Evaluation of plant extracts: *Artemisia afra* and *Annona muricata* for inhibitory activities against *Mycobacterium tuberculosis* and Human Immunodeficiency virus

By

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In accordance with Rule G4.6.3, I hereby declare that the above-mentioned dissertation represents my own unaided work and that it has not previously been submitted for assessment to another university or for another qualification.

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Date

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ABSTRACT

Mycobacterium tuberculosis and Human Immuno-Deficiency Virus (HIV) have a high prevalence in South Africa. The development and spread of drug resistant tuberculosis is a serious problem which is exacerbated by tuberculosis (TB) co-infection in HIV patients. Traditional medicinal plants like *Annona muricata* and *Artemisia afra* are used for respiratory ailments and antiviral therapies respectively. The aim of this study was to evaluate *Annona muricata* (ethanolic extract) and *Artemisia afra* (ethanolic and aqueous extracts) for inhibitory activities against *M. tuberculosis* and HIV. *In vitro* bioassays for anti-TB activity included: microplate alamar blue assay (MABA), flow cytometry and ρ-iodonitrotetrazolium chloride assays while anti-HIV activity was determined using an HIV-1 reverse transcriptase colorimetric ELISA kit and an HIV-1 integrase colorimetric immunoassay. Cytotoxicity of plant extracts were assessed by the MTT assay on Chang Liver and HepG2 cells. Potential synergistic effects were determined using the basis of Combination Index. Potential interactions of plant extracts with drug metabolic pathways were evaluated with the Glutathione-S-Transferase assay kit as well as the CYP3A4 assay kit. *A. muricata* ethanolic extract exhibited anti-TB activity with MIC 125 µg/mL. MABA was shown to be the most sensitive and effective method for the detection of anti-TB activity. *Artemisia afra* aqueous extract showed HIV-1 reverse transcriptase inhibition exhibiting >85% inhibition at 1 mg/mL while the ethanolic extracts of *A. afra* and *A. muricata* showed inhibition of HIV-1 integrase activity at >86.8% and >88.54% respectively at concentrations >0.5 - 4 mg/mL. The aqueous extract of *A. afra* displayed inhibition of HIV-1 integrase >52.16% at 0.5 mg/mL increasing to 72.89% at 4 mg/ml of the extract. *A. muricata* was cytotoxic at an IC_{50} of 30 µg/mL and 77 µg/mL on Chang Liver and HepG2 cells respectively, whilst *A. afra* aqueous and ethanol extracts were not cytotoxic to both cell lines. The ethanolic extract of *A. muricata* showed both antagonistic and synergistic properties at various IC values, when used in conjunction with rifampicin. *A. afra* ethanolic extract interrupted GST activity while aqueous extracts of *A. afra* and *A. muricata* had a slight effect. All extracts interrupted CYP3A4 activity, however the ethanolic extracts of *A. muricata* and *A. afra* showed greater inhibition than the aqueous extract of *A. afra*. These extracts should be investigated further as they could be an important source of compounds for treatment of *M. tuberculosis* and HIV respectively.

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AIDS	Acquired immune deficiency syndrome
ADC	Albumin-dextrose-catalase
ATCC	American Type Culture Collection
ARV	Antiretroviral
Anti-DIG-POD	Anti-digoxigenin-peroxidase
ATP	Adenosine Triphosphate
Bsc	Bachelor of science
$BaCl_2$	Barium Chloride
CO_2	Carbon dioxide
CDC	Centers for disease control
CLSI	Clinical Laboratory Standards Institute
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
°C	Degrees Celsius
CI	Combination Index
C-terminus	Carboxyl terminus
C-3	Carbon 3
CYP3A4	Cytochrome 3A4
CDNB	1-Chloro-2,4-dinitrobenzene
DMSO	Dimethyl sulfoxide
DOH	Department of Health
DOTS	Directly observed treatment
ddH_2O	Double distilled water
DPBS	Dulbecco's Phosphate Bufferred Saline
deCIPhR	Dual enhancement of Cell Infection to Phenotype Resistance
DNA	Deoxyribonucleic acid
dUTP	Digoxigenin Uridine Triphosphate
DS	Donor Substrate
EMB	Ethambutol
XDR-TB	Extensively drug resistant TB
EMEM	Eagles Minimum Essential Medium
ELISA	Enzyme Linked Immunosorbent Assay
FDA	Fluoroscein diacetate
FRET	Fluorescent Resonance Energy Transfer
FBS	Foetal Bovine Serum
g	grams
xg	gravity
Gag	Group specific Antigen

