IN VITRO ANTIMYCOBACTERIAL ACTIVITY OF FICUS SYCOMORUS ON SOME MYCOBACTERIUM TUBERCULOSIS STRAINS

BY

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DEPARTMENT OF BIOCHEMISTRY

FACULTY OF LIFE SCIENCES

AHMADU BELLO UNIVERSITY ZARIA

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DEPARTMENT OF BIOCHEMISTRY

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DECLARATION

I declare that the work in this dissertation entitled "IN VITRO ANTIMYCOBACTERIAL ACTIVITY OF *Ficus sycomorus* ON SOME *Mycobacterium tuberculosis strains*" has been carried out by me in the Department of Biochemistry. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree at this or any other Institution.

Abubakar MOHAMMED SONG _____ _____

Signature Date

CERTIFICATION

This dissertation entitled "IN VITRO ANTIMYCOBACTERIAL ACTIVITY OF *Ficus sycomorus* ON SOME *Mycobacterium tuberculosis STRAINS*" By Abubakar MOHAMMED SONG meets the regulations governing the award of Master degree of the Ahmadu Bello University, and is approved for its contribution to knowledge and literacy presentation.

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ABSTRACT

The anti-mycobacterial activity of *Ficus sycomorus* (stem bark, root, leaves and fruits) was studied *in vitro* against standard *Mycobacterium tuberculosis* strains using standard Nitrate Reductase Assay techniques with isoniazid and rifampicin as controls. The results revealed that the n-hexane fruit extract had activity against all the tested *Mycobacterium tuberculosis* strains, the activity was observed as follows: 400, 200, and 100 µg/mL against susceptible strain, 400µg/mL against isoniazid resistant (R-INH) and rifampicin resistant (R-RIF) strains. The n-hexane leaves, stem bark and root extracts had no activity and also the methanol and aqueous extracts of fruit, stem bark, root, and leaves of *Ficus sycomorus* showed no activity against all the tested *Mycobacterium tuberculosis* strains. The n-hexane fruit extract were subjected to constitutive phytochemical analysis of which saponins and alkaloids had the highest percentage content of, (16.67±0.04 %) and (6.00±0.02 %) respectively. The n-hexane fruit extract was then partially fractionated using thin layer chromatography and Colum chromatography where four fractions A-D were obtained, of which B-D were more active than A. The minimum inhibitory concentration were determined from fraction B-D where fraction B had the lowest minimum inhibitory concentration against all the tested *Mycobacterium tuberculosis* strains. The minimum inhibitory concentrations were observed at 6.25µg/mL against susceptible strain and R-RIF, and 100µg/mL against R-INH. Therefore, these findings indicate that *Ficus sycomorus* could be used as herbal drug not only against *Mycobacterium tuberculosis* but also against Multi-Drug resistant tuberculosis.

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LIST OF ABBREVIATIONS

AFB	Acid fast bacilli
AIDS	Acquired immune deficiency syndrome
ATCC	American type culture collection
BCG	Bacilli calmet guirine
Bp	Base pair
BSL3	Biosafety laboratory three
C	Cytosine
CO_2	Carbon dioxide
CR	Complement receptors
CNS	Central nervous system
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DST	Drug susceptibility testing
EMB	Ethambutol
FTIR	Fourier Transform Infra-red Spectroscopy
G	Guanine
Gly	Glycine
HRZE	Isoniazid, Rifampicin, Parazinamide, Ethambutol
His	Histidine
HIV	Human Immunodeficiency Virus
INH	Isoniazid
KatG	Catalase peroxidase gene
LJ	Löwenstein-Jensen
Leu	Leucine
Lys	Lysine
LD_{50}	Lethal dosage
MDR-TB	Multidrug-resistant tuberculosis
MIC	Minimal inhibitory concentration
MTC	*Mycobacterium tuberculosis* complex
M-TB	*Mycobacterium tuberculosis*
ml	Milliliters
MDR	Multi drug resistant
MTBC	*Mycobacterium tuberculosis* complex
NALC	N-acetyl-L-cysteine
NaOH	Sodium hydroxide
NTLCP	National Tuberculosis and Leprosy Control Programme
NTM	Non-tuberculous mycobacteria
NRA	Nitrate reductase assay
PG	Proline glutamine

PPG	Proline proline glutamine
Pro	Proline
PZA	Pyrazinamide
R	Resistant
R-RIF	Resistant to rifampicin
R-INH	Resistant to isoniazid
RFLP	Restriction fragment length polymerase
RIF	Rifampicin
rRNA	Ribosomal ribonucleic acid
RpoB	RNA polymerase B
RRDR	Rifampicin resistant-determining region
Ser	Serine
SUS	Susceptible
STR	Streptomycin
TB	Tuberculosis
TB-HIV	Tuberculosis- Human Immunodeficiency Virus
Tyr	Tyrosine
TLC	Thin layer chromatography
TCM	Traditional Chines Medicine
UV	Ultra violet
Val	Valine
WHO	World Health Organization
WT	Wild type
ºC	Degree
%	Percentage
µl	Microlitres
µmol	Micromoles

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

The use of traditional medicine has expanded globally and gaining popularity. Traditional medicines are used not only for primary health care of the poor in developing countries, but also in countries where conventional medicine is predominant in the national health care system (Sule *et al*., 2011). Medicinal plants contribute significantly to rural livelihoods apart from the traditional healers practicing herbal medicine; many people are involved in collecting and trading medicinal plants (Sospeter *et al*., 2013). The investigation of efficacy of plant-based drugs is receiving great attention because of the few or no side effects, cheap and easy availability (Kumara, 2001; Chatterjee *et al.*, 2011). The rich flora of West Africa is linked to two different environments: the rain forest along the coast and the savannah in the hinterland. It is well known that plants are significant sources of drugs and drug leads (Cragg *et al.,* 1994; Schumacher *et al.,* 2011). According to WHO, 25% of pharmaceutical drugs are made from plants that were first used in traditional medicine (WHO, 2003). Considering the high costs of synthetic drugs and their various side effects, the search for alternative products from plants used in folklore medicine is further justified. It is believed that plants which are rich in a wide variety of secondary metabolites belonging to chemical classes such as sterols, alkaloids, glycosides, saponins, flavonoids, tannins, and phenols generally possess in their anti-microbial activities (Cowan, 1999).

Ficus sycomorus belongs to the family of Moraceae comprises about 755 fig tree species worldwide (Van-Noort *et al*., 2007) commonly known as “Baure” in Hausa, “Tarmu” in Kanuri and “Kamda” in Babur/Bura. Extracts obtained from the fruits, leaves, stem bark, and root of

Ficus sycomurus usually administered in the form of infusions, decoctions, tinctures, syrups and lotions have been used in the treatments of wide range of diseases and disorders in various African countries (WHO, 1992).Stem-bark extracts of this plant are mixed ration for cows in order to increase milk flow (Bello*et al.,* 2013). The dried fruits of this plant are taken orally by adult human beings in Venda (Cyprus) for the management of tuberculosis (Arnold *et al.,* 2002).It has also been reported for the treatment of snake bites, jaundice, chest pains, dysentery, cold, coughs and throat infections (Sofowara, 1993). Roots extracts are also recommended for cough related cases including tuberculosis, cold, and other related cases (Morayi, 2013). Among other medicinal use is the aqueous extract of *Ficus sycomorus* stem-bark, which was reported to contain pharmacologically active substances such as gallic tannins, saponins, reducing sugars, alkaloids and flavone aglycones, and was relatively safe in rats with LD_{50} of 720 mg/kg, causing no hematological, hepatic and renal toxicities (Sandabe, 2002; Sandabe *et al.,* 2006).Folkloric information is available on the use of *Ficus sycomorus* extracts in the treatment of infertility and sterility in humans (Malgras, 1992; Pakia and Cooke, 2003; Kone and Atindehou, 2008). *Ficus sycomorus* is widely valued for spiritual and sacred purposes. Such use ca n be traced back to ancient Egypt, and it is often mentioned in the Holy Books.

Tuberculosis (TB) is a disease caused by a set of closely related mycobacterial strains such as *Mycobacterium tuberculosis, M. bovis, M. africanum, M.canetti, M. caprae, M.microti and M. pinnipedii* known collectively as the *M. tuberculosis* complex (MBTC) (El ouarti *et al.,* 2012*).*Tuberculosis continues to cause more deaths worldwide among other infectious diseases. The World Health Organization (WHO) estimated in 2011, that there were 8.7 million cases of TB, with nearly 1 million deaths among HIV-negative cases and 430,000 deaths associated with

HIV infection (WHO, 2012). More than 99% of deaths and 95% of new TB cases occur in middle- and low-income countries (Palomino, 2012). In 2012, 8.6 million incidents of new and relapse cases of active TB disease occurred with an estimated 1.1 million (13%) of incident TB-HIV co infected patients. The majority of TB cases worldwide were in the South-East Asia (29%), African (27%) and Western Pacific (19%) regions. India and China alone accounted for 26% and 12% of total cases, respectively (WHO, 2013).

Tuberculosis is a major public health problem in Nigeria. It is among the ten leading causes of mortality among men and women of reproductive age (15 – 49 years) and hospital admissions across public and private health institutions in the country. Nigeria has one of the highest burdens for TB in the world and remains a major target in the global control of the disease (WHO, 2010). In 2011 an estimated 280,000 cases of TB (68%incident cases) were reported from Nigeria which corresponds to a prevalence rate of 280 per 100,000 populations according to the WHO global tuberculosis report of 2012 (Gambo *et al.*, 2013). Based on 2012 survey, Nigeria ranked 3rd among the higher tuberculosis burden countries in the world (WHO, 2014).

Currently, TB is treated with an initial two months regimen comprising multiple antibiotics-isoniazid, rifampicin, pyrizinamide and ethambutol (HRZE) to ensure that the mutants resistant to even a single drug do not emerge, but these drugs are potentially toxic with series of side effects. In the next four months of the treatment only isoniazid and rifampicin are administered to eliminate any persisting tubercle bacilli. Isoniazid (INH) together with Rifampicin (RIF) forms the cornerstone of a short chemotherapy course for tuberculosis treatment. Mutation at codon 315 of *katG* gene is most prevalent in isoniazid and RNA polymerase (*RpoB*) genes of Rifampicin resistant *Mycobacterium tuberculosis* and is high in area with high TB incidence. Therefore treatment failures which may either be as a result of relapse or default cases that may

be due to adherence mainly pose a challenge in the treatment of tuberculosis which will eventually lead to multi-drugs resistant tuberculosis (MDR) (WHO, 2010).

The prevalence of Multi Drug Resistant Tuberculosis (MDR-TB) in Africa remains largely unknown but is estimated to be between 1–4% among new and 4–17% among re-treatment TB cases (Wright *et al.,* 2009). The high number of TB cases per year in each of the high burden African countries (WHO, 2009) by itself implies that even a limited prevalence of MDR-TB represents a significant pool of potentially infectious MDR-TB cases. Timely detection of these cases is crucial for patient management and control of further MDR transmission. Nigeria, the most populous country in Africa, has one of the largest burdens of TB. Clinical response to treatment is often less than optimal and WHO estimates that 2.2% and 9.4% of new and re-treatment TB cases have Multi drug resistant TB (MDR-TB), thus, Nigeria ranking 15th among the high MDR-TB burden countries. The Nigerian TB and Leprosy Control Program (NTBLCP) has given high priority to improve the identification and treatment of cases with MDR-TB and has recently commissioned the development of diagnostic and treatment facilities in referral centers across the country. Drug susceptibility testing (DST) capacity however remains limited because most of these facilities are concentrated in large urban centers (Levott *et al.,* 2013).

MDR-TB is a form of TB that is difficult and expensive to treat because it fails to respond to two important first-line drugs, most specifically, rifampicin (RIF) and isoniazid (INH). Data from more than 100 countries collected during 2009 show that 5% of all TB cases have MDR-TB (WHO, 2010).

1.2 Statement of the Problem

The current lengthy drug regimen and the emergence of drug resistant strains have resulted in resurgence in research efforts to address the urgent need for new anti-TB drugs. Moreover the current chemotherapy relies on drugs which were developed five decades ago and the bacteria have started to show resistant to it (Ramaswamy *et al.,* 1998). There are several reasons that justify the need to search for new drugs for TB, for instance, the improvement of current treatment by shortening its duration to get efficient treatment for MDR TB and to eradicate the latent infection. Thus, the development of new drugs for shortening the duration of the treatment and to fight against multidrug resistant tuberculosis strains is urgent. The challenge of discovering new, urgently needed anti-TB drugs from natural sources requires a truly interdisciplinary research (Kumar *et al.,* 2011). Innovative natural products and chemistry tools have to be developed and employed in order to meet these demands. Recent acceptance of natural herbal medicines as an alternative form of health care and development of multiple drug for resistant bacteria is on the increase.

1.3 Justification

Ficus sycomorus has been shown to contain high pharmacologically active phytochemicals such as tannins, saponins, reducing sugars, alkaloids, flavanoid and phenolic, compounds that are active against both gram positive and negetive bacteria, fungi and Virus. These phytochemicals also demonstrate to cause no haematological, hepatic and renal toxicities (Sandabe, 2002; Sandabe *et al.,* 2006),

In Nigeria, a large percentage of the populace depends on herbal medication because the commercially available orthodox medicines are becoming increasingly expensive and out of

reach (Lawal *et al*., 2012). The primary benefits of using plant-derived medicine are that they are relatively safer than synthetic alternatives, offering profound therapeutic benefits and more affordable treatment (Lawal *et al*., 2012).

1.4 Aim and Objectives of the Study

1.4.1 Aim

The aim of this study is to evaluate the anti-mycobacterial activity of *Ficus sycomorus* by *in vitro* screening against some selected *Mycobacterium tuberculosis* strains.

1.4.2 Objectives

1. To determined the anti-mycobacterial activity of aqueous, methanol and n-Hexane extract of leaves, stem-bark, roots, and fruits of *Ficus sycomorus* against Multidrug resistant *Mycobacterium tuberculosis* strains
2. To determined the phytochemical constituents of the most active extract in aqueous, methanol and n-hexane of *Ficus sycomorus*
3. To partially fractionate the extracts with the most active anti-mycobacterial activity from two (2) above using Thin Layer and column Chromatography.
4. To determined the Minimum inhibitory concentration of the most active fraction

1.4.3 Null hypothesis

- *Ficus sycomorus* extracts have no activity against some *Mycobacterium tuberculosis* strains.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1Tuberculosis

Tuberculosis (TB) is a chronic infectious airborne disease caused by the tubercle bacillus *M. tuberculosis* (Cole *et al.*, 1998). TB infections are characterized by the growth of rod-shaped bundles of the *M. tuberculosis*, which in susceptible animals, including humans, produce microscopic "tubercles" consisting of chronic granulomas, some with caseous necrosis. Lung tissue is frequently infected, but other parts of the body can be involved (Knechel, 2009). Factors that contribute to the development of tuberculosis disease are complex and not completely understood but suppression of cell-mediated immunity plays a key role (Caws *et al.*, 2008).

A number of studies have explored a wide range of natural products with strong activity against *M. tuberculosis* of which include, *Ricinus communis* (Ladda *et al*., 2012), *Alliu sativum*, *Zingiber officinale* (Mann *et al*., 2007). Roots of *Calpurnia aurea* are used for lung tuberculosis (Tadeg, 2004). Additionally it was reported that fruits/seeds of *Ocimum basilicum* and *Ocimum americanum,* leaves of *Eucalyptus camaldulensis*, *Artemisia afra* and *Artemisia abyssinica* are also used for treatment of cough and tuberculosis (Bekalo *et al.,* 2009). Leaves of *Aloe pubescens,* root of *Indigofera amorphoides* are reported to be some of the medicinal plants locally used to treat tuberculosis and other respiratory illness and other diseases (Lulekal *et al.,* 2008). it was reported that *Kanahia laniflora* and Aloe species were locally used for treatment of TB (Seifu, 2004).

