-MS spectrum showing molecular ion peak at *m/z* 291 ($M+H^+$). Other analyses included ^1H NMR, ^{13}C NMR and TLC to obtain an R_f value of 0.8 in ethylacetate: MeOH: H_2O, 6:3:1 mobile phase. All these analyses together with the molecular weight indicated the compound had a molecular formula $C_{15}H_{14}0_6$, suggesting it is a flavonoid. Figure 49 shows the proposed structure for F10-3 to be an epicatechin with the elucidation information outlined.

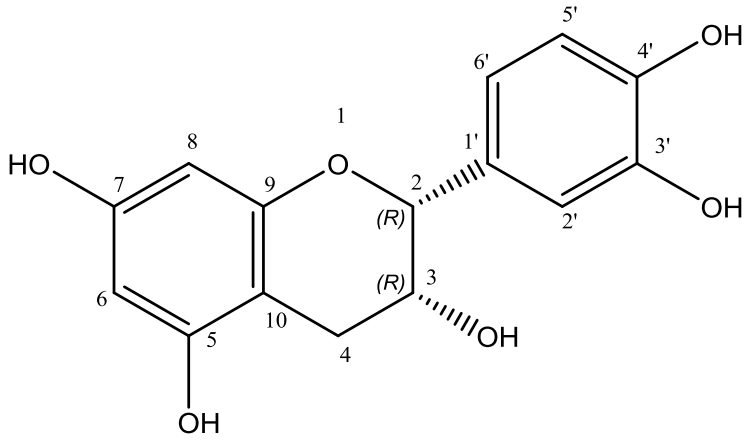


Figure 49: Proposed structure of F10-3 as Epicatechin

The ^1H NMR spectrum (Figure 50) evidenced two aromatic rings with a pair of meta-coupled aromatic signals on ring A(δ_H 5.95, H-6) and (δ_H 5.92, H-8) and a set of protons in the ABD system on ring B (δ_H 6.98, d, *J* =1.6 Hz, H-2'; δ_H 6.81, dd, *J* = 2, 8.4 Hz, H-5'; δ_H 6.77 d, *J*=8, H-6'). On the C ring, the characteristic signals included two non-equivalent double doublets of methylene (δ_H 2.86 dd, 2.73 dd) and two oxymethines (δ_H 4.8, H - 2; δ_H 4.18, H - 3).

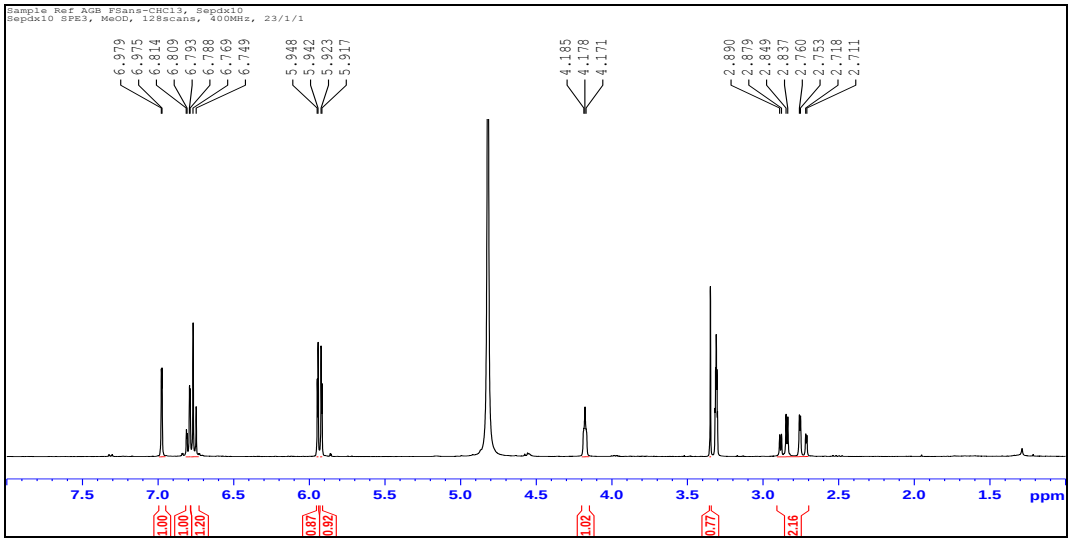


Figure 50: ^{1}H NMR spectrum of F10-3 analysed in methanol – d_4

The ^{13}C NMR spectrum (Figure 51) revealed the presence of 12 carbon signals in the aromatic region which falls between 110 and 160 ppm. Two oxymethines at δ_C 79.97 and 67.51 and one methylene at δ_C 29.34 were also revealed as in the proton signals, which were confirmed in the DEPT-35 analysis (Figure 52). The DEPT-135 spectrum showed seven positive signals, one negative signal to confirm the methylene mentioned earlier, and six quaternary carbons (δ_C 158.08, 157.78, 157.48, 145.88, 145.03 and 132.38) all in the aromatic region. The ^{1}H NMR, ^{1}C NMR and DEPT-35 data suggested that the compound was a flavonoid of the type flavan-3-ol.