Gag-Pol	Group specific Antigen Polypepdtide
GST	Glutathione-S-Transferase
HAART	Highly Active Antiretroviral Therapy
HTS	High-throughput screening
h	hours
HIV	Human immunodeficiency virus
HRP	Horse Radish Peroxidase
HSD	Honestly Significant Difference
INH	Isoniazid
IC	Inhibitory Concentration
iFIGS	Infection format of Fusion Induced Gene Stimulation
Log	Logarithmic
MABA	Microplate Alamar Blue Assay
mg	Milligram
mL	Millilitre
mM	Millimolar
MIC	Minimum inhibitory concentration
min	Minutes
MDR-TB	Multi-drug resistant TB
Mono-THF	Mono-Tetrahydrofuran
MRP	Multiple Resistance Protein
MTT	4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
m	Metres
ng	Nanograms
nm	Nano-meters
nM	Nanomolar
ND	Not Determined
NEAA	Non-essential Amino Acids
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NARTI	Nucleoside analogue Reverse Transcriptase Inhibitor
NYU	New York University
N-terminus	Amino terminus
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NHLS	National Health Laboratory Services
N	Normal
NMMU	Nelson Mandela Metropolitan University
OFX	Ofloxacin

OA	Oleanolic Acid
INT	*p*-Iodonitrotetrazolium chloride
PE	Port Elizabeth
PZA	Pyrazinamide
P-gp	P-glycoprotein
PI	Protease Inhibitor
PAPS	3’-phosphoadenosine 5’-phosphosulfate
pg	Picogram
R	Rand
RIF	Rifampicin
RT	Reverse Transcriptase
RFU	Relative fluorescence units
NaOH	Sodium Hydroxide
spp.	Species
SM	Streptomycin
-SH	Sulfhydral
sec	Second
SD	Standard Deviation
SRB	Sulforhodamine B
SULT	Sulfotransferase
TB	Tuberculosis
TS	Target Substrate
TMB	3,3', 5,5'-tetramethylbenzidine
µL	Microlitres
µm	Micrometres
µM	Micromolar
UV	Ultraviolet Light
vol/vol	Volume/volume
w/v	Weight/volume
WHO	World Health Organization

CHAPTER ONE

LITERATURE REVIEW

1.1 INTRODUCTION

Mycobacterium tuberculosis and Human Immuno-Deficiency Virus (HIV) have a high prevalence in South Africa. Despite the fact that more than 40 years has been placed behind anti- tuberculosis (TB) chemotherapy, TB still remains one of the main infectious killers worldwide (Luo *et al.,* 2011). There were 9.4 million new cases (including 1.1 million cases among people with HIV) and 1.7 million deaths from TB (including 380 000 people with HIV) in 2009 (WHO, 2010). HIV has been estimated to have infected 34 million people worldwide, where South Africa's HIV epidemic remains the largest in the world, with an estimated 5.6 million HIV positive people as of 2009. This exceeds the number of people living with HIV in the entire Asian region (www.who.int/hiv/pub/progress_report2011/en/index.html).

The use or mis-use of anti-tuberculosis drugs has led to an increasing prevalence of multi-drug resistant (MDR) and extreme drug resistant (XDR) *M. tuberculosis* strains, emphasising the need for novel and improved drugs for treatment of TB. There is a renewed and increasing interest in plant medicine as these are seen as potential sources of new compounds for drug development (Luo *et al.,* 2011).