2.2 Classification of the Genus *Mycobacterium*

In 1896, Lehmann and Neumann classified the tubercle bacillus under the binomial genus and species term *Mycobacterium tuberculosis* (Grange, 2009). The *Mycobacterium* genus includes more than 120 different species and is classified under the order of Actinomycetalis, with its own family, Mycobacteriaceae. Within the *Mycobacterium* genus, the pathogens known to cause serious diseases in mammals are those causing tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*Mycobacterium leprae*) (Ryan *et al.,* 2010). The genus name is derived from the Greek word, "*myces*"- which means "fungus" and relates to the mould-like manner in which mycobacteria grow in liquid culture (Kerr, 2004). There are two distinct subgroups in the *Mycobacterium* genus: slow growers and rapid growers (Tortoli, 2003) with the latter being closely related to the *Nocardia* genus. The slow growing populations include members of the *Mycobacterium tuberculosis* Complex (MTBC) which are the causative agents of tuberculosis in animals and humans (Ryan *et al.,* 2010). Species included in the MTBC are; *M. tuberculosis* (the major cause of human tuberculosis), *M. bovis* (cattle, deer), *M. africanum* (human), *M. canetti* (human), *M. caprae* (goats), *M. microti* (vole), *M. pinnipedii* (seal) (Grange, 2009). The MTBC species are grouped by their similarity at the nucleotide level and 16S rRNA sequences but differs largely in their host tropisms, phenotypes and pathogenicity (Brosch *et al,* 2002). Rapid growers usually have two copies of 16S rRNA, while slow growers have only one copy (Tortoli, 2003). With the exception of MTBC, *Mycobacterium leprae* (the causative agent of Leprosy)and *Mycobacterium ulcerans* (the causative agent of Buruli ulcer) all other *Mycobacterium* species are grouped together and called them non-tuberculous mycobacteria (NTM) (Grange, 2009).

2.3 Structure of Mycobacteria

Mycobacteria are mostly straight or curved rods which are non-sporing and do not have flagellae (Knechel, 2009). The morphology of mycobacteria in clinical specimens varies between species and is affected by exposure to antimicrobial therapy (Grange, 2009). Mycobacteria are Gram-positive and acid-fast bacilli (AFB) (Brosch *et al.,* 2002). The typical size of mycobacteria is 0.5μm by 3μm and they have a complex cell wall structure which is core to their survival (Knechel, 2009). The cell wall, (figure 1) has a high lipid content which includes mycolic acids and a trehalose-mycolic acid component called cord factor which has been identified as an important virulence factor. Below the cell wall is the cell membrane which has two phospholipid layers (Cole *et al.,* 1998). The innermost layer of the cell wall gives the bacterium its rigidity and is made up of a net-like macromolecule comprising peptidoglycan and long polysaccharide chains crossed linked with short four amino acid peptides (Dean *et al.,* 2001). To the peptidoglycan or murein layer, mycolic acids (long chain fatty acids) are attached giving the thick cell wall its rigidity (Tortoli, 2003). This thick lipid wall provides a barrier responsible for the physiological properties of *M. tuberculosis* which enables it to be resistant to antibiotics and resistant to host defense mechanisms (Knechel, 2009). *M. tuberculosis* contains a guanine (G) + cytosine (C) - rich genome (Cole *et al.,* 1998).

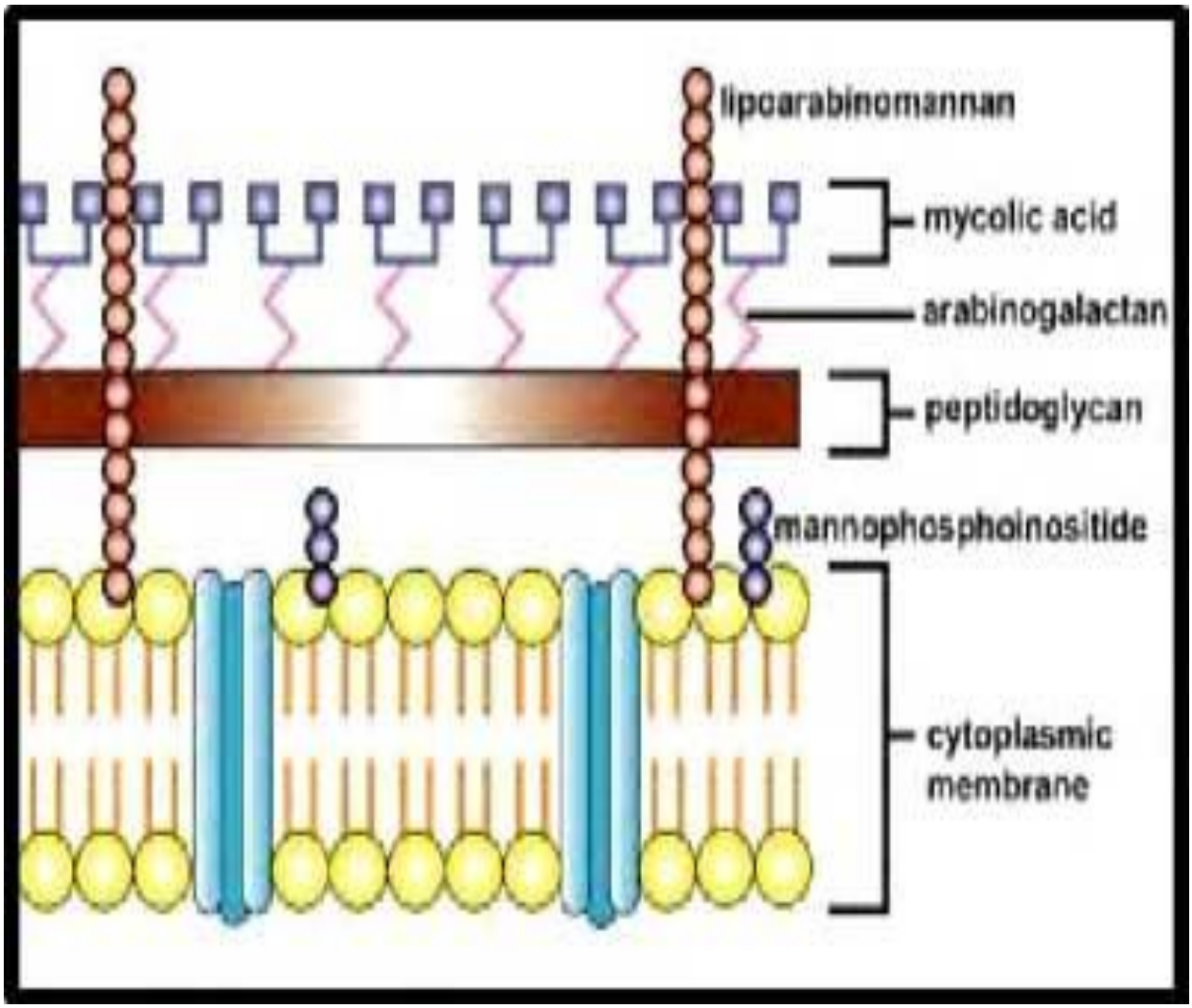


Figure 2. 1: Cell wall of Mycobacteria (www.studyblue.com)

2.4 Growth and Metabolic Characteristics of Mycobacteria

Most mycobacterial species, both slow and rapid growers are able to grow on simple media due to their undemanding nutritional requirements. Clinical cultivation of mycobacteria in laboratories is usually done on Löwenstein-Jensen (LJ) medium (Ryan, 2004). *M. tuberculosis* grows in small buff-coloured colonies resembling small cauliflowers or bread crumbs. It is a slow grower and takes between 6-8 weeks to produce a full lawn of growth (Kent *et al.*, 1985). The commercial liquid-based mycobacterial culture system such as Becton Dickson BACTEC MGIT 960 system is also used and is preferred as it takes 8-12 day for bacterial growth instead of weeks (Whitelaw *et al.*, 2009).

2.5 The *M. tuberculosis* Genome

The type strain of *M. tuberculosis*, H37Rv or ATCC was strained in 1905 and is referred to as the TB reference strain. It is used worldwide in biomedical research applications due to its virulence in animal models (Cole *et al.*, 1998). Sequenced in 1998, H37Rv genome consists of $4.4x10^6$ base pairs (bp) with about 4000 genes (Smith, 2003). The large genome is made up of 65% G + C content which is relatively constant throughout indicating that it evolved with little or no other bacterial DNA inclusion from other genera (Grange, 2009). It is rich in repetitive DNA, especially with insertion sequences, new multigene families and duplicated housekeeping genes (Cole *et al.*, 1998).

In comparison to other bacteria, the majority of the genes are involved in lipid synthesis and metabolism. There are approximately 250 enzymes associated with lipid metabolism of *M. tuberculosis* (Grange, 2009). An unusual feature of the *M. tuberculosis* genome is the coding of the Pro-Glu (PG) and Pro-Pro-Glu (PPG) glycine-rich proteins (Cole *et al.*, 1998) whose functions are unknown but are found in the cell membrane and cell wall. It is hypothesized that these proteins contribute to the antigenic variance of *M. tuberculosis* during infection (Smith, 2003).

2.6 The Origins and evolution of *Mycobacterium tuberculosis*

Evidence suggests that tuberculosis would have already infected and caused disease in human ancestors several thousand years (roughly 15000-20000 years) ago (Sreevatsan *et al.*, 1997). Based on evidence of tuberculosis found in early hominids in East Africa during that period, it is suggested that tubercle bacilli emerged in Africa (Gutierrez *et al.*, 2005; Grange, 2009).

The genus *Mycobacterium* is thought to have originated like other aerobic actimomycetes from soil bacteria with some species (*M. tuberculosis* and *M. leprae*) evolving to live in mammals (Smith, 2003). Prior to sequencing, *M. tuberculosis* was thought to have resulted from a species jump from animals to humans (Brosch *et al.*, 2002; Grange, 2009). Domestication of cattle was initially presumed to have allowed the Mycobacterium pathogen found in livestock to evolve to humans (Cole, 1998; Smith *et al.*, 2009). Already in 1898 small differences were observed between the tubercle bacilli strain from humans and cattle. Strains recovered from cattle were referred to as bovine tubercle bacilli (*M. bovis*) (Grange, 2009) and were hypothesized to be the evolutionary precursor of *M. tuberculosis* (Smith *et al.*, 2009). However, detailed genomic sequencing (Cole *et al.*, 1998) shows a different picture. MTBC differ in phenotypic characteristics but display extreme genetic homogeneity, 99.95% sequence similarity (Brosch *et al.*, 2002) with a high degree of conservation in their house keeping genes and no significant

trace of genetic exchange among species. It is therefore believed that MTBC are the clonal progeny of a single ancestor (Brosch *et al.,* 2002; Gutierrez *et al.,* 2005). Research shows that the ancestral member of the complex, *M. prototuberculosis*" is about 3 million years old. Recent studies have suggested that *M. bovis* and *M. tuberculosis* have evolved separately around the same time (Sreevatsan *et al.,* 1997).

2.7 Pathogenesis and Virulence of *M. tuberculosis*

The majority of tuberculous infections result from inhalation of airborne droplet nuclei carrying the causative bacteria (Ryan *et al.,* 2010). Infection is established by interactions between *M. tuberculosis* and the host macrophages (Eley *et al.,* 2009). The first survival success of *M. tuberculosis,* relies on avoiding the killing action of the host's phagocytes (Ryan *et al.,* 2010). Phagocytosis of tubercle bacilli by antigen-presenting cells in human lung alveoli initiates a complex infection process by *Mycobacterium tuberculosis* and a potentially protective immune response by the host. *M. tuberculosis* has devoted a large part of its genome towards functions that allow it to successfully establish latent or progressive infection in the majority of infected individuals (Ryan *et al.,* 2010). The failure of immune-mediated clearance is due to multiple strategies adopted by *M. tuberculosis* that blunt the microbicidal mechanisms of infected immune cells and formation of distinct granulomatous lesions that differ in their ability to support or suppress the persistence of viable *M. tuberculosis*. It has been reported that mycobacterial sulphatides, derivatives of multiacylated trehalose 2-sulphate, have the ability to inhibit phagolysosomal fusion (Mehta *et al*., 2006). *In vitro* studies demonstrated that *M. tuberculosis* generates copious amounts of ammonia in cultures, which is thought to be responsible for the inhibitory effect. *M. tuberculosis* is equipped with numerous immune evasion strategies, including modulation of antigen presentation to avoid elimination by T cells (Mehta*et*

al., 2006). Protein secreted by *M. tuberculosis* such as superoxide dismutase and catalase are antagonistic to reactive oxygen species (ROI). Mycobacterial components such as sulphatides, Lipo arabino mannan (LAM) and phenolic- glycolipid I (PGL- I) are potent oxygen radical scavengers (Eley *et al.,* 2009). *M. tuberculosis*-infected macrophages appear to be diminished in their ability to present antigens to CD4+ T cells, which leads to persistent infection (Harding *et al.,* 2010) . Another mechanism by which antigen presenting cells (APCs) contribute to defective T cell proliferation and function is by the production of cytokines, including TGF-β, IL-1040 or IL-641 (Harding *et al.,* 2010).

2.7.1 Primary infection

The initial encounter referred to as primary tuberculosis infection typically occurs in the macrophages (Eley *et al.,* 2009). The inhaled pathogenic organism (stage one) deposited in the middle and lower lobes of the alveoli which are recognized by the alveolar macrophage complement receptors (CR1, CR3, CR4). The host's cell-mediated innate immune response is activated and the pathogen is phagocytosed (Ryan *et al.,* 2010). Following phagocytosis by the macrophages, *M. tuberculosis*-specific adaptive cell mediated immunity develops (Eley *et al.,* 2009).

The destruction of *Mycobacterium* is dependent on the host phagocytes intrinsic microbicidal capacity and the ingested *Mycobacterium* virulence factors. Immediate killing of the mycobacterium leads to no infection setting in, but those mycobacterium that escape the initial intracellular killing by interfering with the acidification of the phagosome and affecting lysosomal enzyme activity, making it less effective will multiply and be released from the macrophages (Eley *et al.,* 2009). The *Mycobacterium* will then proceed to attract blood monocytes and other inflammatory cells to the lung, which is referred to as stage two of

pulmonary tuberculosis (Van Crevel *et al.*, 2002). It was usually assumed that tuberculosis results from a single infection with single *M. tuberculosis* strain. However, there is evidence to suggest that mixed infections occur (Warren *et al.*, 2004; Van Rie *et al.*, 2005; Cohen *et al.*, 2011).

2.7.2 Reactivation

In tuberculosis, organisms that are not killed during primary infection enter a dormant or stationary phase as a result of oxygen and nutrient deprivation known as Latent tuberculosis. Latent tuberculosis is present in one third of the world's population with less than 10% developing active disease (Bezuidenhout *et al.,* 2009; Eley *et al.,* 2009). Reactivation of the dormant infection occurs once the host experiences immunosuppression for a variety of reasons and may lead to the transmission of disease (Knechel, 2009). *M. tuberculosis* reactivates at aerobic sites which favors growth of the aerobe MTB. Even though mycobacterial resuscitation-promoting factors have been identified, the mechanism of reactivation of these dormant foci is still poorly understood (Ryan *et al.,* 2010). In developing countries with large immune-suppressed populations and/or settings of high TB incidence, the risk of re-exposure within the population is high and it is therefore difficult to determine whether recurrent TB disease can be attributed to reactivation or re-infection (Horsburgh, 2008). HIV-infected gold miners are at higher risk of TB recurrence with a substantial portion resulting from re-infection (Charalambous *et al.*, 2008)

2.7.3 Pulmonary tuberculosis

Pulmonary tuberculosis is s type of TB that primarily affects the lungs, initially which could be asymptomatic or it may present with clinical systemic symptoms (Ryan, 2010). These clinical features being cough, chest pain, haemoptysis, fever and uncommonly breathlessness (Hopewell,

2008). Pulmonary TB patients co-infected with HIV experience weight loss and fever more than those patients who are HIV-negative. On the other hand, cough and haemoptysis are less common in HIV-positive patients. Unexplained productive coughing in patients for 2-3 weeks should be screened for TB since the sensitivity of cough as a predictor for TB is very high (Maher, 2009).

2.7.4 Extra-pulmonary tuberculosis

Extra-pulmonary tuberculosis is a type of TB disease that affects all other organs apart from the lungs (Knechel, 2009). The high frequency of extra-pulmonary TB is among HIV-infected population which results from failure of the immune system to contain the infection, thereby assisting haematogenous spread and infection of one or multiple non-pulmonary sites (Hopewell, 2008). Patients with extra-pulmonary tuberculosis present with the same systemic features, with clinical signs and symptoms related to the pathology at the local site of disease (Maher, 2009).