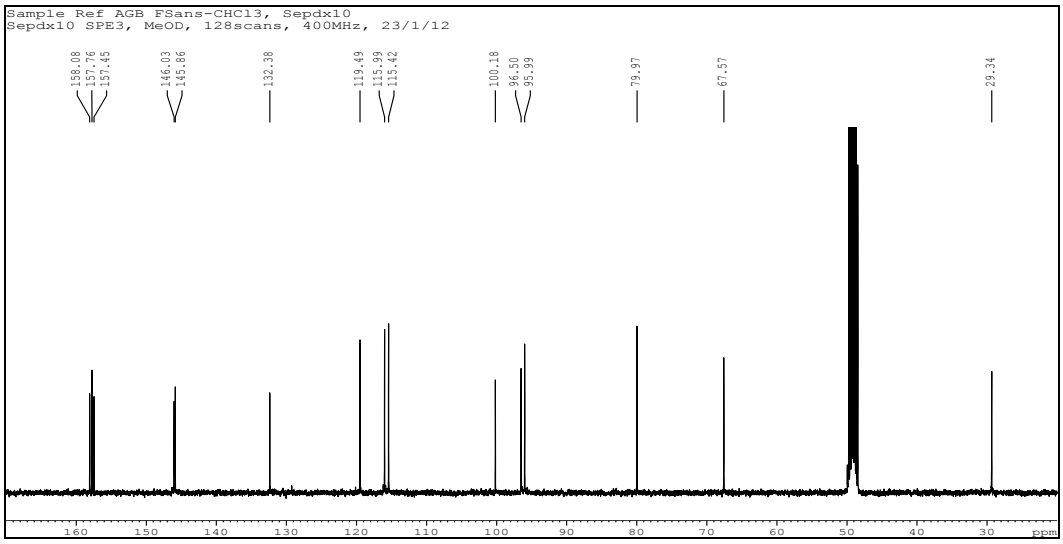


Figure 51: ^{13}C NMR of F10-3 methanol – d_4

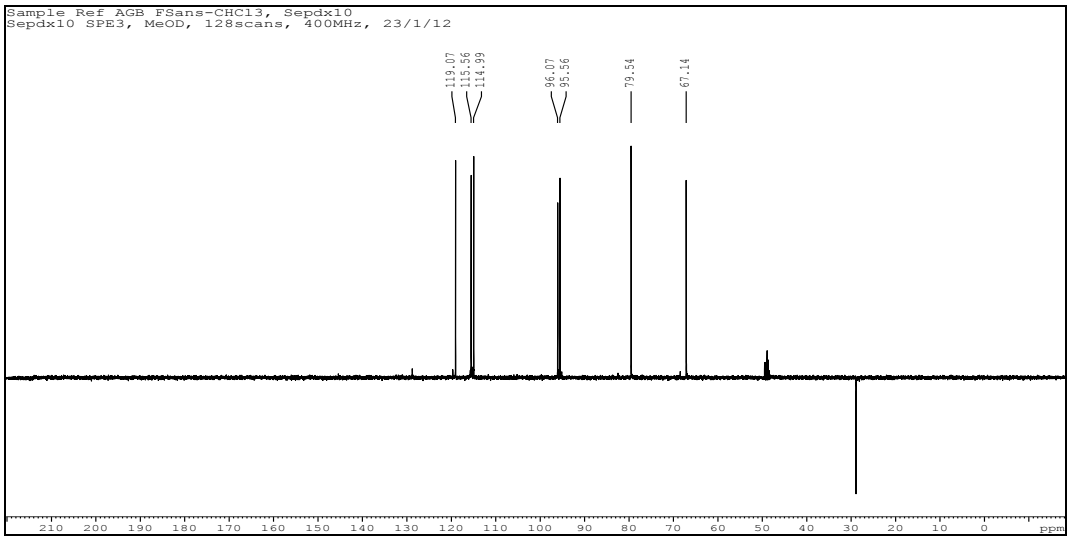


Figure 52: Dept-135 spectrum for F10-3

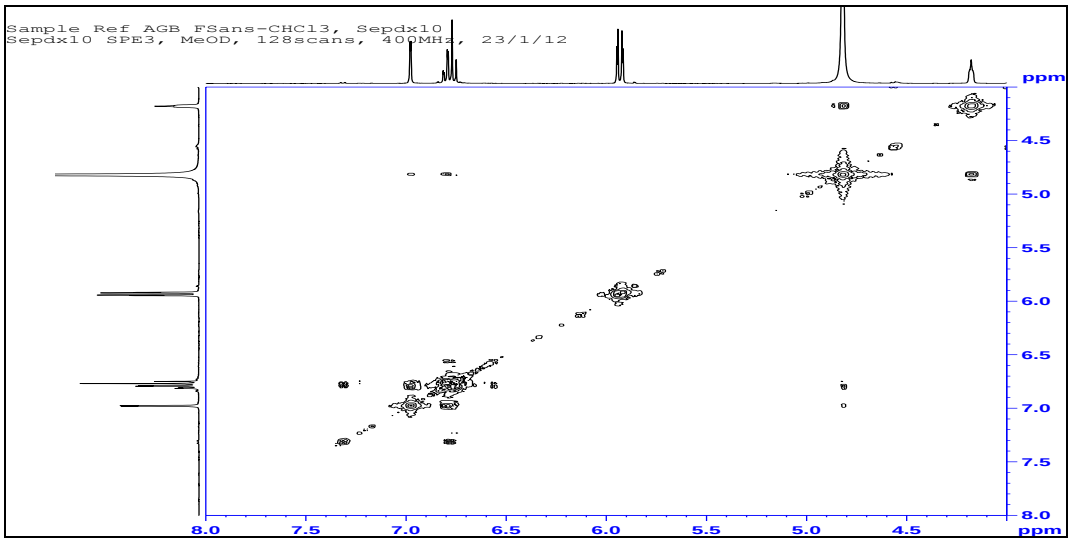


Figure 53: Cosy spectrum of F 10-3

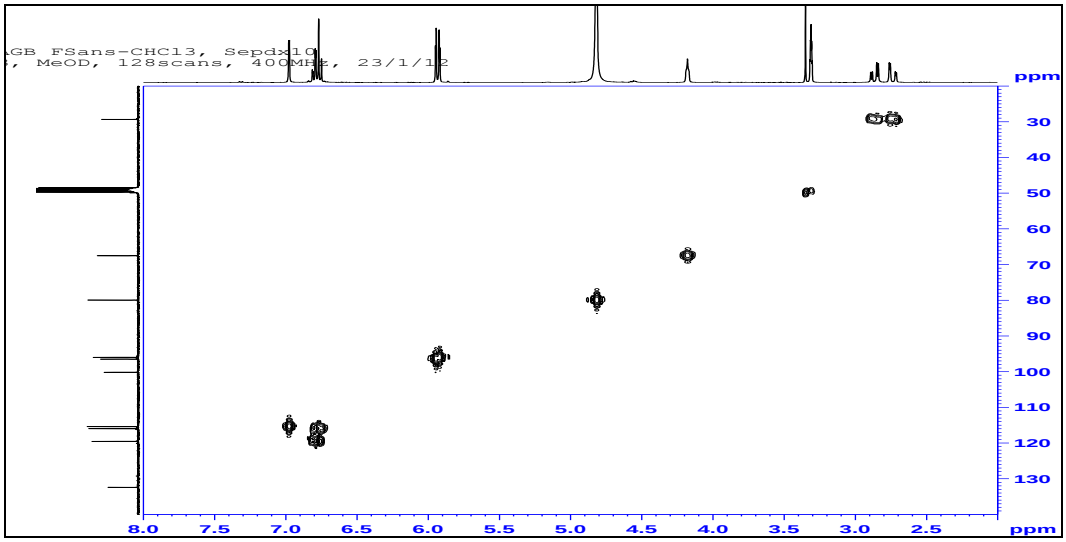


Figure 54: HMQC spectrum of F 10-3

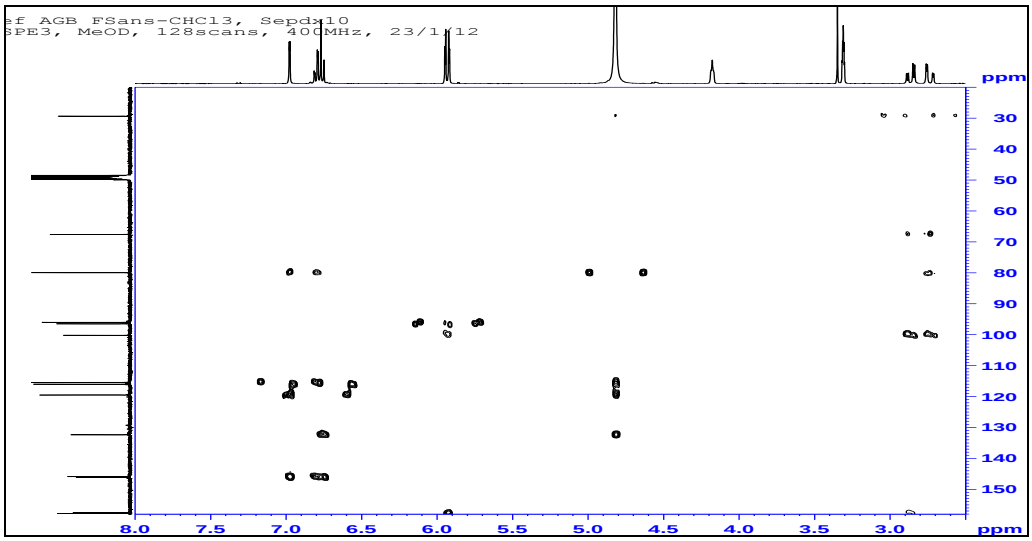


Figure 55: HMBC spectrum of F 10-3

Table 25: ^{1}H (400 MHz) and ^{13}CNMR (100 MHz) spectral data of F10-3 recorded in methanol – d_4