Many plant species are used in traditional South African medicine to alleviate symptoms of TB and several lead compounds have been reported for further research following *in vitro* antimycobacterial activity evaluation. However, more research is needed on the assessment of anti-mycobacterial efficacy of plants against *M. tuberculosis* both *in vitro* and *in vivo* (McGaw *et al.,* 2008). Current antiretroviral drugs are vitally important to improve the quality and prolong the life of HIV/AIDS patients. However, these drugs have many disadvantages including resistance, toxicity, limited availability, high cost and lack of any curative effect. Thus, it is important to search for improved antiretroviral agents which can be added to or replace the current drugs in the anti-HIV spectrum (Klos *et al.,* 2009).

In this study, two plants, namely *Annona muricata* and *Artemisia afra* were investigated for inhibitory activity against *M. tuberculosis* and HIV. *A. afra* is one of the most popular and commonly used herbal medicines in Southern Africa. However, only limited research has been conducted on this species which is rich in terpenes. It has been reported that *A. afra* exhibits some antiviral activity or immune boosting properties (Liu *et al.,* 2009). There are few reports on *A. muricata,* which indicate that all parts of the tree have been used to treat multiple illnesses (Boyom *et al.,* 2011). The leaves are especially used as an antispasmodic, sedative, also for respiratory tract conditions (coughs, grippe, asthma, catarrh and asthenia) (Asprey and Thornton, 1955).

Due to a variety of compounds that could possibly be present in *A. afra* and *A. muricata*, it is important to understand what major compounds are present in these plants and the potential effects they could have on the existing treatment drug-metabolizing pathways. Many HIV/AIDS patients use traditional plant remedies in conjunction with drug therapies provided by clinics and hospitals which may result in drug-herbal pharmacokinetic and pharmacodynamic interactions (Brown *et al.,* 2008; Balayssac *et al.,* 2005; Patel *et al.,* 2011). Some pharmacokinetic interactions include changes in the absorption of the co-administered drug, thus affecting its bioavailability, its therapeutic effectiveness or by producing drastic side effects (Brown *et al.,* 2008; Balayssac *et al.,* 2005).

1.2 *MYCOBACTERIUM TUBERCULOSIS*

1.2.1 Epidemiology and Pathogenesis

Tuberculosis in humans is caused by *M. tuberculosis*, which is a non-motile, rod-shaped bacterium (2 - 4 µm in length and 0.2 - 0.5 µm in width), with non-pathogenic forms being found as part of the normal microflora of humans. *M. tuberculosis* has been classified as a facultative intracellular parasite, with a generation time of between 12 - 18 hours (Black, 2011). Tuberculosis can be diagnosed, by clinical evaluation (fever, night sweats, loss of weight and coughing), the tuberculin skin test, chest X-rays and microbiological analysis (Dev Pandey *et al*., 2008 and Van Helden *et al*., 2006).

Tuberculosis is acquired by the inhalation of respiratory secretions containing tubercle bacilli. *M. tuberculosis* multiplies slowly within the host's macrophages stimulating a host immune response, resulting in large amounts of fluid being released into the lung tissue producing pneumonia-like symptoms. In some instances, *M. tuberculosis* can enter the lymphatic and circulatory systems, which can lead to the perforation of blood vessels resulting in the production of bloody sputa, one of the most important and defining symptoms of tuberculosis. Extrapulmonary tuberculosis can be found within the urogenital tract, meninges, lymphatic system, spleen and bones, and the spinal cord (Bauman, 2011).

South Africa is ranked third in the world with tuberculosis infections and is one of the 22 high burden countries that contribute approximately 80% to the total global TB cases. The development and spread of drug resistant tuberculosis is a serious problem for South Africa, and is responsible for the largest number of multi-drug resistant TB (MDR-TB) cases in the world and the largest reported outbreak of extensively drug resistant TB (XDR-TB) (WHO, 2010). The total number of MDR- and XDR-TB cases recorded for the different provinces of South Africa, during 2004 - 2011, were Limpopo (MDR-TB: 943 and XDR-TB: 27), Western (MDR-TB: 11946 and XDR-TB: 386) and Eastern Cape (MDR-TB: 9154 and XDR-TB: 962); KwaZulu-Natal (MDR-TB: 11393 and XDR-TB: 1499) and Gauteng (MDR-TB: 6994 and XDR-TB: 237) (Koornof *et al.,* 2011 and www.doh.gov.za/docs/policy/2011/policy_TB.pdf).