The location of extra-pulmonary tuberculosis with the most severe disease is the central nervous system which results in meningitis or space occupying tuberculomas. Tubercular meningitis is fatal in most cases if untreated (Knechel, 2009). The acute dissemination of *M. tuberculosis* into the blood stream is referred to as miliary tuberculosis and the infection can spread throughout the body, leading to multi-organ involvement (Wang *et al.,* 2007). Miliary TB progresses rapidly and can be fatal if not treated early. It is difficult to diagnose due to its systemic and nonspecific signs and symptoms (Knechel, 2009). Tuberculosis lymphadenopathy is the most common extra-pulmonary form of tuberculosis and most commonly presents with cervical lymph node enlargement (Maher, 2009). Other sites for TB infections include the bone joints, pleura and genitourinary systems (Knechel, 2009).

2.8 Diagnosis of tuberculosis

The diagnosis of TB relies on the detection of TB bacteria in sputum, aspirates, biopsies or blood (Doherty, 2008). The bacteria can be detected by means of microscopy or culture but the sensitivity of these methods are reliant on the quality of the specimen, concentration of bacteria and the volume of the specimen received (Whitelaw *et al.,* 2009). In addition, MTBC grows slowly resulting in longer turnaround times for culture, thus, there is need for rapid new molecular diagnostic tests (Afanas‟ ev *et al.,* 2007).

2.8.1 Culture

Bacterial culture on solid or liquid media is the gold standard for diagnosis of *M. tuberculosis*. Cultures are also used to distinguish *M. tuberculosis* from other *Mycobacteria* (Doherty, 2008) and to perform drug sensitivity testing (Wood, 2007). Prior to growing the bacteria on culture media, the specimen needs to be digested and decontaminated to remove other faster growing contaminating bacteria or fungi. The most commonly used decontamination and digestion methods are the sodium hydroxide (NaOH) method and the *N*-acetyl-*L*-cysteine-sodium hydroxide (NALC-NaOH) method (Whitelaw *et al.*, 2009). During the decontamination process, some mycobacteria may be killed (WHO, 1998). The bacterium in the specimen is concentrated by centrifugation to optimise yield. Once the specimen is decontaminated, it is inoculated onto one or more culture media to achieve growth.

These are different types of media used for *Mycobacterium* growth, (Whitelaw *et al.*, 2009);

A. Solid media
    I. Lowenstein-Jensen (LJ) - an egg-based medum
    II. Middlebrook 7H10 or 7H11 - agar-based medium,
    III. Dubos agar-based medium

B. Liquid media

I. Middlebrook 7H9 broth

II. Dubos Tween albumin broth

Although solid media are used widely due to the low cost, there are advantages in using liquid media for culturing TB (Doherty, 2008). One of the key advantages is the rapid growth of the bacteria in liquid media (8-12 days to give a positive growth), compared with solid media (takes 6-8 weeks) (Wood, 2007). The isolation rates of the bacteria from liquid culture are higher especially when using the commercial liquid-based mycobacterium culture system such as Becton Dickson BACTEC MGIT 960 system (Whitelaw *et al.*, 2009). The MGIT 960 tubes contain 7ml of modified 7H9 broth and the system is based on the consumption of oxygen by the growth of MTB which produces fluorescence when illuminated by a UV lamp, (Wood, 2007). The key disadvantage of the commercial liquid-culture systems is the high costs of the reagents and the equipment and higher contamination rates than with solid media (Whitelaw *et al.*, 2009).

2.9 Drug Susceptibility Testing (DST)

Determination of resistant to a given drug is performed as an *in-vitro* assay in the laboratory, a process called drug susceptibility testing (DST). Where resources are limited, the WHO recommends a hierarchy of DST that should include at least Rifampicin and isoniazid the two most efficacious drugs that define MDR-TB (Rich *et al.*, 2006). For more than 40 years, DST in the developing countries has relied on conventional *indirect* susceptibility methods on Lowenstein-Jensen (LJ) solid medium (Canetti *et al.*, 1963). Indirect testing involves primary isolation of pure colonies of *Mycobacterium tuberculosis*, which are then used as inoculum for DST. In contrast, direct DST involves inoculation of processed smear positive samples rather

than pure MTB colonies. Results of direct testing are much more rapid and help to triage MDR from non-MDR-TB patients promptly.

2.9.1 Solid Media Culture-Based Tests Nitrate reductase assay *(NRA)*

Mycobacteria tuberculosis has nitro reductase enzymes that catabolically reduce nitrate (NO_3) to nitrite (NO_2), in the reaction pathway:

$KNO_3 + 2e- + H_2 \longrightarrow NO_2 + H_2O + K$

In 1879, Griess, a German chemist working at the University of Marburg, described the diazotization reaction, which now forms the basis for the Griess test for the detection of nitrite (Griess *et al*., 1879). By incorporating 1mg/mL potassium nitrate (KNO_3) in the medium, the reduction of nitrate to nitrite can be detected using the Griess reagent by diazonium reaction. When Griess reagent is added on the 7^{th}, 14^{th} or 21^{st} day of incubation, the nitrite in the medium causes a pink-purplish color. In the presence of rifampicin or isoniazid at the critical concentrations, the appearance of a pink-purple color represents resistant to the drug (Angeby *et al*., 2002). Susceptible strains do not grow, as they are inhibited by the antibiotic thus producing a non-coloured reaction. As the NRA uses the detection of nitrate reduction as an indicator of growth, DST results can be obtained faster using NRA than the visual detection of colonies by propotion method. Progress had been made in the use of the NRA for indirect DST in *M. tuberculosis* showing sensitivity and specificity of 92-100% (Martin *et al*., 2008). The NRA test is technically easy to set and read, and gives a clear cut answer on susceptibility.

2.10 Molecular Basis and Mechanisms of Resistant to First-line Drugs

Resistant of *M. tuberculosis* to anti-tuberculous drugs results from a spontaneous genetic event, occurring at a frequency of 10^{-5} to 10^{-8} (Pfyffer, 2000). *M. tuberculosis* resistant to various drugs

arises independently, which appears to be confined to chromosomal DNA and does not involve transfer of mobile genetic elements (such as plasmids) that occur in other bacteria and Mycobacteria (Pfyffer, 2000). Resistant to the first-line anti-TB drugs occurs through sequential accumulation of mutations in the genes targeted by the drugs (Johnson *et al*., 2007). These mutations occur at the drug target region or at the enzymes that are involved in the activation of the drug (Somoskovi *et al.,* 2001). These drug-resistant strains develop when chemotherapy is disrupted or otherwise inadequate (Ramaswamy *et al.,* 1998).

The molecular mechanisms conferring drug resistant have been described for several of the major first and second line anti-tuberculosis drugs. This understanding has facilitated the recent development of rapid molecular diagnostic tests for identification of drug resistant (Zhang *et al.,* 2009). While about 96% of rifampicin resistant strains have mutations in a defined region of the *rpoB* gene, resistant to isoniazid is more complicated, involving at least two gene *katG* and *inhA* which together account for approximately 65-75% of resistant strains (Ramaswamy *et al*., 1998). For other drugs, known drug resistant mutations occur in a smaller proportion of strains suggesting other mechanisms of resistant. Several studies have shown that the frequency distribution of mutations among drug resistant strains shows geographic variability both between and within countries (Ohno *et al.,* 1996; Miotto *et al.,* 2006; Barnard *et al.,* 2008; Green *et al.,* 2008).

2.10.1 Isoniazid

Isoniazid (INH) is a pro-drug with a simple structure containing a pyridine ring and a hydrazide group requiring activation (Somoskovi *et al.,* 2001; da Silva *et al.,* 2011). Almost all mycobacteria and prokaryotes other than *M. tuberculosis* are resistant to INH (Ramaswamy *et al.,* 1998). INH is activated by the catalase/peroxidase enzyme encoded by *katG* which prevents

the synthesis of mycobacterial mycolic acids by inhibiting the NADH-dependent enoyl-ACP reductase coded by *inhA* (da Silva *et al.,* 2011). The basis of resistant to INH is more complex and is based on a genetic system involving a large number of gene regions such as *katG, inhA, kasA, aphC, oxyR* and less common *iniA, iniB* and *iniC* genes (Afanas'ev *et al.*, 2007; Wei-Wei *et al.,* 2007). Studies have shown that *katG* covers a wide range of 50-95% of INH resistant in strains while *inhA* resistant is present in 20-35% of strains (Wei-Wei *et al.,* 2007; Aslan *et al.,* 2008).

Molecular genetic studies have correlated INH resistant with loss of catalase-peroxidase activity which is controlled by the *katG* gene (Ramaswamy *et al.,* 1998). Mutations in the catalase-peroxidase gene, usually at codon 315, prevent the conversion of the INH pro-drug into its active form (Wei-Wei *et al.,* 2007). The promoter region of the *inhA* operon comprises two genes, *fabG1 (mabA)* and *inhA* (Afanas'ev *et al*., 2007; Ramaswamy *et al*, 1998). The metabolic activity of the operon is to code for β-ketoacyl reductase and the fatty-acid enoyl-acyl carrier protein reductase which participate in mycolic acid biosynthesis (Afanas'ev *et al.,* 2007). Missense mutations in the promoter region (position -15T or -8C) result in over expression of the target for the active INH radical leading to INH resistant (Afanas'ev *et al.*, 2007; Evans *et al*., 2009). Mutations in the *katG* gene code predominantly for high-level resistant to INH while low-level resistant is determined mainly by mutations present in the 315 codon *inhA* gene (Barnard *et al.*, 2008; Van Deun *et al.*, 2010). Resistant to the anti-tuberculosis drug ethionamide is also cause by mutations in the *inhA* gene. Further research is required to better understand the mechanisms responsible for INH resistant in the other genes (Afanas'ev *et al.,* 2007).

2.10.2 Rifampicin

Rifampicin (RIF) is a lipophylic ansamycin with efficient antimicrobial activity and is considered a key drug in the treatment of tuberculosis (da Silva *et al.,* 2011). The activity of RIF involves binding to the β-subunit of RNA polymerase resulting in inhibition of transcription (Wei-Wei *et al.,* 2007). Resistant to RIF is caused by amino acid substitutions as a result of missense mutations, or deletions or insertions in this region encoding the β-subunit of the RNA polymerase enzyme (Aslan *et al.,* 2008; Ramaswamy *et al.,* 1998). Resistant to RIF is largely confined to mutations present in an 81 base pair hotspot region on the *rpoB* gene (Ramaswamy *et al.*, 1998; Aslan *et al*, 2008). This drug resistant region is known as the RIF resistant-determining region (RRDR) (Afanas'ev *et al.*, 2007; WHO, 2007; Evans *et al.,* 2009). The RRDR spans from codon 507 to 533 of the *rpoB* gene and more than 95% of resistant strains harbour mutations in this region (Afanas'ev *et al.,* 2007; Wei-Wei *et al.,* 2007; Evans *et al.*, 2009;).

The characterization of the *M. tuberculosis rpoB* gene showed a wide variety of distinct alterations in the RRDR in epidemiologically unrelated patient strains (Ramaswamy *et al.*, 1998). Studies have commonly documented missense mutations at codon 531 (Ser), 526 (His) resulting in amino acid replacements. Amino acid substitutions Ser513Leu and His526Tyr 32 were most common (Afanas'ev *et al.,* 2007). Several research groups have investigated the relationship between RIF MIC levels and structural changes found in the *rpoB* gene (Ramaswamy *et al.,* 1998). They had found a strong correlation between *rpoB* variants and RIF MICs. High-level drug resistant to rifampicin, rifabutin and rifapentine had amino acid substitutions at codons 513, 526 and 531, whereas, amino acid substitution at positions 514, 521 or 533 conferred low-level resistant to RIF. In RIF resistant strains that lacked changes in the RRDR, the molecular mechanisms involved are unknown (Ramaswamy *et al.*, 1998).

2.11 Traditional Medicine Benefits and Challenges

Traditional Medicine is defined by the WHO, as the sum total of knowledge or practices whether explicable or inexplicable, used in diagnosing, preventing or eliminating a physical, mental or social disease which may rely exclusively on past experience or observations handed down from generation to generation, verbally or in writing(WHO, 2010). It also comprises therapeutic practices that have been in existence often for hundreds of years before the development of modern scientific medicine and are still in use today without any documented evidence of adverse effects. The explicable form of Traditional Medicine can be described as the simplified, scientific and the direct application of plant, animal or mineral materials for healing purposes and which can be investigated, rationalized and explained scientifically. Plants are one of the most important sources of medicine. Plant derived compounds (phytochemicals) have been attracting much interest as natural alternatives to synthetic compounds (Kalimuthu *et al*., 2010). Extracts of plants are used for the treatment of various diseases and this forms the basis for all traditional systems of medicine (Kalimuthu *et al*., 2010). The treatment and control of diseases by the use of available medicinal plants in a locality will continue to play significant roles in medical health care implementation in the developing countries (Ekundayo *et al*., 2011). The use of these medicines as therapeutic agents however is limited. This is due to various challenges such as drug solubility, stability, adsorption and toxicity. In addition, some of these drugs are expensive and generally unavailable to citizens of developing countries, especially those residing in the rural areas (Sule *et al*., 2011). The shortfalls in the use of chemotherapeutic agents as control agents in fungal diseases, further encourages the use of plants as a form of alternative medicine.

Medicinal plants have been found as important contributors to the pharmaceutical, agriculture and food industries. With the onset of the synthetic era, pharmaceutical industries are producing

a lot of synthetic drugs that help to alleviate the chronic diseases. With the passage of time many associated with frequent use of synthetic drugs become prominent like severe side effects and resistant of microbes against these drugs (Kalimuthu *et al*., 2010).

On the other side synthetic drugs are expensive and a large population cannot afford these drugs. In recent times research on medicinal plants has been intensified all over the world. Recently there is an emerging trend in research to support the biological activities of medicinal plants (Ekundayo *et al*., 2011). Many scientific researchers have been reported about the efficacious and chemotherapeutic role of medicinal plants in the treatment of diverse diseases.

2.12 Discovery of Drug from Herbal Medicine

Plants, which have formed the basis of traditional medicine for thousands of years, were originally instrumental to early pharmaceutical drug discovery and industry. Hence, the 8 history of drug discovery and even drug chemistry is inexorably bound to the plant kingdom and the process of deriving drugs from plant sources is certainly not new (WHO, 2013).

2.12.1 Drug outside Africa

In 1785, the English Physician, discovered the medicinal uses of Foxglove (Digitalis purpurea) which gave rise to digoxin, an indispensable cardiac drug; while Ergot of Rye (Claviceps purpurea) was discovered as the foremost natural uterine stimulant while the analgesic morphine was produced from opium poppy flower (Papaver somniferum) by a French scientist; Strychnine from Strychnox nuxvomica was straind as a CNS stimulant in the 19th century; quinine for malaria fever from Cinchona officinalis was reported during the same century. Ephedrine (from Ephedra sinica) was discovered for asthma from the traditional Chinese medicine (TCM) in the 20th century (Enwonwu, 2003). The first British Pharmacopoeia of 1863 contained descriptions

of 187 crude drugs including Digitalis, Datura, Belladona and Hyoscyamus. There is therefore little or no doubt that ethnographic (ethnopharmacognosy) research can provide important clues leading to new drugs for the modern pharmacies. It is the herbal medicine (among other alternative therapies available in the U.K.) that has seen the biggest interest in recent years. About 85% of Japanese doctors prescribe herbal medicines and two-thirds of the Indian populations use Ayurvedic medicines (Enwonwu, 2003). There has been about 26-fold increase in the budgetary allocation (USD 52million) for complementary and alternative therapies by the US congress within 1992 to 1998 and this was doubled (USD 104 million) in 2002 (Enwonwu, 2003). As a whole, the annual market value of herbal drug products is close to USD 43 billion which is more than the total annual budget of many Africa countries (Enwonwu, 2003).