Position	**^{1}H (ppm)**	**J/Hz**	**^{13}C**
1	-	-	
2	4.75 s	-	79.95
3	4.1 m	-	67.5
4a	2.76 dd	2.8	29.33
4b	2.89 dd	4.4	29.33
5	-	-	157.44
6	5.95 d	2.4(*m*)	96.55
7	-	-	157.75
8	5.92 d	2.4(*m*)	96.2
9	-	-	157.75
10	-	-	100.21
1’	-	-	132.5
2’	6.98 d	1.6(*m*)	115.98
3’	-	-	145.87
4’	-	-	146.08
5’	6.81 dd	2, 8.4 (*m*,*o*)	115.35
6’	6.77 d	8 (*o*)	119.45

3.5.5 Investigation of the aq. MeOH subextract

The chromatographic fractionation of 10 g aqueous methanol subextract of the leaves of *F. sansibarica* was performed by a series of techniques, which included reversed phase VLC and SPE by step-gradient mobile phase of 100% water to 100% methanol in 10% increments. Three fractions A, B and C were realized. ^{1}H NMR analyses for the fractions revealed that fraction A (6 g) was characterized by very polar white crystalline principles, which were dominated with peaks in the δ_H 3 - δ_H 5 region likely to arise from anomeric protons of sugars. Fraction B revealed a strong presence of aromatic signals with peaks arising from chemical shifts δ_H 6.98, δ_H 6.814, δ_H 6.77, δ_H 5.95 and δ_H 5.92. These signals are equivalent to the catechin-type of flavonols. Fraction C on the other hand was also dominated with aromatic signals slightly further downfield (δ_H 8 - δ_H 6) compared to fraction B, and dense anomeric proton signals arising between δ_H 5 - δ_H 3. The fractionation scheme of the aqueous methanol subextract is illustrated in Figure 56 below.

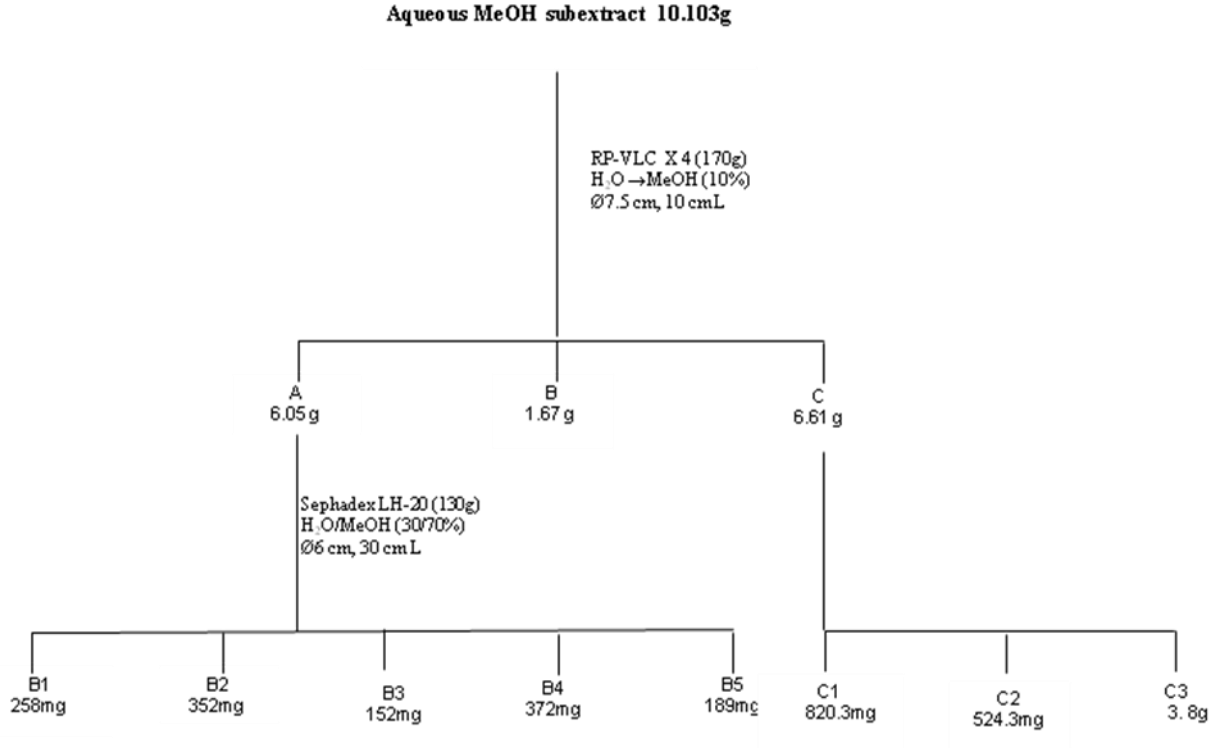


Figure 56: Fractionation scheme of the aq.methanol subextract.

Upon testing these fractions for FAS-II enzymes inhibition, fractions B and C showed rather significant activity, while fraction A was not active at all (Table 26). Fraction B was more active than C against all the three FAS-II enzymes tested. Fractions B and C were further subjected to chromatographical isolations.

Fraction B (1.67g) was loaded onto a sephadex L-20 column with the mobile phase consisting of water and methanol in the ratio of 30% to70%. Five fractions were obtained which were very closely related, however due to small quatities realised, we did not proceed with the separation to get pure compounds

Fraction C was also subjected to further isolation by preparatory HPLC with an isocratic gradient mobile phase consisting of methanol and acetonitrile. The conditions were as outlined in Chapter 2.4.1.5. The fractionation yielded three fractions; C1, C2, and C3. These fractions, upon being tested against FAS-II enzymes exhibited activity (Table 26) with IC_{50} values which ranged from 0.75 μg/ml to 6.0 μg/ml. Fraction C2 was the most active with IC_{50} values of 4 μg/ml, 4.6 μg/ml and 0.75 μg/ml against FabG, FabI and FabZ respectively. According to the HPLC-UV spectra of this fraction, it showed richness in compounds related to apigenin glucoside derivative and is the fraction from which another pure compound proposed to be either apigenin-8-C-glucoside or its isomer was isolated. As it was mentioned in Section 3.4.7, FabZ was the most sensitive to the fraction (IC_{50} of 0.75 μg/ml), and apigenin glucosides being large molecules fail to enter the narrow active site tunnel of the enzyme tending to block the entrance of the tunnel. This eventually inhibits the substrate from accessing the active site (Zhang *et al*., 2008).

A series of flavonoids have been published to be inhibitors of *Plasmodium falciparum* (*Pf*FabZ) (Tasdemir *et al*. 2006) and *Helicobacter pylori* (*Hp*FabZ) (Zhang *et al*., 2008) FabZ enzyme, in which apigenin show activity against FabI and FabZ respectively. It is however, reported that the activity against FAS-II enzymes increased as the aglycone became more hydroxylated (Tasdemir 2006). Suffice to say that correlations of little alterations in the structures of flavonoids e.g., positional changes of OH groups or glycosidation, bring about dramatic changes in their biological effects. Since our isloltaed compound (C2-4) has a similar parent chemical structure as that of apigenin, it would be assumed that its *in vitro* activity increases due to the increase of hydroxyls in the

structure. In this case, we can argue that comparing the activities of a glycoside to its respective aglycone is not feasible as the glycosidic residue can be crucial for their activity or can only improve pharmacokinetic parameters.