1.2.2 Treatment and Drug Resistant Tuberculosis (TB)

Drug-resistant tuberculosis is defined as the resistance of *M. tuberculosis* to at least one first-line anti-tuberculosis drug (CDC, 2010). Likewise extensively drug resistant TB (XDR-TB) is defined as resistance to at least rifampicin and isoniazid (MDR-TB), in addition to any fluoroquinolone, and at least one of the three following injectable drugs capreomycin, kanamycin, and amikacin used for MDR-TB treatment (Streicher *et al.,* 2012). The treatment of active tuberculosis involves the use of one or more drugs, administered for a minimum of six months under strict clinical management (Bapela *et al*., 2006; Matsumoto *et al*., 2006).

The standard first-line antibiotics used include isoniazid (INH), rifampicin (RIF), ethambutol (EMB), streptomycin (SM) and pyrazinamide (PZA) (Bapela *et al*., 2006;

Johnson *et al.,* 2006). Isoniazid is a very efficient mycobactericidal drug, and is capable of significantly reducing the transmission of tuberculosis, while rifampicin, plays an important role in the prevention of TB re-activation, as it is capable of killing off metabolically active *M. tuberculosis* cells (Suresh *et al*., 2006 and Sharma and Mohan, 2004). Resistance to either isoniazid or rifampicin can be managed by using other first-line antibiotics; but with the emergence of multi-drug resistance, treatment protocols have been further complicated as second-line drugs are now required.

Table 1.1: Treatment guidelines of MDR-TB and XDR-TB (Dept. of Health, S.A; Streicher *et al.,* 2012).

MDR-TB		XDR-TB	
Intensive Phase	Continuation Phase	Intensive Phase	Continuation Phase
Kanamycin (IM)		Capreomycin (IM)	
Ethionamide	Ethionamide	Ethionamide	Ethioamide
Pyrazinamide	Pyrazinamide		
		ρ-aminosalicyclc acid	ρ-aminosalicyclic acid
Ofloxacin	Ofloxacin	Moxifloxacin	Moxifloxacin
Terizidone or cycloserine	Terizidone or cycloserine	Terizidone or cycloserine	Terizidone or cycloserine

Unfortunately, second-line antibiotics have many limitations making them unsuitable for short treatment programmes. MDR-resistant patients require prolonged treatments with antimicrobial drugs that are less effective, more expensive, slightly more toxic, and have the possibility of leading to undesirable drug interactions, as some antiretrovirals and anti-TB drugs cannot be used simultaneously (Matsumoto *et al*., 2006 and Sharma and Mohan, 2004).

However, for the first time in 40 years, a large number of pharmaceutical companies have focussed on drug discovery to develop new chemical entities using either target based or phenotypic screens (Villemagne *et al.,* 2012). There are at least ten compounds in clinical trials, four of which are existing drugs that are being re-developed or re-purposed for TB treatment and then there are six new compounds that are being developed for TB treatment. It is unfortunate that majority of these drugs

are still present in preclinical testing (Villemagne *et al.,* 2012 and Zhenkun, 2010). Currently there are three drugs which have great promise for the release for public use as they are proving to be very effective in phase 3 trials, namely Bedaquiline, Delaminid and PaMZ. Bedaquiline and Delaminid, have shown to be effective at shortening the duration of treatment against MDR-TB where PaMZ, a combination therapy using a novel drug moxifloxacin and pyrazinamide, has shown effectiveness against TB, with 99% of TB bacteria killed within two weeks as well as some forms of drug-resistant TB (http://blogs.sun.ac.za/news/2012/07/25/new-tb-drugs-bring-hope-in-fight-against-tb/).