2.12.2 Evidence within Africa

Current supports from some relevant organizations on the other hand, the orthodox medicine, as currently made available today in Nigeria (as in most African countries), so long as every nook and corner of our rural populations in Africa cannot yet be provided with basic health care needs including full-time resident medical personnel, the practice of conventional medicine has failed woefully (Elujabo, 2003). Although wherever, modern health facilities exist, traditional medicine is incomparable. Therefore, the most workable health agenda for Nigeria is the institutionalization of traditional medicine in parallel (not in complete fusion) with orthodox medicine, within the national health scheme in order to move the health agenda forward. Effective health agenda for Nigeria can never be achieved by orthodox medicine alone unless it is complemented by traditional medicine practice (Elujabo, 2003).

2.12.3 The value of plants used in ethno medicine for drug discovery

Medicinal plants provide a rich source of raw materials for primary health care in Africa and other parts of the developing world. According to Fabricant and Farnsworth (2001) the goals of using plants as sources of therapeutic agents are:

i. To strain bioactive compounds for direct use as drugs;

ii. To produce bioactive compounds of novel or known structures as lead compounds for semi synthesis to produce patentable entities of higher activity and/ or lower toxicity;

iii. To use agents as pharmacologic tools;

iv. To use the whole plant or part of it as an herbal remedy. Notable examples were quinine from Cinchona pubescens, reserpine from Rauvolfia serpentine and taxol from Taxus spp.

During the last few decades, there has been a resurgence of interest in plants as source of medicines and of novel molecules for use in the elucidation of physiological/biochemical phenomena. There was worldwide green revolution, which is reflected in the belief that herbal remedies are safer and less damaging to the human body than synthetic drugs. Furthermore, underlying this upsurge of interest in plants is the fact that many important drugs in use today were derived from plants or from starting molecules of plant origin: digoxin/digitoxin, the vinca alkaloids, reserpine and tubocurarine are some important examples (Iwu *et al*., 1999).

For centuries, people have used plants for healing. Until recently, plants were important sources for the discovery of novel pharmacological active compounds, with many drugs being derived directly or indirectly from plants. Many modern drugs have their origin in the ethno-pharmacology (Badami *et al*., 2003), Indeed, traditional medicine is a potential source of new drugs and as a source of cheap starting products for the synthesis of known drugs. Some examples include reserpine from Rauwolfia species, viablastine from Catharanthus roseus (Sofowora, 1993).

2.13 The Plant Ficus sycomorus

2.13.1 Taxonomy

Kingdom:	Plantae
Phylum:	Tracheophyta
Subphylum:	Spermatophyta
Class:	Magnoliopsida
Order:	Rosales
Family:	Moraceae
Genus:	*Ficus*
Species:	*sycomorus*

2.13.2 Common names

English:	fig. Sycamore
Hausa:	Baure
Kanuri:	Tarmu
Babur/Bura:	Kamda

2.13.3 Botanic Description

Ficus sycomorus is a large, semi-deciduous spreading savannah tree, up to 21 (max. 46) m, occasionally buttressed. Bark on young stems pale green with a soft powdery covering; on older stems, grey-green, fairly smooth, with scattered grey scales and pale brown patches where scales have fallen off. Slash pale pink with heavy latex flow (Orwa *et al*., 2009).Leaves broadly

(ob)ovate or elliptic, base (sub) cordate, apex rounded or obtuse, margin entire or slightly repand -dentate, 2.5-13 (max. 21) x 2-10 (max. 16) cm, scabrous above, petiole 1-5 cm, 5-7 pairs of yellow lateral veins, lowest pair originating at the leaf base (Orwa *et al*., 2009). Figs in leaf axils or on up to 10 cm leafless branches on old wood, solitary or paired, globose or (ob)ovoid, yellow-red to reddish-purple when ripe, up to 3.5 x 5 cm, pubescent or almost glabrous. The fruit is a large edible fig, 2–3 cm in diameter, ripening from buff-green to yellow or red. They are borne in thick clusters on long branchlets or the leaf axil. Flowering and fruiting occurs year-round, peaking from July to December. The bark is green-yellow to orange and exfoliates in papery strips to reveal the yellow inner bark. Like all other figs, it contains a latex (Orwa*et al*., 2009).



Figure 2.2: *Ficus sycomorus* fruit (Orwa *et al*., 2009).

2.13.4 Ecology and Distribution

Ficus sycomorus is a common savannah tree that grows in high water table areas. Often found along watercourses such as streams and rivers, swamps and waterholes. The sycamore fig is sensitive to frost but can withstand some cold. It is found in afro- montane rain forests and undifferentiated afro-montane forests, especially along edges and in clearings, riverine forests, riparian woodland, secondary evergreen bush land; left as single trees in farmland and occasionally seen as single trees on rocky outcrops. Widely distributed among the natives of Angola, Benin, Botswana, Burundi, Cameroon, Congo, Cote d'Ivoire, Democratic Republic of Congo, Djibouti, Egypt, Eritrea, Ethiopia, Gambia, Ghana, Guinea, Israel, Kenya, Lesotho, Madagascar, Mozambique, Namibia, others are Nigeria, Rwanda, Saudi Arabia, Senegal, Somalia, South Africa, Sudan, Swaziland, Syrian Arab Republic, Tanzania, Uganda, Zambia, and Zimbabwe (Orwa *et al*., 2009).

2.13.5 Uses of *Ficus sycomorus*

Ficus sycomorus also known as *Fig Sycamore* is an important medicinal and nutritional plant of the family moraceae comprises about 755 fig tree species worldwide (Vannoort *et al*., 2007) . It is a common savannah tree that grows in high water table areas. Often found along watercourses such as streams and rivers, swamps and waterholes, native to tropical regions of Africa. Extracts obtained from the fruits, leaves, stem bark, and root bark usually administered in the form of infusions, decoctions, tinctures, syrups and lotions have been used in the treatments of wide range of diseases and disorders in various African countries (WHO, 1992).

Leaves and Fruit extracts of this plant are mixed ration for cows in order to increase milk flow (Rajaram *et al.,* 2013). Extract of fruits, leaf, roots and stem barks to treat various ailments such as cough, diarrhea, skin infections, stomach disorders, liver disease, epilepsy, tuberculosis,

lactation disorders, helminthiasis, infertility, sterility and diabetes mellitus (Igbokwe *et al*., 2010; Adoum *et al*., 2012).The plant has also been reported to be a potent antimicrobial agent against ciprofloxacin resistant *Salmonella* typhi (Adeshina *et al*., 2010). In country like Palestine, milky sap from *F. sycomorus* stem bark is used for treating skin diseases while the decoction of the stem bark is used for problems of the gastrointestinal tract. It is further used as seasoning; the leaf are dried and added to cake as a condiment, eaten raw or cooked as soup. Dry branches of the species are collected and used as fuel (Auda, 2012).The dried fruits of the plant are taken orally by adult human beings in Venda (Cyprus) for the management of tuberculosis (Arnold and Galamain, 2002). It has also been reported for the treatment of snake bites, jaundice, chest pains, dysentery, cold, coughs and throat infections (Sofowara, 1993). Roots extracts are also recommended for cough related cases including tuberculosis, cold, and other related cases (Morayi, 2013). Among other medicinal uses, the aqueous extract of *Ficus sycomorus* stem-bark has been reported to contain pharmacologically active substances such as gallic tannins, saponins, reducing sugars, alkaloids and flavone aglycones, and was relatively safe in rats with LD_{50} of 720 mg/kg, causing no hematological, hepatic and renal toxicities (Sandabe, 2002; Sandabe *et al.,* 2006),. Folkloric information is available on the use of *Ficus sycomorus* extracts in the treatment of infertility and sterility in humans (Malgras, 1992; Pakia and Cooke, 2003; Kone and Atindehou, 2008).

2.15 Phytochemicals

Phytochemicals are chemical compounds formed during the plants normal metabolic processes often referred to as "secondary metabolities." Phytochemicals are naturally occurring, biologically active chemical compounds in plants. The prefix "phyto" is from a Greek word meaning plant. In plants, phytochemicals act as a natural defense system for host plants and provide colour, aroma and flavour. More than four thousand (4, 000) these phytochemical

compounds have been discovered to date and it is expected that scientists will discover many more (Okwu, 2004). Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts which are precursors for the synthesis of useful drugs (Sofowora, 1993).

2.15.1 Types of phytochemicals

The thousands of phytochemicals that have been discovered are grouped based on function and sometimes source. These grouping include the widely studied, alkaloids, flavonoids, phyto-oestrogens, phytosterols and carotenoids (Sofowora, 1993).

2.15.2 Phenolic compounds

Phenols are a member of a group of aromatic chemical compounds with weakly acidic properties and are characterized by a hydroxyl (OH) group attached directly to an aromatic ring. The simplest of phenols derived from benzene is also known as phenol and has the chemical formula C_6H_5OH. The presence of phenols is considered to be potentially toxic to the growth and development of pathogens (Okwu and Ndu, 2006). The structural classes of phenolic compounds include the polyphenolic (hydrolysable and condensed tannins) and monomers such as ferulic and catechol (Okwu, 2004). Polyphenols might interfere in several of the steps that lead to the development of malignant tumours, may play a role in inactivating carcinogens and inhibiting the expression of mutagens (Okwu, 2004).

2.15.3 Flavonoids

Flavonoids are 15-carbon compounds generally distributed throughout the plant kingdom. They are known to be synthesized by plants in response to microbial infection and have been found in vitro to be effective against a wide array of microorganisms (Harborne and Baxter, 1999).

Flavone with the molecular formula, $C_{15}H_{10}O_2$, is a commonly found plant flavonoid. Flavonoids are potent water-soluble super antioxidants and free radical scavengers which prevent oxidative cell damage, have strong anti-cancer activities and protects against all stage of carcinogens. Flavonoids in the body are known to reduce the risk of heart diseases. In terms of anti-cancer activity, they inhibit the initiation, promotion and progression of tumors (Okwu, 2004). In recent times, plant flavonoids have attracted attention as potentially important dietary cancer chemo-protective agents (Hertog *et al*., 1993).

2.15.4 Saponins

Saponins are glycosides of both triterpenes and steroids that are characterized by their bitter or astringent taste, foaming property, haemolytic effect on red blood cells and cholesterol binding properties (Okwu, 2005). Saponins have been shown to possess both beneficial (lowering cholesterol) and deleterious (cytotoxic and permeabilization of intestinal epithelium) properties and to exhibit structure dependent biological activity. In medicine, it is used to some extent as an expectorant and an emulsifying agent (Harborne and Baxter, 1999).

2.15.5 Glycosides

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different varieties of organic hydroxy (occasionally thiol) compounds (invariably monohydrate in character), in such a manner that the hemi-acetal entity of the carbohydrate must essentially take part in the condensation (Firn, 2010). Glycosides are colorless, crystalline carbon, hydrogen and oxygen-containing (some contain nitrogen and sulfur) water-soluble phyto constituents, found in the cell sap. Chemically, glycosides contain a carbohydrate (glucose) and a non-carbohydrate part (aglycone or genin) (Firn, 2010). Alcohol,

glycerol or phenol represents aglycones. Glycosides are neutral in reaction and can be readily hydrolyzed into its components with ferments or mineral acids. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone or pharmacological action. The rather older or 26 trivial names of glycosides usually has a suffix „in" and the names essentially included the source of the glycoside, for instance: strophanthidin from Strophanthus, digitoxin from Digitalis, barbaloin from Aloes, salicin from Salix, cantharidin from Cantharides, and prunasin from Prunus. However, the systematic names are invariably coined by replacing the "ose" suffix of the parent sugar with "oside". These groups of drugs are usually administered in order to promote appetite and aid digestion. Glycosides are purely bitter principles that are commonly found in plants of the Genitiaceae family and though they are chemically unrelated but possess the common property of an intensely bitter taste. The bitters act on gustatory nerves, which results in increased flow of saliva and gastric juices. Chemically, the bitter principles contain the lactone group that may be diterpene lactones (example andrographolide) or triterpenoids (example. amarogentin). Some of the bitter principles are either used as astringents due to the presence of tannic acid, as antiprotozoan, or to reduce thyroxine and metabolism. Examples include cardiac glycosides (acts on the heart), anthracene glycosides (purgative, and for treatment of skin diseases), chalcone suppressant in various preparations. Excessive ingestion of cyanogenic glycosides can be fatal. Some food stuffs containing cyanogenic glycosides can cause poisoning (severe gastric irritations and damage) if not properly handled (Sarker and Nahar, 2007).

2.15.6 Tannins

Tannin are general descriptive name for a group of polymeric/phenolic substances capable of tanning leather or precipitating gelatin from a solution, a property known as astringency

(Harborne and Baxter, 1999). They are divided into two groups, namely hydrolyzed and condensed tannins. Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose, while the numerous condensed tannins (often proanthocyanides are derived from flavonoid monomers (Harborne and Baxter, 1999; Okwu, 2005). Many physiological activities such as stimulation of phagocytic cells, host mediated tumor activity and wide range of anti-infective action have been assigned to tannins (Okwu, 2004). Tannins (tannic acid) are a mixture of esters of gallic acid with glucose whose exact composition varies according to its source. Tannic acid is an astringent that precipitates albumin (Okwu, 2004).

2.15.7 Alkaloids

Alkaloids are a class of chemical compounds containing a nitrogen ring. These are the largest group of secondary chemical constituents made largely of ammonia compounds comprising basically of nitrogen bases synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring, most containing oxygen. The compounds have basic properties and are alkaline in reaction, turning red litmus paper blue. In fact, one or more nitrogen atoms that are present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid. The degree of basicity varies considerably, depending on the structure of the molecule, and presence and location of the functional groups (Sarker and Nahar, 2007). They react with acids to form crystalline salts without the production of water (Firn, 2010). Majority of alkaloids exist in solid such as atropine, some as liquids containing carbon, hydrogen, and nitrogen. Most alkaloids are readily soluble in alcohol and though they are sparingly soluble in water, their salts of are usually soluble. The solutions of alkaloids are intensely bitter. These nitrogenous compounds function in the defense of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics,

and poisons due to their potent biological activities. In nature, the alkaloids exist in large proportions in the seeds and roots of plants and often in combination with vegetable acids. Alkaloids have pharmacological applications as anesthetics and CNS stimulants (Ngbede *et al*., 2008). More than 12,000 alkaloids are known to exist in about 20% of plant species and only few have been exploited for medicinal purposes. Other important alkaloids of plant origin include the addictive stimulants caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine. Amino acids act as precursors for biosynthesis of alkaloids with ornithine and lysine commonly used as starting materials. Alkaloids are produced by a large variety of organisms, including bacteria, fungi, plants, and animals, and are part of the group of natural products (also called secondary metabolites). Many alkaloids can be purified from crude extracts by acid-base extraction. Many alkaloids are toxic to other organisms. They often have pharmacological effects and are used as medications, as recreational drugs, or in entheogenic rituals. Examples are the local anesthetic and stimulant cocaine; the stimulant caffeine; nicotine; the analgesic morphine; the antibacterial berberine; the anticancer compound vincristine; the anti-hypertension agent reserpine; the cholinomimeric galatamine; the spasmolysis agent atropine; the vasodilator vincamine; the anti-arhythmia compound quinidine; the anti-asthma therapeutic ephedrine; and the antimalarial drug quinine. Although alkaloids act on a diversity of metabolic systems in humans and other animals, they almost uniformly invoke a bitter taste (Ngbede *et al*., 2008).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Chemicals/Reagents

N-Hexane (JHD, AR; Lobal Chem, India), Dimethyl sulphure oxide DMSO (Park Scientific Ltd, Northampton, UK), Ethylacetate (Qualikems), 2% (Loba Chem, India), 1% n-1-napthyl-ethylenediamine dihydrochlorid (Sigma-Aldrich, St. Louis, MO, USA),Hydrochloric acid, potassium nitrate, Methanol (Merck-Germany), TLC silica gel 60 F254 pre-coated plates (Merk-Germany), Silica gel-60 for column (60-120µm in size), Sulphuric acid (Sigma-Aldrich, St. Louis, MO, USA) and Anisaldehyde (Sigma-Aldrich, St. Louis, MO, USA). All chemicals and reagents used were of analytical grade.

3.2 Equipment

UV lamp (Sigma-Aldrich, St. Louis, MO, USA),Spectrophotometer (Sigma-Aldrich, St. Louis, MO, USA), Water bath (HHS, Mc Donald Scientific International), biosafety level three, biosafety cabinet, others include conical flasks, falcon tubes, pasture pipette, oven, incubator, inspissator, eggs mixer and autoclave.