Table 26: IC_{50} values in µg/ml of the RP-VLC aq. MeOH subextracts

Fraction	FAB G	FAB I	FAB Z
A	Na	na	na
B	3	2.5	4.4
C	4.8	2.5	8
B1	15	3.8	9
B2	5	2.8	5.8
B3	12	6.4	na
B4	4	2.9	3.7
C1	5.5	2.8	6
C2	4	4.6	0.75
C3	5	4.8	3.2
EGCG	0.23		0.25
Triclosan		0.027	

na- not active

Fraction C2 was then further separated into five subfractions by preparatory HPLC, with conditions as follows; The gradient elution was set at 0-5 % A (0-10 mins), 5-40% A (10-15 mins), 40-100% A (15-25 mins), 100-5% A(25-26 mins), 5% (26-30 min), with solvent A being methanol and solvent B was acetonitrile. The other conditions remained the same as outlined in Chapter 2. However, the extract was set at a concentration of 40 µg/ml. From this analysis five fractions were obtained, labelled C2-1, C2-2, C2-3, C2-4 and C2-5. And a pure compound C2-4 was realized, which was obtained as a yellow amorphous solid. Due to the very small amount obtained and time constraints, we were unable to test it for activity against FAS-II enzymes, but was analysed by ^{1}H NMR, ^{13}C NMR, DEPT COSY, HMQC and HMBC. The NMR analyses of Fraction C2-5 was found to be similar to C2-4, however when analysed by HPLC, two distinct peaks appeared at RT 17.5 and RT 19.3. This baseline chromatographic separation could imply a compound and its isomer isolated together. This fraction was also subjected to spectroscopic analysis for structure elucidation, and the structure proposed was the same as that of compound C2-4, only that it had more baseline peaks. Both samples were analysed for accurate mass measurement on the [M+H] + ions and the results obtained

were 433.1132 for C2-4 and 433.1149 for C2-5, however, with the same theoretical mass of 433.1135. C2-4 was proposed to be either apigenin-8-*C*-glucoside (vitexin) or apigenin-6-*C*-glucoside (isovitexin). However, from the analyses, we can tentatively say that fraction C2-5 could be a mixture of apigenin-8-*C*-glucoside with its isomer (apigenin-6-glucoside) or that it had a lot other impurites. This was evidenced by the two peaks that appeared in the HPLC analysis

3.5.5.1.1Structure elucidation of compound C2-4

Compound C2-4 was isolated from the aqueous methanol subextract of the leaves *of F. sansibarica*. It was obtained as a yellow amorphous solid. The TLC analysis of C2-4 on silica gel 60 F_{254} plate showed a yellow spot with R*f* = 0.43 (with mobile phase of $CHCl_3$: MeOH, 6:4) after spraying with 4% vanillin/H_2SO_4 reagent and heating for about 5 minutes (Figure 57).

The accurate mass measurement on the [M+H] + ion was 433.1132, with Cortisone being used as an internal standard, [M+H] + =361.2015 in this experiment. Moreover, a direct infusion of the sample in positive ESI-MS mode gave a prominent molecular ion of *m/z* 433 $[M+H]^+$, thus again suggesting a Mr = 432. Interestingly, The fragmentation pattern of the isolated compound included a peak at *m*/*z* 313 $[M+H-120]^+$ indicating a glycoside attached by a *C*-linkage.

In a HPLC analysis under the conditions described previously in Section 2.3.1.5, it was eluted at RT 43.39 min and it showed a similar UV spectra and diagnostic shifts as the standard apigenin-7-*O*-glucoside which eluted at RT 53.4 min. Generally, flavone *C*-glycosides elute faster than their corresponding iso-glycosylflavones and *O*-glycosides (Andersen and Markham, 2006). There were two maxima of the UV spectra at λ=268.6 nm and λ=336.5 nm, while those of apigenin-7-*O*-glucoside appeared at λ=262 nm and λ=333 nm. At this point we hypothesised a flavone *C*-glycoside. Vitexin and isovitexin have been previously identified in many *Ficus* species although not yet in *F. sansibarica*.

Figure 57: TLC analysis plate of C2-4

For further elucidation and confirmation of the structure, ^{1}H NMR, ^{13}C NMR and 2D NMR experiments were performed initially in Methanol-d_4 (MeOD), then in DMSO-d_6

The ^{1}H NMR spectrum (Figure 58) revealed two signals with 'ortho coupling' at δ 6.93 (2H, **d**, J=8.5 Hz) and 7.85 (2H, **d**, J=9Hz), which could be preliminary assigned to the H-2′, H-3′, H-5′ and H-6′ of the B ring of an hypothetical apigenin *C*-glycoside.

This spectrum also evidenced a possible anomeric hydrogen signal at δ_H = 4.9, correlated to chemical shifts δ_c 72.7 (HMQC), δ_c 80.4, δ_c 109.5, δ_c 162.2 and δ_c 165.7 (HMBC) which were assigned to ring A, thus consistent with the presence of a sugar moiety. We may assume this signal to be a doublet, although the signal is obstructed by a water residue peak which arises at chemical shift δ_c 4.8)

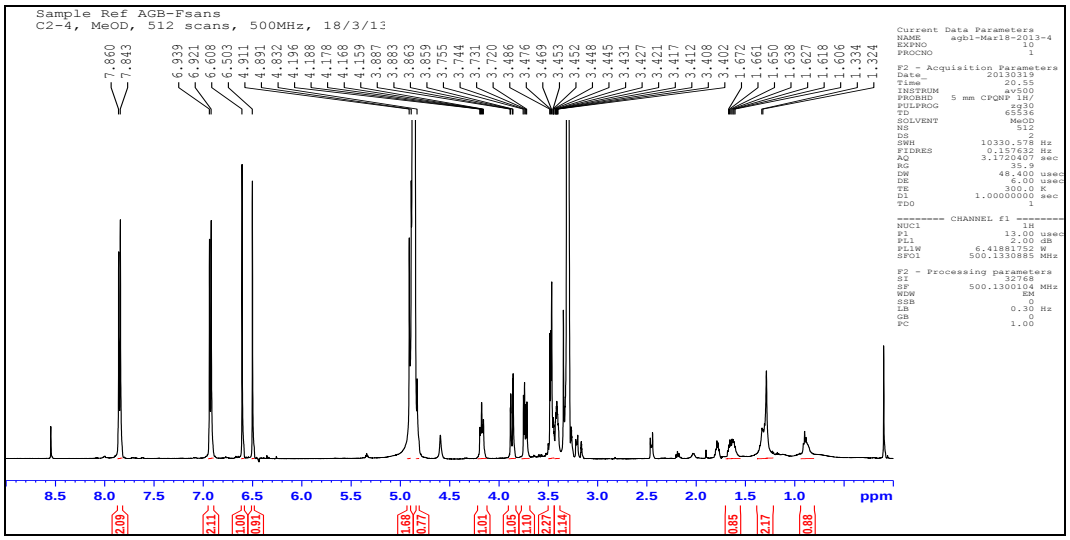


Figure 58: ^{1}H NMR spectrum of C2-4 analysed in methanol – d_4

The structural assignment of C2-4 was further substantiated by its ^{13}C NMR spectrum (Figure 59) which revealed the presence of 21 carbon signals with an indication of 15 carbons arising from the flavonol skeleton with plausible chemical structure of $C_{21}H_{20}O_{10}$. In addition, the DEPT-135 spectrum in Figure 60 revealed eight quaternary carbons (δ_C 184.1, 166.3, 163.1, 162.2, 158.9, 123.3, 109.5 and 104.0) and one methylene (δ_C 63.21). The HMQC (Figure 62) and HMBC (Figure 63) spectra were analysed to identify the C-H and C-C-H connectivity respectively. The B-ring carbons resonate at 123.3 (C1'), 129.6 (C2' and C6'), 117.3 (C3' and C5') and 163.0 (C4').

The findings of a δ_H 6.6 linked directly to δ_C 104.0 and correlated with δ_C 184.1 (C=O) and δ_C 166.3 conforms with C3, C4 and C2 of a C-ring structure, respectively. All these data are consistent with previous data of apigenin derivatives analysed in MeOD (Svehliková *et al.*, 2004) (Table 26).