1.3 HUMAN IMMUNODEFICIENCY VIRUS (HIV)

1.3.1 Epidemiology and Pathogenesis

Human immunodeficiency virus (HIV) is a lentivirus that causes acquired immunodeficiency syndrome (AIDS), a condition in humans resulting in the progressive failure of the immune system. Infection with HIV occurs by the transfer of blood, semen, vaginal fluid, pre-ejaculate or breast milk. Within these body fluids, HIV is present as both free virus particles and virus within infected immune cells. This infection is regarded as a communicable disease where sexual transmission is the most common mode of HIV transmission (UNAIDS Report on the Global Aids Epidemic, 2010). The rate of new infection worldwide is estimated at 2.7 million per year (UNAIDS, 2010).

HIV infects vital cells in the human immune system such as helper T cells (specifically $CD4^+$ T cells), macrophages and dendritic cells. When $CD4^+$ T cell numbers decline below a critical level, cell mediated immunity is lost and the body becomes progressively more susceptible to opportunistic infections (Cunningham *et al.,* 2010).

1.3.2 Treatment Regimes

Treatment for HIV ranges from the use of nucleoside reverse transcriptase inhibitors (NRTI's), highly active antiretroviral therapy or otherwise known as HAART, which introduced protease inhibitors and then the non-nucleoside reverse transcriptase inhibitors (NNRTI's) (Karim and Karim, 2010). HAART provides combinations consisting of at least three drugs belonging to at least two types of antiretroviral agents. Typically, these are two nucleoside analogue reverse transcriptase inhibitors

(NARTIs or NRTIs) and either a protease inhibitor or a non-nucleoside reverse transcriptase inhibitor (NNRTI) (NYU Medical Centre, 2009). The NRTI's, such as Zidovudine, Lamivudine and Emtricitabine, are analogues of the nucleosides required for DNA synthesis. They differ in that they lack the 3'-hydroxyl group of the ribose ring and when they become phosphorylated they can be incorporated into the growing DNA strand, however once this occurs, DNA synthesis halts due to the absence of the 3' hydroxyl group (Karim and Karim, 2010).

NNRTI's, such as Nevirapine and Efavirenz, do not undergo chemical modification in order to become active and the different members in this group have diverse chemical structures. They are relatively small and act by binding non-competitively to the active receptor site of the reverse transcriptase enzyme. This causes a change in the three dimensional structure of the enzyme and thus impairment of the polymerase activity (Karim and Karim, 2010).

Protease inhibitors (PI's), such as Ritonavir, Indinavir and Amprenavir, are also structurally diverse molecules which require no chemical modification in order to become active. They are metabolized in the liver and the gut by the P450 enzyme system, which results in multiple interactions with other hepatically metabolized drugs (Karim and Karim, 2010). These drugs target the HIV encoded protease enzyme, which is responsible for cleaving the HIV protein precursor transcript into its subunits prior to the virion assembly and the export of the new virus from the infected cell. They bind to the central, active cleavage site of the HIV protease heterodimer (Karim and Karim, 2010).

There is currently no available vaccine or cure for HIV or AIDS, however a number of trials have been conducted using two different vaccines in sequence or in combination, now being referred to as the prime boost strategy, including many trials combining a DNA plasmid with a viral vector, based on either poxvirus or adenovirus, combining DNA plasmid with protein, or combining a protein vaccine with a viral vector (Fast and Kaleebu, 2010). In general, the prime boost trials have shown a synergistic effect of the two components. RV144, the most recent efficacy trial in Thailand showed 30% reduced incidence of infection in the group that received a prime boost vaccine (Veronin and Phogat, 2010 and Fast and Kaleebu, 2010). A vaginal gel containing

tenofovir, a reverse transcriptase inhibitor, was shown to reduce HIV infection rates by 39% in a trial conducted in South Africa (Karim and Karim, 2010).

1.4 *IN VITRO* BIOASSAYS FOR EVALUATION OF PLANT EXTRACTS

1.4.1 Inhibition of *M. tuberculosis*

1.4.1.1 Microplate Alamar Blue Assay (MABA)

Microbroth dilution susceptibility tests in 96-well microplates offers the advantages of small sample requirements, low cost and high-throughput including the potential for automation (Pauli *et al.,* 2005). Microplate Alamar Blue assay (MABA), a colorimetric drug-susceptibility testing method uses an oxidation/reduction indicator dye, alamar blue that changes colour from blue to pink to indicate bacterial growth and can be read visually without the need for instrumentation (Collins and Franzblau, 1997). The reduced form of the dye can also be quantitated colorimetrically by measuring absorbance at 570 nm (and subtracting absorbance at 600 nm; the peak for the oxidised form), or fluorimetrically by exciting at 530 nm and detecting emission at 590 nm (Collins and Franzblau, 1997).