3.3 Plant Collection

Fresh leaves, Root, Stem bark and Fruit of *Ficus sycomorus* were obtained from Saye village of Zaria Local Government Area, Kaduna State, Nigeria. The sample was authenticated by comparison with herbarium specimen with voucher number 081 at the herbarium of the Department of Biological Sciences, Ahmadu Bello University Zaria.

3.3.1 Aqueous, methanol, and n-hexane extract preparation

The leaves, root, stem bark and fruit of *Ficus sycomorus* were washed with distilled water and dried at room temperature. The samples were grinded using a pistol and mortar. Exactly 100 g of dried powdered leaves, root, stem bark and fruit were extracted in 500mL of Distilled water, methanol, and n-hexane respectively by maceration within 48hrs. The solution of extract was gently evaporated to dryness in a water bath at 40°C. The extract was stored in a refrigerator at 4°Cuntil when required (Akujobi *et al.,* 2004).

3.4 Standard *Mycobacterium tuberculosis* strains

American Type Culture Collection (ATCC) standard strains of *Mycobacterium tuberculosis*, were obtained from National Tuberculosis and Leprosy Training center Saye, Zaria. ATCC 27294 (susceptible to isoniazid and rifampicin), ATCC 35822 (resistant to isoniazid), and ATCC 35838 (resistant to rifampicin).

3.5 Screening of methanol, aqueous, and n-hexane extracts for anti-mycobacterial activity

The anti-mycobacterial activity of aqueous, methanol, and n-hexane extracts were determined using Nitrate reductase assay (NRA). The anti-mycobacterium activity of these extracts were tested on three different standard strains of *Mycobacterium tuberculosis* ATCC27294, ATCC 35822, and ATCC 35838 which are susceptible to both isoniazid and rifampicin, isoniazid resistant, and rifampicin resistant, respectively.

3.6 Media preparation

Lowenstein Jensen media base was prepared according to manufacturer's instructions. The culture media was prepared by weighing 37.4g of commercial Lowenstein Jensen medium powder and dissolving in 600mL distilled water then12mL glycerol was added and mixed

thoroughly, the solution was heated with frequent agitation just until the media boils, the solution was autoclaved at 121°C for 30 minutes and cooled in order to prevent contamination. One liters of homogenized eggs was also added, mixed and the media was then dispensed into 15mL falcon tubes and were inspissated at 85°C for 45 minutes; then incubated at 37°C for 48 hours for sterility checks (Concepcions *et al.*, 2001).

3.6.1 Preparation of Lowenstein- Jensen medium with KNO_3 and drugs

Two grams of potassium nitrate was dissolved in 10 mL of sterile distilled water. 1ml of prepared KNO_3 was added to 200mL of the drug-containing and drug-free LJ medium, dissolved by stirring, aliquoted and then inspissated for 45 minutes at 85°C. After inspissation, the media was incubated for 48 hours at 37°C for sterility check before use. For NRA, the bottles containing LJ with antibiotics and KNO_3 were used for the test while LJ with KNO_3 only were used as positive control (Canetti *et al.,* 1963).

3.7 Nitrate Reductase Assay method

Nitrate Reductase Assay was performed as described by Golyshevskaia *et al.* (1996) and Angeby *et al.*(2002) for anti-mycobacterium activity. The standard critical concentrations of 0.2µg/mL for INH, 40µg/mL for rifampicin were used as negative controls and different extracts concentrations (400, 200, 100, 50, 25, 12.5, and 6.25µg /mL) of each test extract sample of *Ficus sycomorus* were used. To the all plain and drug LJ media, potassium nitrate (KNO_3 1mg/mL) were added, and growth of *M. tuberculosis* strains was observed in the form of pink color, which indicate the reduction of nitrate to nitrite. For each batch of test, three control bottles were prepared for inoculation of *M. tuberculosis* strains

For each concentration of the extract, 0.2 mL of inoculum suspension was inoculated into the bottles containing LJ medium with potassium nitrate. Similarly, 0.2 mL of inoculum suspension was also inoculated into the standard drugs, while 0.2 mL of the1:10 inoculums were inoculated into drug-free media which served as positive control. Falcon tubes in triplicates were utilized for each test sample extracts, standard anti-tuberculosis drugs and control bottles. After inoculation all the bottles were incubated at 37^0C for a maximum of twenty one days.

Five hundred micro liter of a mixture of three reagents (100µl of concentrated HCl, 200µl of 2% sulphanilamide and 200µl of 1% n-1-napthyl-ethylenediamine dihydro chloride, ratio 1:2:2), known as Griss reagent was added to one drug-free (positive control) bottle after 7,14 or 21 days of incubation. When the color of control bottle changed to pink, then bottles with drugs were further tested for presence of the bacteria.

3.8 Quantitative phytochemical

Total flavonoids, tannins, alkaloids, phenols and saponins were quantitatively determined from the n-hexene extract of leaves, Root back, Stem back and Fruit of the *Ficus sycomorus.*

3.8.1 Determination of tannins

Tannins determination was done according to the method described by Gracelin *et al*.(2013), with slight modifications. Five mL of 50% DMSO was added to 0.15 g of the extract fraction taken in a 25 mL flask and then shaken for 1 hr. It was filtered into a 25 mL volumetric flask and made up to the mark with distilled water. Then 5 mL of the filtrate was pipette out into a test tube and mixed with 2 mL of 0.1 M $FeCl_3$ in 0.1 N HCl and 0.008 M potassium ferrocyanide. . The absorbance of the different concentrations of each extract was measured against a reagent (distilled water) blank at 605 nm by using a spectrophotometer within 10 minutes. The total

tannins content of each extract was determined using a conventional method and external calibration with slope of standard tannic acid (0.00042). All samples were analyzed in triplicate, using the formula below,

$$\frac{A \times DF \times GF}{W} \times 100$$

Where, A Absorbance
DF Dilution factor
GF Gradient factor (Slope of standard tannic acid curve)
W Weight of sample (Gracelin *et al*. 2013),

3.8.2 Determination of flavonoids

Flavonoids determination was done according to the method described by (Mir *et al*., 2013), with slight modifications 0.15g of extract fraction was extracted with 10mL of 80% methanol at room temperature. The mixture was then filtered through a filter paper in to a pre-weighed 20mL beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The percentage flavonoids were calculated in triplicate by difference using the formula below, (Mir *et al*., 2013).

$$\frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100$$

3.8.3 Determination of saponins

Saponins determination was done according to the method described by (Mir *et al*., 2013),with slight modifications 0.15 grams of the extract fraction was added to 10 mL of 20% methanol and kept in a shaker for 30 minutes. The extract was heated over a water bath for 4 hours at 55°C. The mixture was then filtered and the residue re-extracted with another 20 mL of 20 % methanol. The combined extracts were reduced to approximately 4mL by incubating in water bath at 90°C. The concentrated mixture was then transferred into a 50 mL separating funnel, and extracted twice using 10 mL diethyl ether. Ether layer was discarded while aqueous layer was retained and

20 mL n-butanol was added to it. Then the n-butanol extract was washed twice with 5 mL of 5 % aqueous sodium chloride (NaCl). The remaining solution was heated in a water bath at 55°C. After the evaporation, samples were dried in oven at (40°C). The saponins content was calculated as percentage of the initial weight of sample taken (Mir *et al*., 2013). Using the formula below,

$$\frac{\text{weight of residue}}{\text{weight of sample}} \times 100$$

3.8.4 Determination of Phenols

Phenols determination was done according to the method described by Gracelin *et al*.(2013), with slight modifications. The extract fraction was boiled with 5 mL of ether to extract the phenol components for 15 minutes. Exactly 1 mL of the extract was pipette into a 20 ml flask, and then 5 mL of distilled water was added. Exactly 1 mL of ammonium hydroxide solution and 2 mL of concentrated amyl alcohol were added. The samples are made up to 20 mL mark with ether and left to react for 30 minute for color development. The absorbance of the different concentrations of each extract was measured against a reagent blank at 505 nm by using a spectrophotometer. The total phenols content of each extract was determined using a conventional method and external calibration with slope of standard tannic acid (0.00042). All samples were analyzed in triplicate, using the formula below,

$$\frac{A \times DF \times GF}{W} \times 100$$

Where, A Absorbance
DF Dilution factor
GF Gradient factor (Slope of standard tannic acid curve)
W Weight of sample (Gracelin *et al*. 2013),

3.8.5 Determination of Alkaloid

Alkaloids determination was done according to the method described by **(**Harborne, 1973), with slight modifications. 0.15 grams of the extract fraction was weighed into a 25 mL beaker and 20 mL of 10% acetic acid in ethanol was added, covered and allowed to stand for 4 hrs. The mixture was filtered and the extracts were incubated at 40°C to evaporation on a water bathto one-quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation stops. The whole solution was allowed to settle and the precipitate solution was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed and the difference taken using the following formula below,

$$\frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100$$ **(**Harborne, 1973)

3.9Fractionation of n-hexane *Ficus sycomorus* fruit extract

3.9.1 Column chromatographic separation

One hundred grams (100g) of silica gel G (60- 120µm mesh size) was carefully packed using wet method in a heavy walled glass tube leaving sufficient head space. The column was allowed to settle for some hours to allow the silica gel (stationary phase) settle sufficiently. Three grams (3g) of the crude extract pre-adsorbed on silica gel was loaded onto the packed adsorbent and allowed to stabilize for 3 hrs before elution began. Gradient elution of the solvents (mobile phase) started with n-Hexane (100%) as the first or initial eluent. Subsequently, the polarity was increased, n-hexane and ethyl acetate was added gradiently as n-Hexane: ethyl acetate (9:1), (8:2), (1:1), (100% ethyl acetate), and ethylacetate: Methanol (9:1). Fractions of 20 ml each were collected and allowed to evaporate at room temperature. Each fraction was numbered in accordance with how they were collected respectively. The column fractions were monitored on TLC, and visualized with U.V light. P-anisaldehyde and 10% sulphuric acid were the general

detecting reagents used. Fractions that showed the same spot color and with the same Rf value were combined together in one beaker. Then it was allowed to dryness under room temperature.

3.10 Determination of minimum inhibitory concentration (MIC) for fraction B

The MIC was evaluated from fractions B which had the most activity on *Mycobacterium*. The method used was the macro-tube dilution method described by Adeniyi *et al*.,(1996). The fractions were serially diluted from the solutions of 10000µg/mL to obtained varying concentrations (200, 100, 50, 25, 12.5 and 6.25µg/mL). The concentrations were incorporated into the prepared LJ media base and 6ml each was dispensed into falcon tubes, then inspissated at 85^0C for 45 minutes and inoculated with 0.2ml each with standardized *Mycobacterium* strains. Another set of three test tubes containing only LJ media base were used as negative controls. Also test tubes containing standard anti-tuberculosis drugs and test organisms were used as positive controls. All the tested tubes and control tubes were then incubated at 37^0C for a maximum of 21 days. The lowest concentration of extract fraction showing no pink color change (that is no reduction of nitrate to nitrite) means no growth and was regarded as the minimum inhibitory concentration (MIC).

3.11 Statistical Analysis

The results were expressed as mean ± standard deviation and analyzed using SPSS windows version 20. Analysis was performed using one way ANOVA.

CHAPTER FOUR

4.0 RESULTS

4.1 Anti-mycobacterial activity of aqueous, methanol and n-hexane extract of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains

Table 4.1 showed the results of the anti-mycobacterial screening of the n-hexane extract at different concentrations (400, 200, 100, 50, 25, 12.5 and 6.25µg/mL) against the three standard *Mycobacterium tuberculosis* strains (susceptible, isoniazid resistant and rifampicin resistant). Samples from fruit extracts showed inhibitory activity at 400, 200, and 100 µg/mL against susceptible strain (ATCC 27294), 400 µg/mL against isoniazid resistant strain (ATCC 35822) and rifampicin resistant strain (ATCC 35838). There were no pink colors observed and these signify a complete inhibition of the *Mycobacterium tuberculosis* by the fruits extracts. While no activity was observed using Stem bark, roots and leaves, because pink color change was obtained in the slants indicating the conversion of nitrate to nitrite.

Table 4.2 showed the results of the anti-mycobacterial screening of the methanol extract at different concentration (400, 200, 100, 50, 25, 12.5 and 6.25µg/mL) against the three standard *Mycobacterium tuberculosis* strains (susceptible, isoniazid resistant and rifampicin resistant). No activity was observed using all tested extract against all *Mycobacterium tuberculosis* strains.

Table 4.3 showed the results of the anti-mycobacterial screening of the aqueous extract at different concentrations (400, 200, 100, 50, 25, 12.5 and 6.25µg/mL) against the three standard *Mycobacterium tuberculosis* strains (susceptible, isoniazid resistant and rifampicin resistant). No activity was observed using all the tested extracts against all *Mycobacterium tuberculosis* strains.

Table 4.1: Anti-mycobacterial activity of n-hexane extracts of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains.

Conc (µg/mL)	Leaves			Stem			Root			Fruit		
	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF
400	–	–	–	–	–	–	–	–	–	+	+	+
200	–	–	–	–	–	–	–	–	–	+	–	–
100	–	–	–	–	–	–	–	–	–	+	–	–
50	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–
12.5	–	–	–	–	–	–	–	–	–	–	–	–
6.25	–	–	–	–	–	–	–	–	–	–	–	–

Negative control

Conc(µg/mL)	SUB	R-INH	R-RIF
RIF 40	+	+	–
INH 0.2	+	–	+

Control without Drug (positive control = Pass

Key: + = Active (Growth absent), – = Not active (Growth present),
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)

Table 4. 2: Anti-mycobacterial activity of methanol extracts of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains.

Conc (µg/mL)	Leaves			Stem			Root			Fruit		
	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF
400	_	_	_	_	_	_	_	_	_	_	_	_
200	_	_	_	_	_	_	_	_	_	_	_	_
100	_	_	_	_	_	_	_	_	_	_	_	_
50	_	_	_	_	_	_	_	_	_	_	_	_
25	_	_	_	_	_	_	_	_	_	_	_	_
12.5	_	_	_	_	_	_	_	_	_	_	_	_
6.25	_	_	_	_	_	_	_	_	_	_	_	_

Negative control

Conc (µg/mL)	SUB	R-INH	R-RIF
RIF 40	+	+	_
INH 0.2	+	_	+

Control without Drug (positive control) = Pass

Key: + = Active (Growth absent), – = Not active (Growth present),
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)

Table 4. 3: Anti-mycobacterial activity of aqueous extracts of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains.

Conc (µg/mL)	Leaves			Stem			Root			Fruit		
	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF
400	–	–	–	–	–	–	–	–	–	–	–	–
200	–	–	–	–	–	–	–	–	–	–	–	–
100	–	–	–	–	–	–	–	–	–	–	–	–
50	–	–	–	–	–	–	–	–	–	–	–	–
25	–	–	–	–	–	–	–	–	–	–	–	–
12.5	–	–	–	–	–	–	–	–	–	–	–	–
6.25	–	–	–	–	–	–	–	–	–	–	–	–

Negative control

Conc (µg/mL)	SUB	R-INH	R-RIF
RIF 40	+	+	_
INH 0.2	+	_	+

Control without Drug (positive control) = Pass

Key: + = Active (Growth absent), – = Not active (Growth present),
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)

4.4 Quantitative Phytochemistry of *Ficus sycomorus*

Table 4.4 showed the quantitative phytochemical analysis of the *Ficus sycomorus* n-hexane fruit extracts (most active).Tannins, Flavonoids Saponins, Phenols, and Alkaloid, were all present. The highest percentage was obtained in saponins (16.67±0.04) while the lowest percentages were obtained in phenols(0.18±0.10).

Table 4. 4: Quantitative Phytochemical Analysis of n-hexane *Ficus sycomorus* Fruit extract

Phytochemical	% Quantitative value
Tannins	4.16±0.03
Flavonoids	4.00±0.04
Saponins	16.67±0.04
Phenols	0.18±0.03
Alkaloids	6.00±0.02

Mean ± SD of Triplicate Determinations

4.5 Fractionation of n-hexane fruit (most active) extract

4.5.1 Chromatogram of *Ficus sycomorus* n-hexane fruit extract

Plate.1 showed thin layer chromatography (TLC) of *Ficus sycomorus* n-hexane fruit extract developed in n-hexane-ethyl acetate (9:1) using pre-coated thin layer chromatography plate, detected with p-anisaldehyde which revealed 7 major spots.