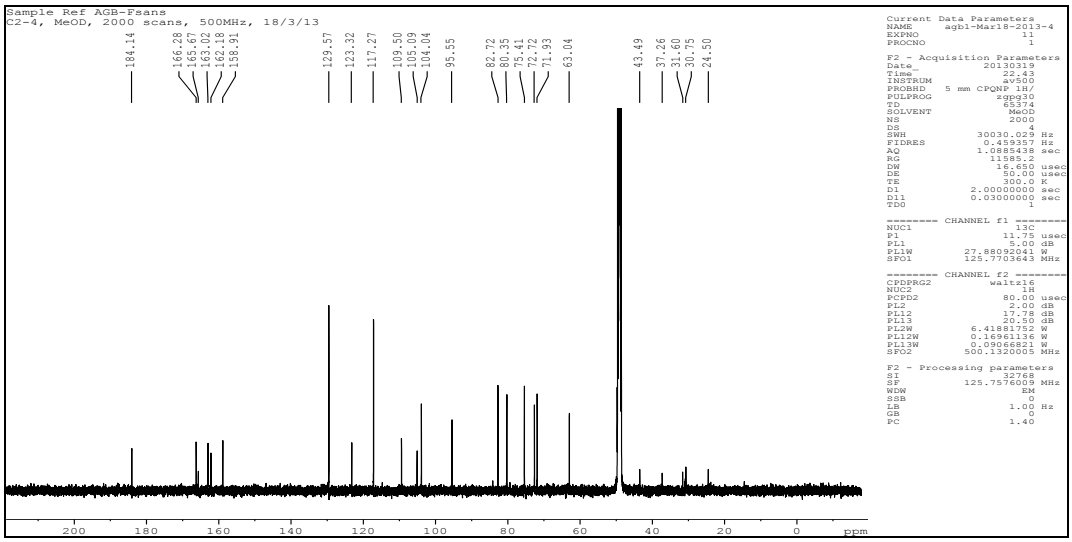


Figure 59: ^{13}CNMR spectrum of C2-4 analysed in methanol – d_4

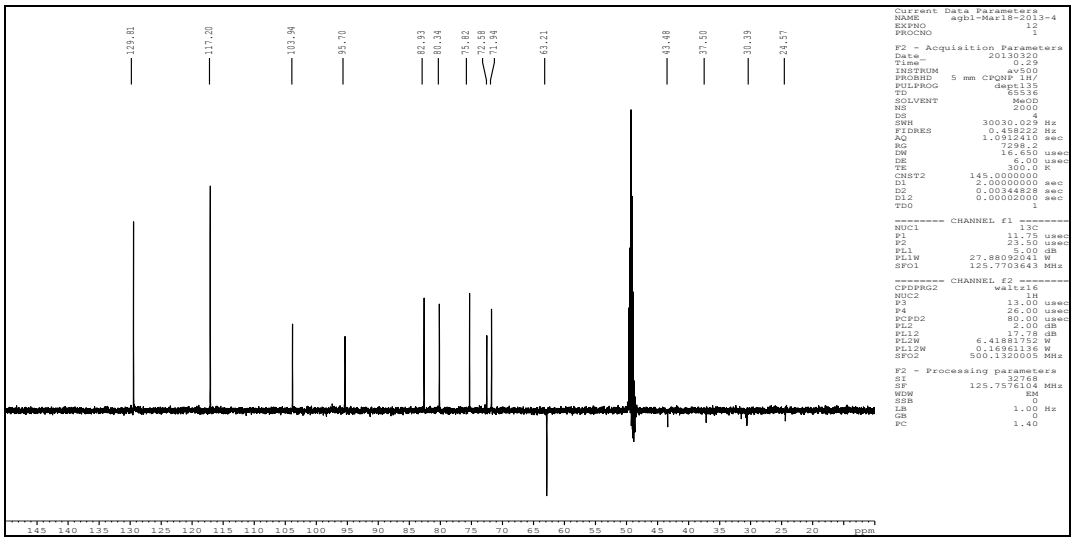


Figure 60: Dept-35 spectrum of C2-4

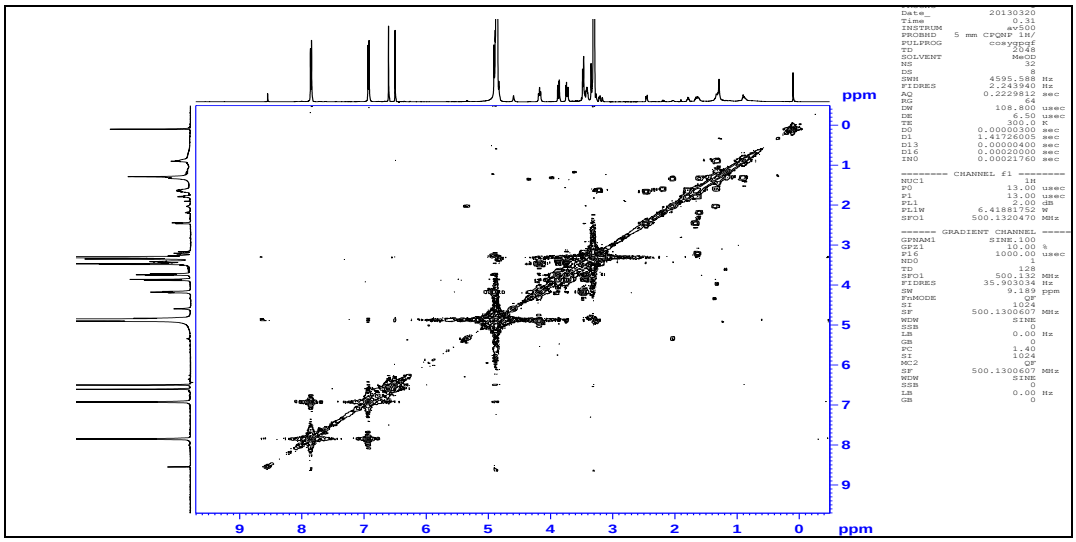


Figure 61: Cosy spectrum of C 2-4

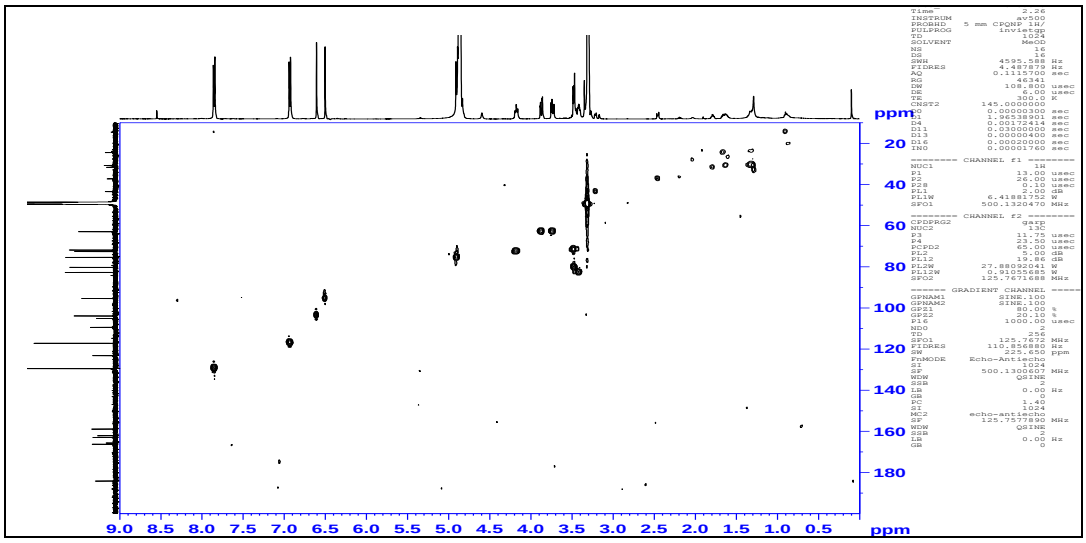


Figure 62: HMQC spectrum of C 2-4

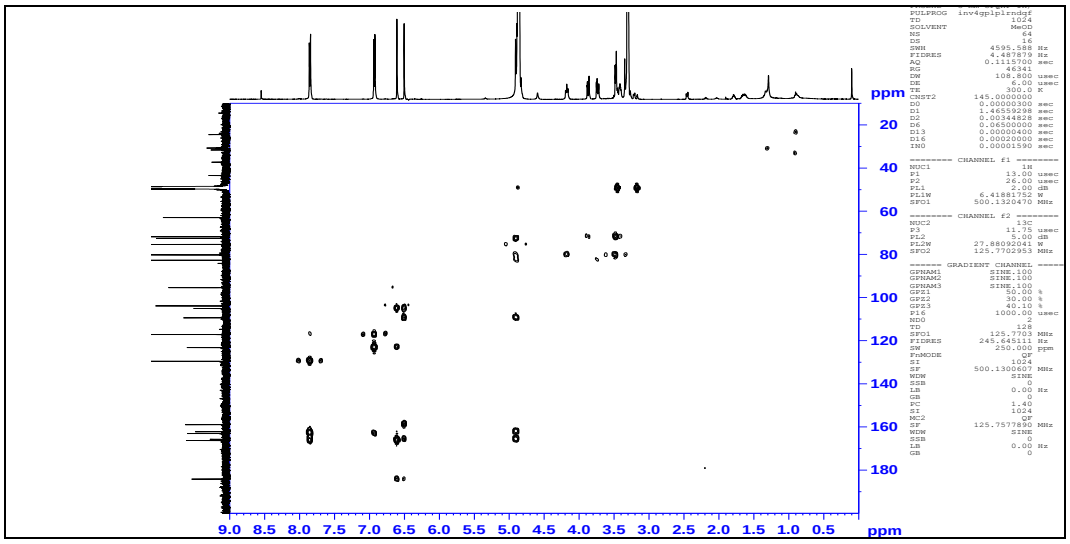


Figure 63: HMBC spectrum of C 2-4

Table 27: Chemical shifts (ppm) reported in literature for a series of five different apigenin-7-*O*-glycosides in MeOD compared with those of C2-4.

	Apigenin-7-*O*-glycosides (Svehliková *et al*., 2004)					**Average**[a]	**C2-4**[a]
C-2	164.5	166.9	166.9	166.8	166.8	*166.38*	166.3
C-3	103.3	103.7	104.1	103.4	104.1	*103.72*	104.0
C-4	182.2	184.2	184.2	182.2	184.2	*183.4*	184.1
C-5	161.5	162.9	163.3	162.7	163.0	***162.68***	**158.9**
C-6	99.8	101.0	100.6	99.6	101.1	***100.42***	**95.6**
C-7	162.9	164.6	164.6	164.5	164.4	*164.2*	165.7
C-8	95.0	95.6	96.3	95.0	96.0	***95.58***	**109.5**
C-9	157.2	159.0	158.8	157.1	158.8	***158.18***	**162.2**
C-10	105.6	106.7	106.7	105.7	106.8	*106.3*	105.1
C-1	121.2	122.2	122.9	121.2	122.8	*122.06*	123.3
C-2 / C-6	*128.8*	*129.5*	*129.8*	*128.8*	*130.0*	*129.38*	129.6
C-3 / C-5	*116.2*	*117.2*	*117.2*	*116.2*	*117.4*	*116.84*	117.3
C-4	*161.3*	*163.8*	*163.3*	*161.5*	*163.0*	*162.58*	163.0

[a]In bold, signals which differ more than 1 ppm.

The elucidation of the A-ring, however, may be interpreted in two ways, depending on the assumption of a *C6*- or *C8*-glycoside (isovitexin or vitexin, respectively). On one hand, the anomeric proton is correlated in the HMQC with δ_C 109.5, and with two more carbons (δ_C 165.67 and δ_C 162.18) according the HMBC experiment. On the other hand, the aromatic proton δ_H 6.5 -which is attached directly with the carbon resonating at δ_c 95.6- shows correlations with 5 carbons, namely δ_C 105, 109.5, 158.9, 165.67 and –although with less intensity- 184.1.

According to all previously reported data on flavones, the chemical shift of the C10 always ranges between 105-106 ppm and so δ_C 105.1 can be confidently assigned to this position (Svehliková *et al.*, 2004).

From here, two scenarios can be envisaged:

1. If we assume that C2-4 is vitexin, then C8 is 109.5, which is not a quaternary carbon as was shown in the DEPT experiment (Figure 60). This carbon does not directly attach to the carbon of the glycoside through. Meanwhile, C7 and C9 could be δ_C 165.67 and δ_C 162.18 or vice versa (Figure 64).

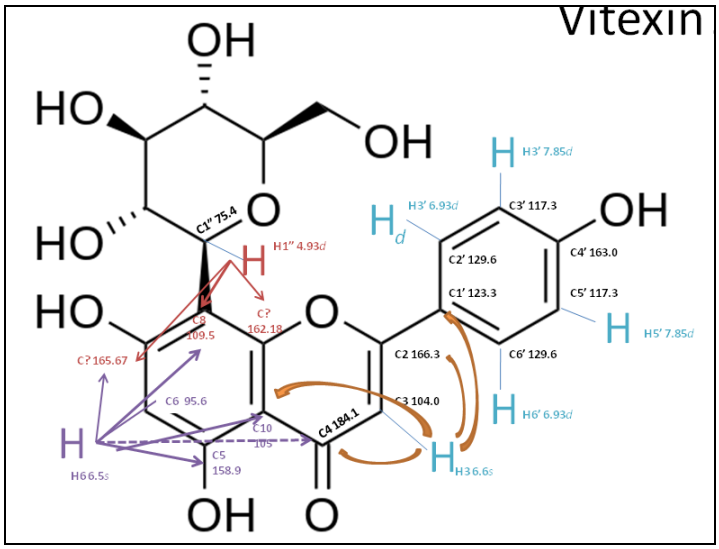


Figure 64: Theoretical allocation of NMR signals to fit the structure of vitexin

2. If we assume that C2-4 is isovitexin, then C8 is 95.6, while C7 and C9 may take any of the values δ_C 165.67 or δ_C 158.9 (Figure 65).

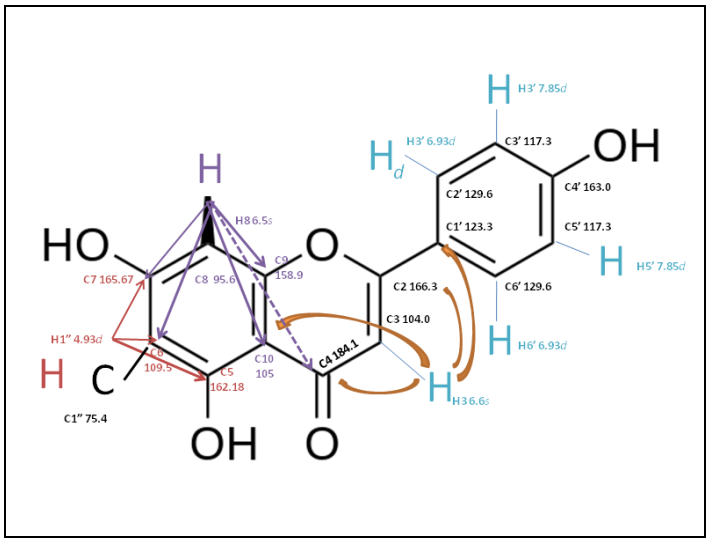


Figure 65: Theoretical allocation of NMR signals to fit the structure of isovitexin

To ascertain the most likely compound, Table 28 summarizes both scenarios together with the compound C2-4 spectral data, which could be equally acceptable in theory.