4.5.2 Column chromatography of the most active extract

Table 4.5showed fractions obtained from n-hexane fruit extracts using silica gel column chromatography that yielded eighty fractions which were pooled together based on their thin layer chromatography profile to give 4 pooled fractions (A-D). A was obtained from 1-27, B 28-38, C 39-56 and D 57-80, of which revealed RF values of 0.43,0.66,0.57 and 0.74 respectively.

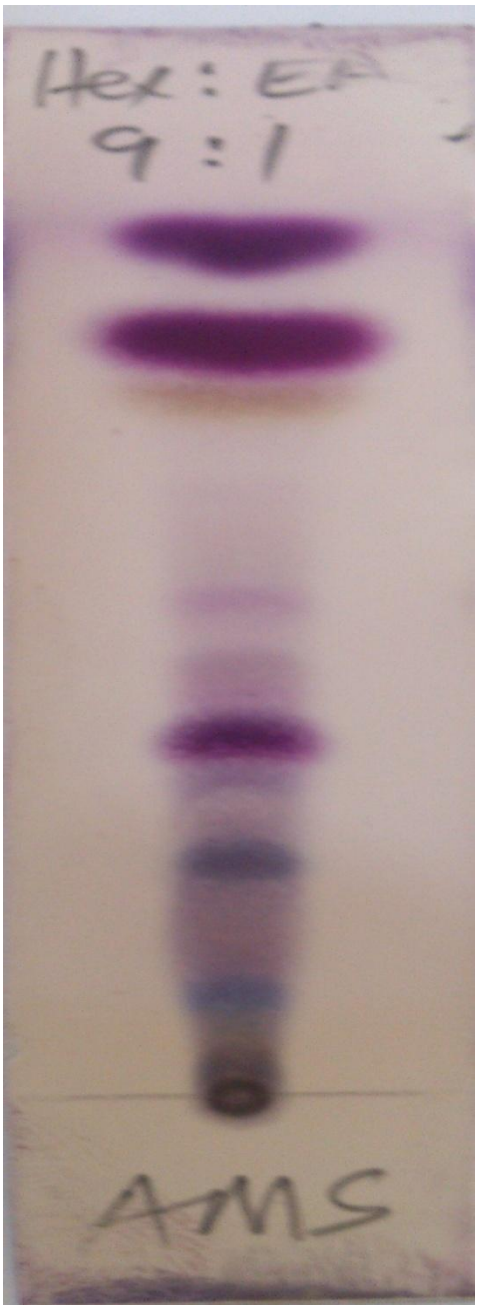


Plate I:TLC of *Ficus sycomorus* n-hexane fruit extract developed in n-hexane-ethyl acetate (9:1) detected with p-anisaldehyde in H_2SO_4

Table 4. 5: Fractions obtained from column chromatographic separation of n-hexane extracts

Number	Fraction	Eluting Solvent
A	1-27	Absolute n-Hexane
B	28-38	9:1
C	39-56	8:2
D	57-80	1:1

Eluting solvent n-hexane:ethylacetate

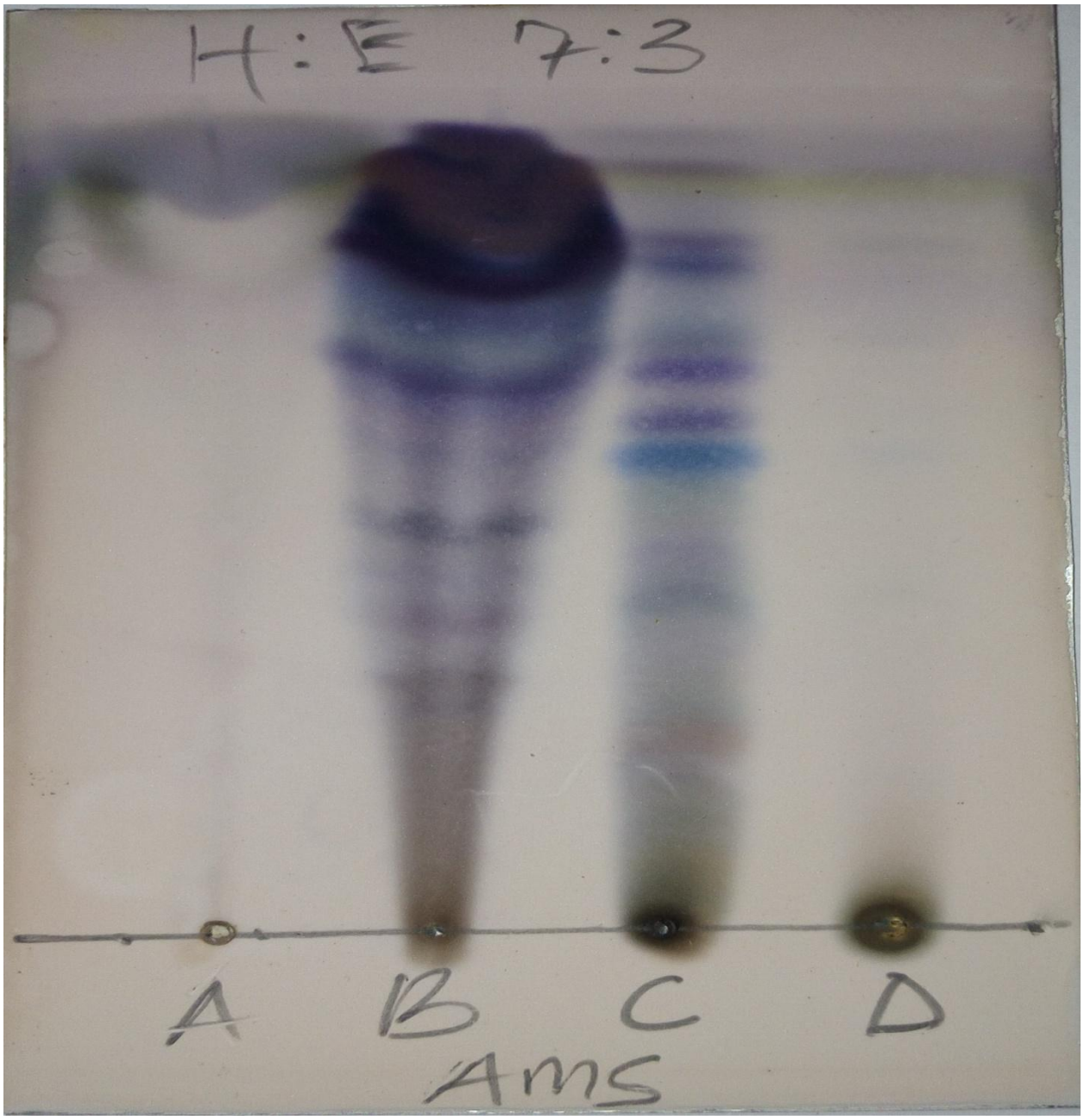


Plate II: Chromatogram of fractions obtained from Column Chromatographic separation of fruit n-hexane fraction of *Ficus sycomorus* in n-hexane-ethylacetate (7:3) detected with p- anisaldehyde in H_2SO_4.

4.6 Antimycobacterial activity of column chromatographic fractions of n-hexane fruit extract of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains

Table 4.6 shows the anti-mycobacterial activities of column chromatographic fractions of *Ficus sycomorus* n-hexane fruit extract against three standard *Mycobacterium tuberculosis* strains. Fractions B, C and D showed antimycobacterial activity at 400 µg/mL against all the tested *Mycobacterium tuberculosis* strains (because no color change was obtained which indicate no conversion of nitrate to nitrite). Except for fraction A, that shows activity to susceptible strains only, but did not show activity against rifampicin and isoniazid resistant strains.

Table 4. 6: Antimycobacterial activity of column chromatographic fractions of n-hexane Fruit extract of *Ficus sycomorus* against three standard *Mycobacterium tuberculosis* strains.

Conc (µg/mL)	A			B			C			D		
	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF
400	+	_	_	+	+	+	+	+	+	+	+	+

Negative control

Conc (µg/mL)	SUB	R-INH	R-RIF
RIF 40	+	+	_
INH 0.2	+	_	+

Control without Drug (positive control) = Pass

Key:

+ = Active (Growth absent), – = Not active (Growth present)

Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)

4.7 Minimum Inhibitory Concentration (MIC) of column chromatographic fractions of n-hexane Fruit extract of *Ficus sycomorus*

Table 4.7 showed minimum inhibitory concentration (MIC) of column chromatographic fractions of *Ficus sycomorus* n-hexane fruit extract. All the fractions showed MIC against all the tested strains. Fraction B had the lowest MIC at concentration of 6.25 µg/mL against susceptible strain (Plate 3) and R-RIF strain (Plate 4), and 100 µg/mL against R-INH strain (Plate 5). While fraction C, at a concentration of 25 µg/mL against susceptible strain (Plate 6). Then lastly, Fraction D, at 100 µg/mL against susceptible strain (Plate 9) and R-RIF strain (Plate 10)

Table 4. 7: Minimum Inhibitory Concentration (MIC) of column chromatographic fractions of n-hexane *Ficus sycomorus* Fruit extract against three Standard *Mycobacterium tuberculosis* strains

Conc (µg/mL)	B			C			D		
	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF	SUB	R-INH	R-RIF
200	+	+	+	+	_	+	+	_	+
100	+	+	+	+	_	+	+	_	+
50	+	_	+	+	_	+	_	_	+
25	+	_	+	+	_	+	_	_	_
12.5	+	_	+	_	_	_	_	_	_
6.25	+	_	+	_	_	_	_	_	_

Negative control

Conc(µg/mL)	SUB	R-INH	R-RIF
RIF 40	+	+	_
INH 0.2	+	_	+

Control without Drug (positive control) = Pass

Key: + = Active (Growth absent), – =Not active (Growth present),

Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)

CHAPTER FIVE

5.0 DISCUSSION

The anti-mycobacterial activity of *Ficus sycomorus* by *in vitro* screening against some selected *Mycobacterium tuberculosis* strains was studied. The results obtained from anti-mycobacterial activity of the aqueous, methanol and n-hexane extracts of leaves, stem-bark, roots, and fruits of *Ficus sycomorus* against *Mycobacterium tuberculosis* strains revealed that the n-hexane fruit extract have the potent antimycobacterial activity in relation to the standard antimycobacterial drugs control (Table 4.1).This activity was obtained against all the three tested *Mycobacterium tuberculosis* strains, which could be due to the non polar extracting properties of n-hexane. Reports from literature show that long-chain unsaturated fatty acids such as oleic and linoleic acid extracted from n-hexane are selective inhibitors of the enoyl-acyl carrier protein reductase, which is a potent inhibitor of cell wall fatty acid synthesis (Zhing*et al.,* 2005). Also n-hexane extract of *Adhatoda vasica* has been reported by Ignacimuthu and Shangumam, (2010) to have antimycobacterial activity. No activity was observed in *Ficus sycomorus* stem bark, root and leaves n-hexane extracts (Table 4.1), which could be due to the bio-availability of the phyto-constituent. No activity was also observed in aqueous and methanol extracts (Table 4.2 and 4.3) which could be due to their polar and semi-polar extracting properties.

Quantitative phytochemical analysis revealed highest percentage content in saponins and alkaloids, (Table 4.4) as such this activity could also be either a function of the individual or the additive effects of the phytochemical constituents. *Ficus sycomorus* n-hexane fruit extracts exhibit secondary metabolites apart from its high nutritive value (Okoronkwo *et al.*, 2014), such secondary metabolites are flavonoids, tannins, alkaloids, saponins and phenols which are found to have antimicrobacterial activity. Studies by Mousa *et al*., (1994), Sandabe, (2002), Sandabe *et*

al., (2006), and Ladda *et al.,* (2012), also report the roles of secondary metabolite in active killing of micro-organisms. Therefore different phytochemicals have been found to possess a wide range of activities, which may help in protection against chronic diseases (Mir *et al*., 2013). Jha *et al.,* (2012) reported six quinazoline alkaloid from *Justicia adhatoda* having significant antimycobacterial activity, and in silicon analysis confirmed that these alkaloids inhibit β-ketoacyl-acyl-carrier protein synthase III, an enzyme involved in the initial step of fatty acid biosynthesis, leading to poor cell wall development and survival of bacilli (Jha *et al.*,2012). To develop drug resistant, the efflux pump in the bacteria release compounds preventing them from reaching their respective targets inside the cell, an alkaloid Piperine atrans-transisomer of 1-piperonyl-piperidine, is an antimycobacterial agent which at 128 μg/mL completely inhibits the efflux pump of *M. Smegmatis* and *M. tuberculosis.*

Another possible mechanism of action of these phytochemicals could be in the inhibition of RNA and protein synthesis in the *Mycobacterium* cell as it was proposed and demonstrated by Sureram, *et al.,* (2012) that an alkaloid bisbenzyl isoquinoline inhibit RNA and protein synthesis in the mycobacterium cell. A flavanoid isobachalcone from the Moraceae have also been reported by Kuete, *et al.*, (2010) to be active against *M. smegmatis* and *M. tuberculosis*

Sharma, *et al.,* (2008), reported that a flavanoid epigallocate-chin gallate/epigallocatechin-3-gallate directly inhibits fatty acid synthesis systems I and II which is the fourth step of the fatty acid elongation cycle is carried out by an enoyl-acyl carrier protein reductase (InhA in*M. tuberculosis*) which catalyses an NADH-dependent reduction of the trans-2-enoyl fatty acyl chain to the saturated fatty acyl chain, by interacting with the residues near the NADH binding site.

The highest percentage content of saponins could also be responsible for the mycobacterial activity because saponins are thought to form complexes with bile and/or cholesterol, therefore preventing absorption of cholesterol by the small intestine of the gastrointestinal tract (Kim *et al*., 2003) as such cholesterol will be unavailable for the formation mycobacterial cell wall. Saponins also have the ability to enhance both humoral and cell mediated immune responses to antigens in humans, where as mycobacterial infection are thought to be successful in an immune compromised host (Iqbal *et al.,* 2006). They have been shown to increase antigen-specific antibody production as well as to induce a strong cytotoxic T lymphocyte response (Iqbal *et al.*, 2006). A complex of cholesterol, saponin and antigen known as an immune stimulating complex can induce both a humoral and cellular immune response in animal models (Naknukool *et al*., 2011). Besides modulating the adaptive arm of the immune system, saponins have been found to modulate innate immune responses as well (Naknukool *et al*., 2011).

Phytochemicals have also been associated with reduction of drug resistant form of bacteria, (stauri *et al*., 2007). Many medicinal plants are considered to be potential antimicrobial crude drugs as well as a source for novel compound with antimicrobial activity, with possibly new modes of action (Dhiman *et al*., 2012).

The lowest MIC of fraction B obtained could also be as the result of more active constitutive phytochemical content which were able to inhibit the mycobacterial growth. Ladda *et al.,* (2012) and Thankamani *et al.,*(2011) reported that the MIC of *Ricinus communis* and *Alstonia scholaris* ware150µg/mL and 100 µg/mL against *M. tuberculosis* respectively.

Therefore n-hexane fruit extract of *Ficus sycomorus* been the most active against both drug-susceptible and drug-resistant strains of *M. tuberculosis* could be a breakthrough in addressing the issues of MDR-Tuberculosis.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The results obtained in this study from (Leaves, stem, root and fruits) of *Ficus sycomorus* against three different standard *Mycobacterium tuberculosis* strains, showed that:

The n-hexane fruit extracts had the highest activity with MIC of 6.25µg/ml on both susceptible and R-RIF strains, and with 100 µg/ml against R-INH strains.

Hence, it is concluded that the n-hexane *Ficus sycomorus* fruit extract has potent anti-*Mycobacterium tuberculosis* activity which are due to the phytochemical constituents present in the plant and could be a function of either the individual or the additive effects of the phytochemical constituents. All these findings justify the anti-mycobacterial activity of *Ficus sycomorus* by *in vitro* screening against some selected *Mycobacterium tuberculosis* strains

Therefore, n-hexane *Ficus sycomorus* fruit extract could be a promising herb drug for Multi-drug resistant tuberculosis if properly explored and standardized, especially in the context of emergence of MDR-TB and even pre XDR-TB, long drug regiment and toxicity associated with synthetic drugs.