Table 28: ^{1}H (500 MHz) and ^{13}CNMR (125 MHz) spectral data of C2-4 recorded in methanol – d_4

					C2-4
Position	**^{1}H (ppm)**	***J* values (Hz)**	**COSY**	**HMBC**	**^{13}C**
2				3, 2', 6'	166.3 s
3	6.59 s			2, 3, 10, 1'	104.0 d
4				3	184.1 s
5				6	162.2 s
6	6.49 s			5,7,8,10	109.5s
7				6, 1"	165.7 s
8				6, 1''	95.6 d
9				1''	158.9 s
10				3, 6	105.1 s
1'				3, 3', 5'	123.3 s
2'	7.85 d	9	3'		129.6 d
3'	6.93 d	8.5	2'	2'	117.3 d
4'				2', 3', 5', 6'	163.0 s
5'	6.93 d	8.5	6'	6'	117.3 d
6'	7.85 d	9	5'		129.6 d
1"	4.90 d		2''	2'',5",7,8,9	75.41d
2"	4.18 m		1'', 3''	1''	72.72 d
3"	3.48 m		2'', 4''	2'', 4''	80.4 d
4"	3.48 m		3'', 5''	3'', 5'', 6''	71.9 d
5"	3.42 m		6''	6''	82.7 d
6"a	3.72 dd	5.5, 7	6''a-6''b, 5''		63.0 t

Burns, Ellis and March (2007) demonstrated that the ^{13}C NMR chemical shift of flavones is diagnostic and sufficient for identification of such a subclass of flavonoids. One of their key observations is that glycosylation is always found to deshield the ^{13}C nuclei at the site of the substitution about 10 ppm without significantly affecting the remaining chemical shifts. Importantly, these authors noticed some cases of wrong assignments of the C9 in literature, where some authors swapped the values of C5 and C9, thus resulting in C5 being below δ_C 160 and C9 above 160. All of them used DMSO-*d.* However, while

the spectra analysed in MeOD-d_4 and DMSO-d_6 show similarities in the chemical shifts, there are slight shifts in which because of the increased hydrogen bonding (Choo *et al*., 2012), the alcohol analysed substrates tend to move downfield more compared to when analysed in DMSO-*d*. Still, literature using MeOD-d_4 reports consistently the value for C9 at below 160 ppm (Svehliková *et al*., 2004).

As a conclusion, the second scenario is more consistent with all these considerations, and therefore it is more likely that C2-4 may be isovitexin.

To unambiguously confirm the identity of C2-4, the sample was dissolved in DMSO-d_6 and further NMR experiments were run. These were done in April 2014. A first attempt with similar number of scans as with the previous experiments resulted in very poor spectra. A second attempt with 4,000 scans yet failed to yield better spectra. Finally, we managed to secure a time slot to run the sample 10,000 scans in August 2014. This time we obtained better spectra but still some of the expected signals were obscured by the noise. Specially the C=O at position 4, which although could be "seen" was not automatically assigned by the software because the noise-to-signal ratio was not favourable. Moreover, the signal at around δ_C 104-105 corresponding to C10 and the signal corresponding to C2 are also missing. This made us to fear that degradation after all the time in DMSO-*d* (4°C) occurred. The HMBC shows the anomeric proton correlating with δ_C 109 and δ_C 160.7. The proton of a phenolic group is now visible at ppm 13.4 correlating with δ_C 102.56, 109 and 160.7 suggesting H-5 or H-7. It is striking though the lack of correlations of a proton signal at 6.2, whilst the proton at 6.5 does correlate with the carbon at 102.56 suggesting it could be H-3.

This time, a standard of vitexin (Sigma-Aldrich) was also analysed in the same solvent and Table 28 shows the assignation of the obtained signals compared with literature values of different flavones C-*β*-D-glucosides. The characteristic signal that make us to think that the structures of C2-4 conforms more with isovitexin than with vitexin is the fact that the carbon appearing at 93 ppm matches with a non glycosylated C8 whilst non glycosylated C6 always are more deshielded (98 ppm).

Table 28: ^{13}CNMR (125 MHz) spectral data of C2-4, vitexin, isovitexin, orientin and homoorientin recorded in DMSO – d_6.

		Flavone *C8*-glycosides				**Flavone *C6*-glycosides**	
Position	**C2-4**	**4 Vitexin[a]**	**Vitexin[b]**	**Vitexin[c]**	**Orientin[b]**	**Isovitexin[c]**	**Homoorientin[b]**
2	-	163.96	163.5	1664.4	164.1	163.8	163.6
3	102.56	102.46	102.2	102.9	102.3	103.9	102.6
4	-	182.12	181.8	182.5	182.0	182.0	181.7
5	160.7	160.4	160.2	160.9	160.3	161.1	160.6
6	109	98.14	97.9	98.7	97.9	108.8	108.9
7	161	162.56	162.2	163.1	162.5	163.5	163.1
8	93	104.05	104.3	105.1	104.5	93.6	93.2
9	156.4	156.01	155.7	156.5	155.9	156.2	156.1
10	-	104.62	103.8	104.5	104.0	104.6	103.4
1’	121.15	121.62	121.3	122.1	122.0	121.5	121.4
2’	128.31	129.0	128.7	129.5	113.9	128.8	113.1
3’	115.96	116.06	115.5	116.2	145.9	115.9	145.8
4’	161	161.15	160.8	161.6	149.7	162.8	149.7
5’	115.96	115.8	115.5	116.2	115.5	115.8	115.7
6’	128.31	129.0	128.7	129.5	119.2	128.8	118.7
1”	73.26	73.4	73.2	73.9	73.3	73.4	72.8
2”	70.6	70.8	70.6	71.3	70.6	70.9	69.9
3”	78.98	78.7	78.4	79.2	78.6	78.6	78.7
4”	70.2	70.5	70.3	71.1	70.5	73.1	70.4
5”	81.48	81.87	81.5	82.3	81.7	81.7	81.4
6”	61.47	61.3	61.0	61.8	61.4	61.3	61.2

a) Standard obtained from Sigma-Aldrich
b) Data reported by Burns, Ellis and March, (2007).
c) Data reported by Muppaneni, (2013).

In any case, it would be the first time that the identification of any of these compounds are reported in *F. sansibarica* . It is however, interesting to note that the identification and isolation of vitexin (apigenin-8-*C*-glucoside), from other plant species is mostly coupled with the identification of its isomer, isovitexin (apigenin-6-*C*-glucoside) and of luteolin-8-*C*-glucoside. These closely related metabolites are difficult to separate and isolate, however, vitexin in particular, has been more frequently isolated from other *Ficus* species, such as; from the ethanol extract of the leaves of *F. microcarpa* (Wang *et al*., 2010) and the leaf aqueous methanol and butanol extracts of *F. deltoidea* (Choo *et al*., 2012; Omar *et al*., 2011). Also the aglycone, apigenin has only been reportedly identified

from some *Ficus* species (Lansky and Paavilainen, 2011). While there is no report of the isolation of apigenin-7-glucoside from the members of the genus *Ficus*, apigenin-6-neohesperidose has been isolated from *F. pumila* (Sirisha *et al.*, 2010; Abraham *et al*., 2008). Vitexin is also reported to be in higher concentrations in the plant species it has been identified than it isomeric form (Choo *et al.*, 2012). It however, falls short of being the identified compound in this thesis with the analysis that has been discussed.

Flavones, in particular apigenin and some of its derivatives are active components in many herbs that have been found to have impact on human health. Apigenin possesses a number of pharmacological activities which include antioxidant, antiviral, anti-inflammatory, anti-cancer and cellular autoimmune properties (Omar *et al*., 2011; Shui and Leong, 2004). Noteworthy, vitexin has shown to be 10-300 times more active than isovitexin in *in vitro* leishmanicidal and antitrypanosomal assays against *Leishmania donovani* amastigotes and *Trypanosoma brucei rhodensiense* trypomastigotes, respectively (Lagnika *et al*., 2009). As these parasites lack the FAS-II enzymes, the activities of vitexin may be due to either multitarget capabilities or unspecific toxicity. The fraction from which C2-4 was isolated exhibited notable activity against FAS-II enzymes. Therefore, the FAS-II inhibitory activity of vitexin and isovitexin warrant further research in order to confirm this mechanism of action, or better to explore their mechanism of action.

4. CONCLUSION

The genus *Ficus* is a promising hub for more phytochemical and biological studies as it has more than 750 species for which many have not yet been studied. As drug discovery research is particularly critical today because of the rise in cases of infectious diseases, the results of the biological screening together with the chemical profiling revealed that the eight *Ficus* species from Zambia have potential for further phytochemical and biological studies. The antimicrobial, antimycobacterial and antiprotozoal results of the screenings provide some evidence for the use of the genus *Ficus* as a source of primary care in this country.

From a phytochemical point of view, the analyses of the crude methanol extract and subextracts did not show significant qualitative differences. Only the HPLC fingerprinting revealed more subtle quantitative differences in terms of phenolic metabolites. The fact that the crude extracts of all the plant parts of *F. sansibarica* and only the root bark of *F. ovata* had predominant apigenin derivatives makes these derivatives be identified as markers for the chemical constituent of these species. In addition, the absence of caffeic acid and catechin/epicatechin derivatives in the fingerprints can also be strong indicators to specific species. For instance; the leaf extracts of *F. sansibarica* and *F. wakefieldii*; the root extracts of *F. sansibarica* and *F. sycomorus* subsp. *Sycomorus* and the leaf extract of *F. ingens* did not exhibit the aforementioned derivatives. Overall, *F. sansibarica* stands-out as the most chemically different species of the eight investigated. Still, these variations may well be due to environmental factors and, therefore, the indiscriminate use of these *Ficus* species may be correlated to a similar phytochemical profile.

In terms of biological activity, our results show that the *Ficus* crude extracts and subextracts show *in vitro* activity against microbial, mycobacterial and protozoal causative agents. Overall, the antibacterial and antifungal value of the Zambian *Ficus* samples was mild and do not seem to explain their traditional use in microbial infections. Unless these herbal extracts have a symptomatic effect or their antimicrobial activities are somehow enhanced *in vivo*, something that would have to be investigated.

On the other hand, most of the *Ficus* species showed moderate antimycobacterial activity. In particular all plant parts of *F. lutea* are active upon the human pathogens. However, *F. ovata* stem bark was the most active and may hold promise.

The screening against *Schistosoma* afforded an interesting result. The activity of most *Ficus* species is retained by the hexane and choloroform subextracts. Among them, the ones from *F. ovata* were not only the most active but may act through a different mechanism from that of Praziquantel, the current first choice drug targeting only the adult forms.

The antiprotozoal (*Leishmanial, trypanosomal* and *Plasmodial*) activities are here attributed to the hexane and chloroform subextracts of the stem back of *F. ovata* which was the most active against all the three protozoa with IC_{50} values lower than 10 µg/ml. Of particular mention, *F. ovata* showed very significant activity against *P. falciparum* with IC_{50} of 1.14 µg/ml and also a favourable selective cytotoxicity index of 13.

As for their *in vitro* activities upon *pf*FAS-II enzymes, we found that there is no species showing consistent significant inhibition of all three enzymes but all the aqueous methanol extracts exhibited a degree of selectivity towards FabG, FabI or FabZ, pointing out to polar compounds as inhibitors of this promising therapeutic target. The bioactivity-guided isolation of active principles against *pf*FAS-II enzymes was attempted on *F. sansibarica.* As discussed earlier in the thesis, this is because the other seven species showed variability in chemical terms but this species (*F. sansibarica*) was different in that apigenin derivatives were more pronounced in the crude extract. In fact, one of the most active fractions which showed significant *pf*FAS-II inhibition with IC_{50} values ranging from 4 µg/ml to 0.75 µg/ml contained likely apigenin *C*-glucosides. From it, a compound (C2-4) was isolated. Spectroscopic analyses may indicate that it is isovitexin. If confirmed, it would be the first time it is reported from the leaf extracts of *F. sansibarica*. However, due to time constrains the activity of apigenin-8-*C*-glucoside upon the enzymes was not performed. What is even more encouraging is that these fractions significantly inhibit all the three enzymes, offering a great advantage over compounds that would selectively act against only one enzyme, in which case resistance against it would easily develop. So the triple inhibition action is very advantageous as it would be

unlikely that resistance would develop against all three enzymes at the same time, seeing that they catalyze different stages of the FAS-II pathway. Another, interesting result is the activity of the isolated epicatechin (F10-3) against FabZ and FabI with IC_{50} values of 3.5 µg/ml and 4.2 µg/ml respectively. Since we did not test it upon the enzymes together with a pure standard, we can only hypothesize that the recorded activity was due to the effect of the main compound and the 'impurities', which in this case creates another door to further investigations.

In summary, we here provide a comprehensive set of data which will serve to establish the true therapeutical potential of Zambian *Ficus* species in the frame of traditional medicinal use. Some of the biological activities are very promising and they serve as baseline data meriting further work as detailed in the next section. Also we provide some directions to further work towards the chemical standardisation, and chemotaxonomy of these similar species, and report for the first time on two of the secondary metabolites for one of the species.

4.1 FURTHER WORK

To discover new potent drugs for various diseases discussed herein this thesis, the combination of biological and chemical screening is not adequate; therefore, bioactivity-guided isolation is required to identify the metabolites responsible for the activity. It is however, imperative to keep in mind that some activity is as a result of synergy. For the FAS-II enzyme inhibition, IC_{50} values should be generated to establish same comparisons with other crude extracts. In particular, FAS-II enzyme activity and cytotoxicity should be performed on the pure compounds that were isolated.

While *in vitro* assays can be sensitive, quick and inexpensive, the results that are obtained may not necessarily predict *in vivo* activity as observed by Wright (2010), therefore, there will be need to further screen the active extracts found in the antibacterial, antimycobacterial, antischistosomal and antiprotozoal screenings using suitable *in vivo* assays, more so for the extracts and subextracts which were found to have antiprotozoal activity, *pf*FAS-II inhibition activity and antischistosomal activity.

From the phytochemical work that has been presented in this thesis, it is apparent that more work needs to be done to profile by identification, the chemical constituents of *Ficus* species, in particular the appealing *F. sansibarica* and also of *F. ovata* which exhibited remarkable activities against all the protozoal and the *Mycobacterium tuberculosis* screening.

With the phytochemical techniques that have been explored in this thesis, and considering that Zambian *Ficus* species plants have not been investigated pharmacologically and phytochemically, an opportunity lies open to further the cause to investigate Zambian *Ficus* as potential sources for drug development.

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6. APPENDIX