6.2 Recommendations

The following recommendations should be taken into consideration:

1. Based on the above findings, the standardization of this part of the plant (in crude form) as anti-mycobacterial herbal remedy might be a promising venture for the development of novel plant-derived anti-mycobacterial drug.
2. Similar studies should be performed and compared the method used in this study with other DST method to evaluate the *Mycobacterium tuberculosis* activity.
3. A study should be carried out to purify and strain the main active compounds in *Ficus sycomorus*
4. An *in-vivo* study should be carried out to evaluate the mycobacterium activity of n-hexane fruit extract of *Ficus sycomorus*.

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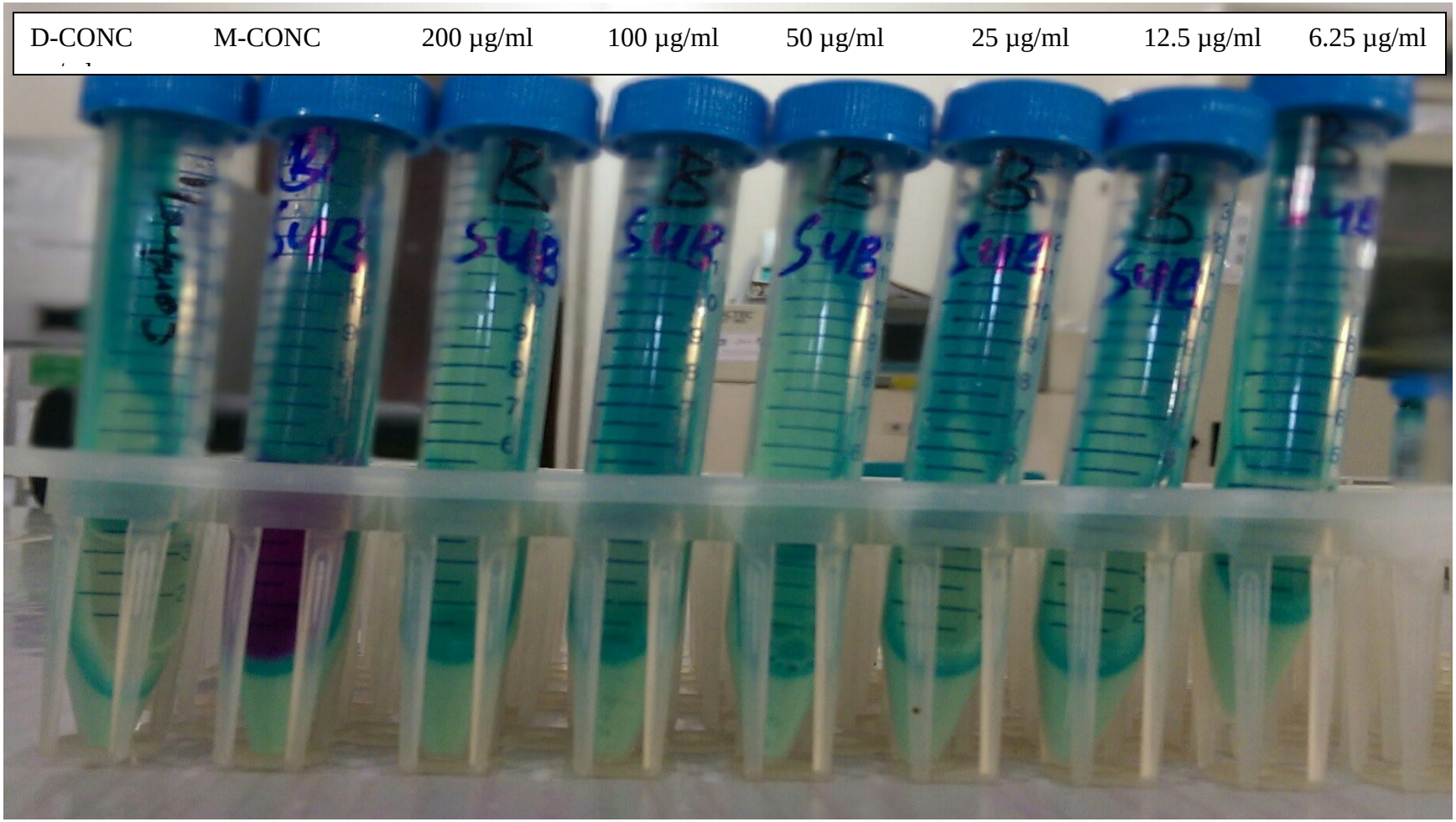
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APPENDICES

Appendix I: Preparation of stock solution of the extract

The stock solutions of the methanol, aqueous, and n-hexane extract were prepared by dissolving 0.1 g of the extract in 10 ml 50%DMSO except for aqueous extract which was dissolved in sterile distilled water to give a concentration of 10,000µg/mL. The stock solution was then reconstituted to a graded concentration of 400, 200, 100, 50, 25, 12.5 and 6.25 µg/mL. They were well labeled and stored at 4^0C until when required.

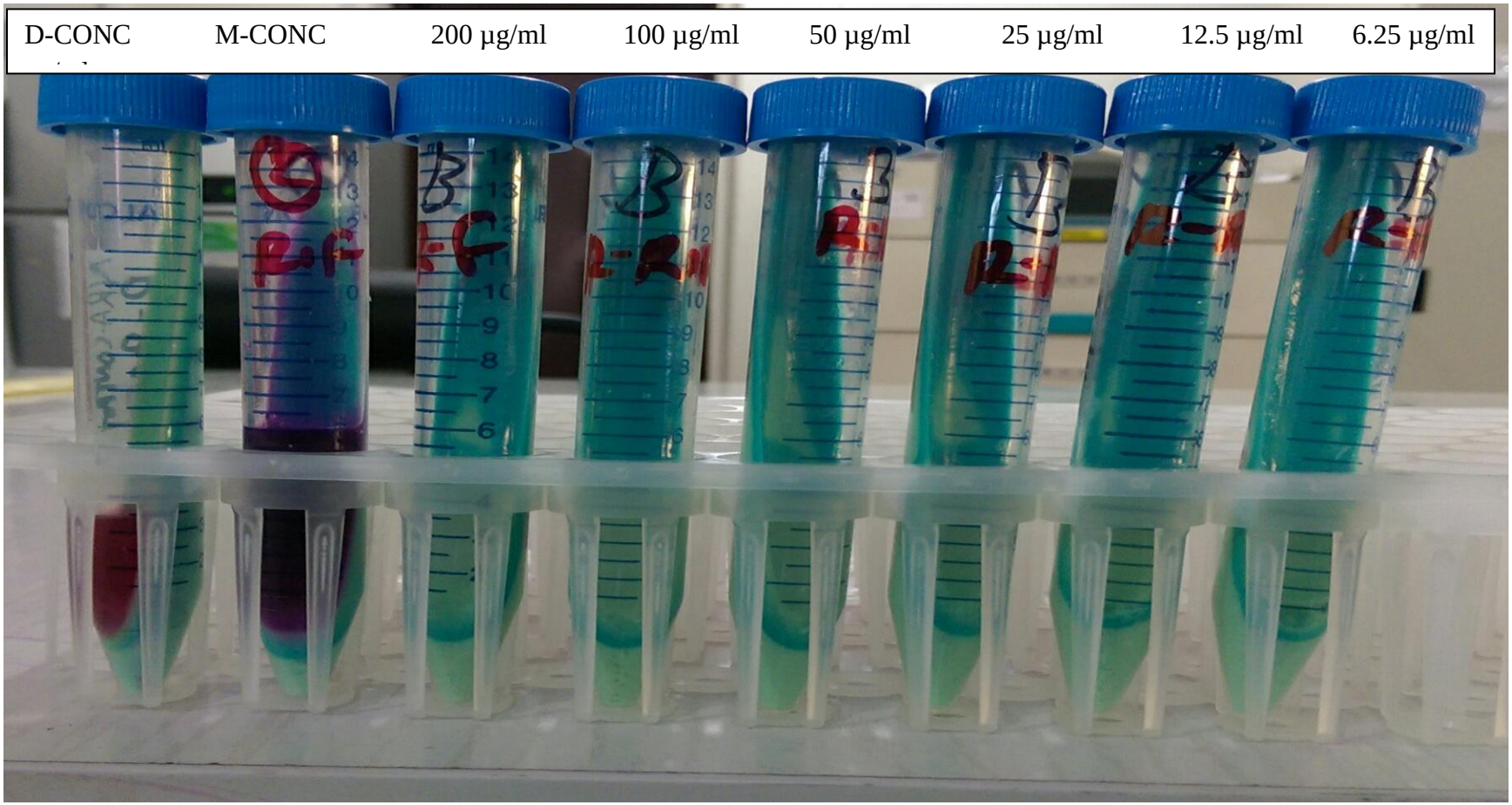
D-CONC	M-CONC	200 µg/ml	100 µg/ml	50 µg/ml	25 µg/ml	12.5 µg/ml	6.25 µg/ml



Appendix II: MIC of Chromatographic fraction B against the susceptible strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix III: MIC of Chromatographic fraction B against the R-RIF strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

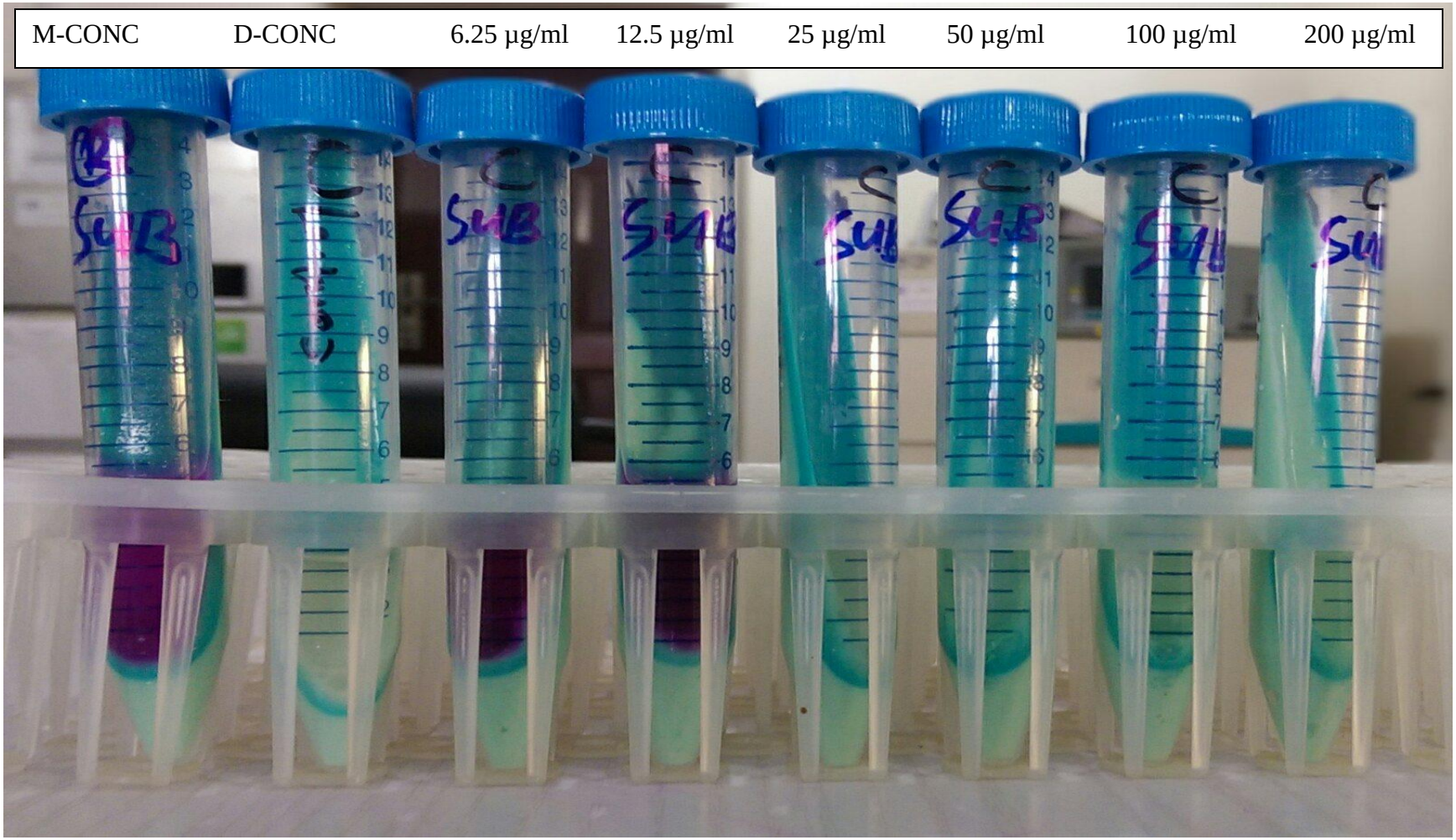
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix IV: MIC of Chromatographic fraction B against the R-INH strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

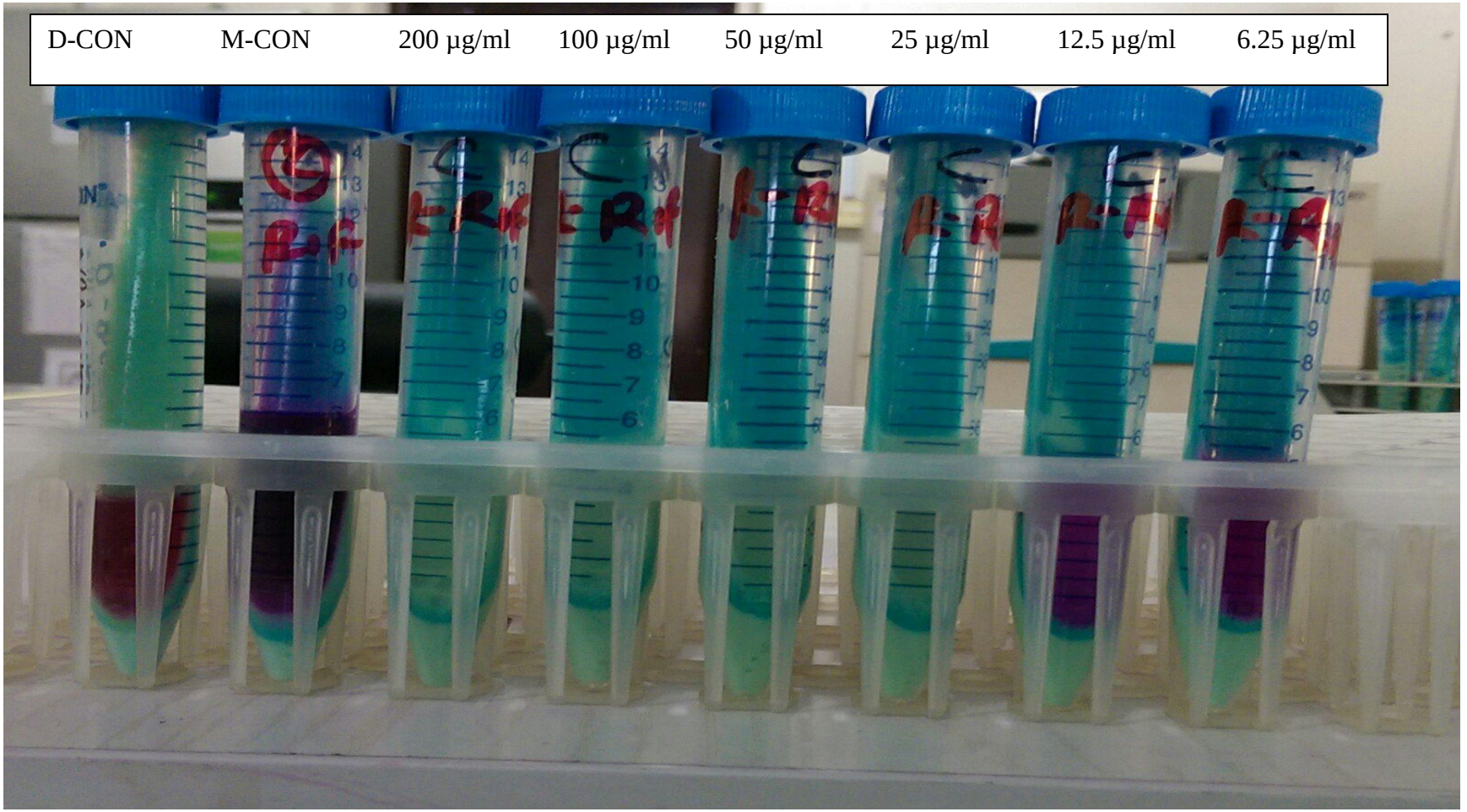
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix V: MIC of Chromatographic fraction C against the susceptible strain of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

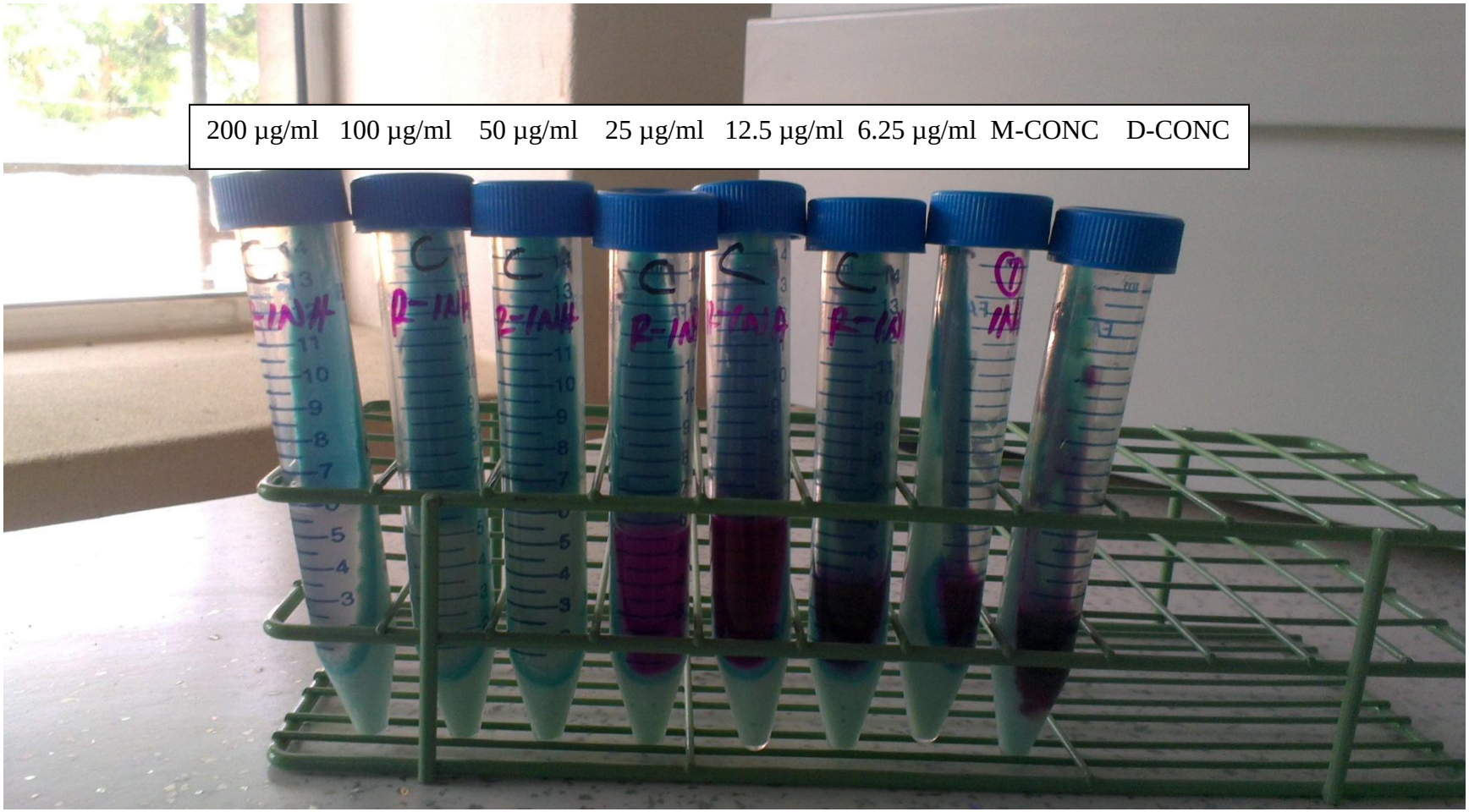
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix VI: MIC of Chromatographic fraction C against the R-RIF strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

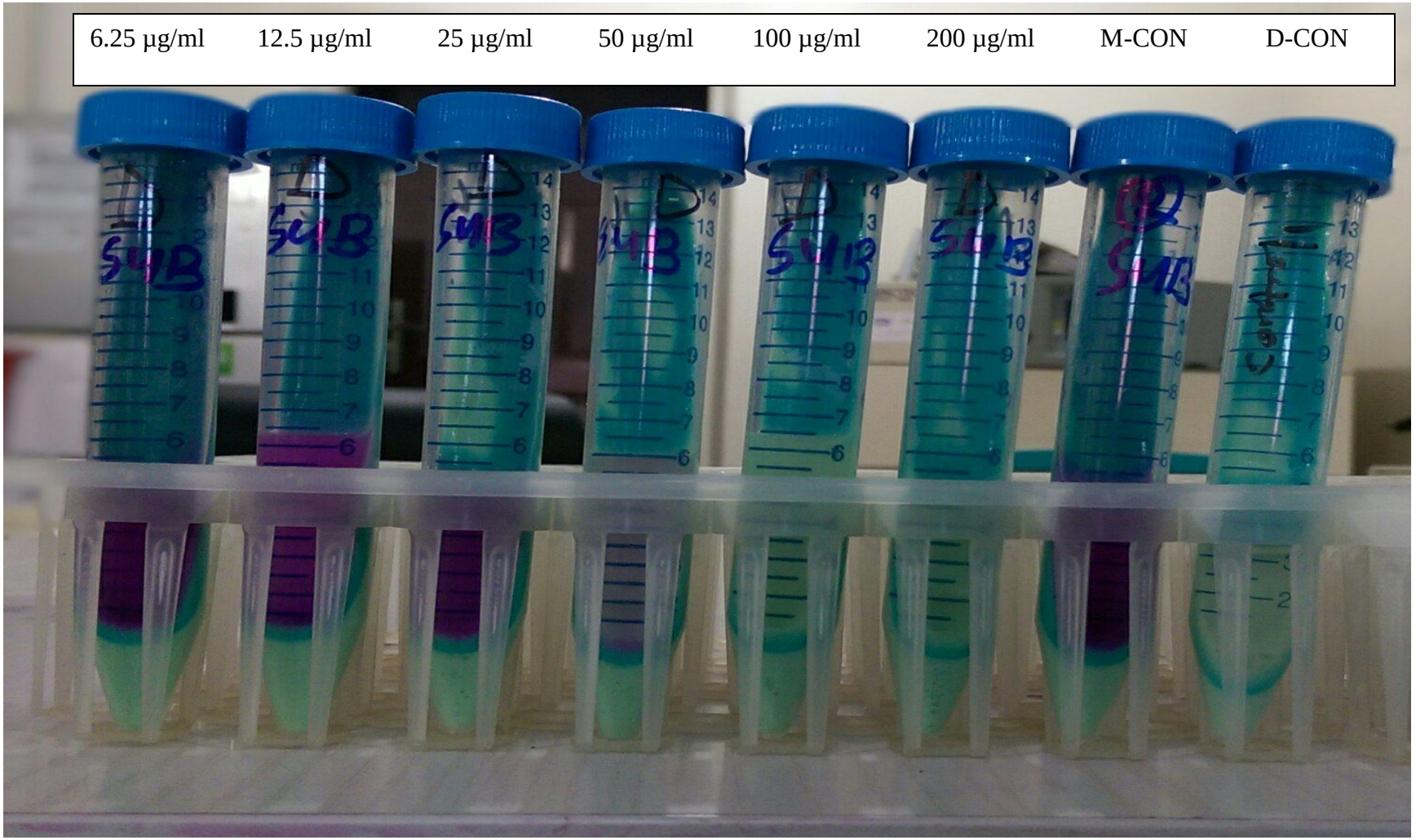
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix VII: MIC of Chromatographic fraction C against the R-INH strain of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

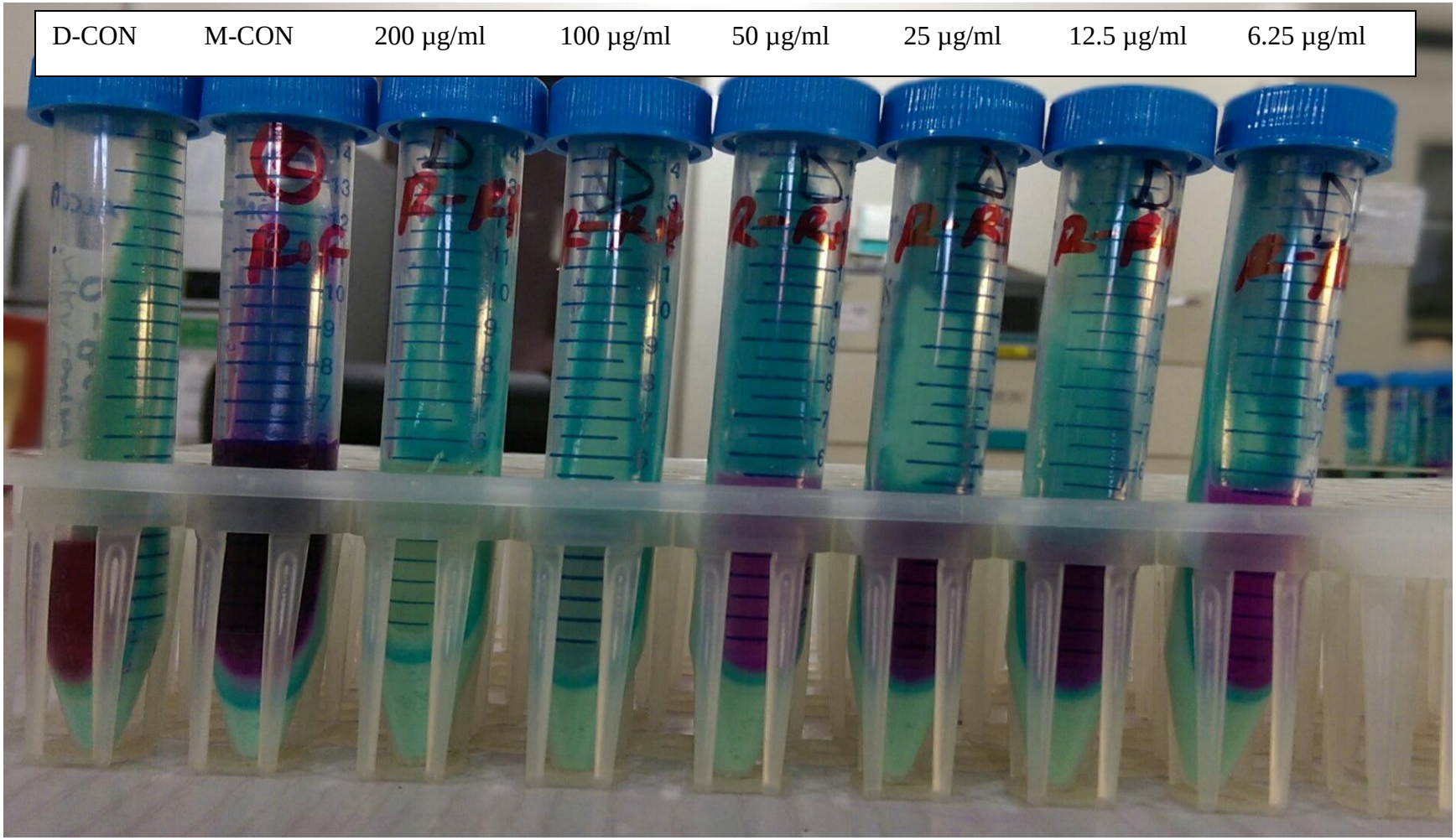
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix VIII: MIC of Chromatographic fraction D against the susceptible strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

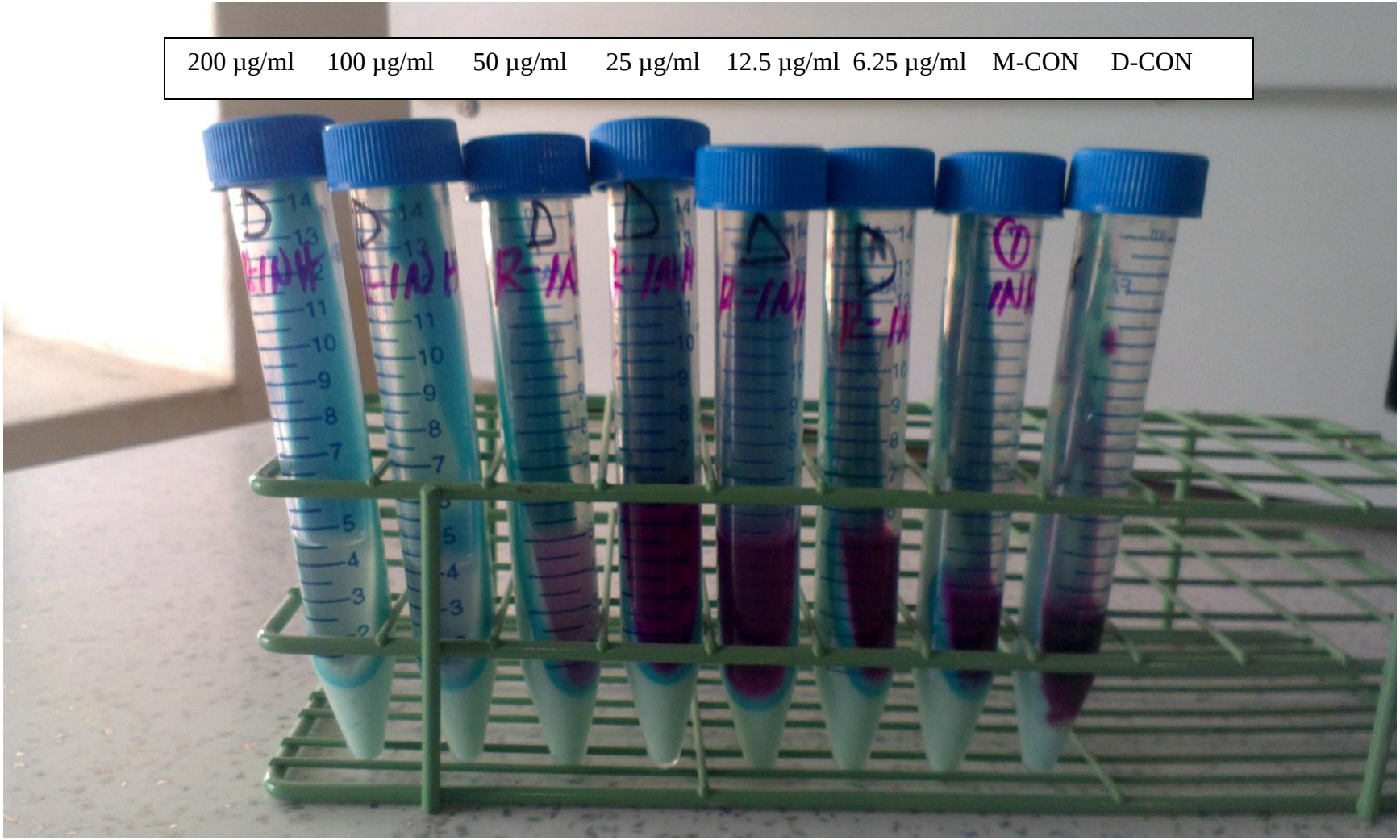
Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix IX: MIC of Chromatographic fraction D against the R-RIF strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)



Appendix X:MIC of Chromatographic fraction D against the R-INH strains of *Mycobacterium tuberculosis*

D-CONC (Drug control), M-CONC (Media control)

Susceptible (SUB), isoniazid resistant (R-INH) and rifampicin resistant (R-RIF)