MABA has been used previously for drug-susceptibility testing of *M. tuberculosis* against antituberculosis drugs and has also been used for screening other antimicrobial agents against *M. avium* and *M. tuberculosis* (Collins and Franzblau, 1997; Franzblau *et al.,* 1998; Bastian *et al.,* 2001). Multiple drug, extract or compound concentrations can be tested using MABA (Gautam *et al.,* 2007; Ananthan *et al*., 2009; Lougheed *et al*., 2009; Green *et al.,* 2010).

1.4.1.2 p-Iodonitrotetrazolium chloride (INT) Assay

This assay is a microplate assay, which is very similar to that of MABA, however it determines the MIC of plant extracts using *p*-Iodonitrotetrazolium chloride (INT) dye. The tetrazolium dye (INT) acts as an electron acceptor and is reduced to a coloured product by biologically active organisms. Viable bacteria reduce the yellow dye to a purple/pink colour and the MIC is defined as the lowest sample concentration that prevents change in colour and exhibits complete inhibition of bacterial growth. When larger volume tube assays were used on plant extract testing, precipitation of insoluble components and the green colour of the extracts made it difficult, if not impossible to

determine the MIC which explains why it is not often used for screening plant extracts (Eloff, 1998).

This assay has been used for detection of antimicrobial activity using control bacteria such as *Bacillus subtilis, Staphylococcus aureus*, *Streptococcus* spp, *Escherichia coli* and *Pseudomonas aeruginosa* (Eloff, 1998; Smith and McFeters, 1997; Kuete *et al.,* 2008) but not for *M. tuberculosis*.

1.4.1.3 Flow Cytometry

Flow cytometry has been used for the detection of *M. tuberculosis* that are resistant or susceptible to antimicrobial agents (Kirk *et al*., 1998; DeCoster *et al*., 2005; Pina-Vaz *et al*., 2005; Fredricks *et al*., 2006). The main advantage of flow cytometry is the shorter time required to obtain a result (Fredricks *et al*., 2006; Kirk *et al*., 1998). Fluorescent dyes such as fluorescein diacetate (FDA) (Norden *et al.*, 1995; Kirk *et al*., 1998; Reis *et al*., 2004) or SYTO16 (Pina-Vaz *et al*., 2005; Govender *et al*., 2010) have been used for detection of resistance in *M. tuberculosis* isolates.

The method is based on the ability of mycobacteria to hydrolyse fluorescein diacetate to free fluorescein via non-specific cellular esterases. Accumulation of fluorescein in metabolically active mycobacterial cells can then be easily detected by using a flow cytometer. By contrast, mycobacteria that are killed or inhibited by antimycobacterial agents hydrolyse significantly less FDA and therefore have reduced levels of fluorescence (Kirk *et al*., 1998). Safety is a primary concern when working with *M. tuberculosis* and has been improved by procedures such as exposure to 10% formaldehyde for one hour that kills the mycobacteria prior to flow cytometry testing without compromising their staining characteristics (Norden *et al.*, 1995; Moore *et al.,* 1999).

Flow cytometry with the fluorescent nucleic acid stain SYTO16 can also be applied to screening of anti-tuberculosis agents against *M. tuberculosis* (Pina-Vaz *et al*., 2005). *M. tuberculosis* cells are grown in the absence or presence of antimycobacterial drugs and heat-killed, stained with SYTO16 and then analysed by flow cytometry. An isolate was considered sensitive whenever the number of fluorescent particles in the drug-containing medium was reduced in comparison to the untreated growth control (Pina-

Vaz *et al*., 2005). Although the use of heat killed cells stained with SYTO16 ensures biosafety, the small size of *M. tuberculosis* and their tendency to aggregate makes analysis fairly challenging (Fredricks *et al*., 2006; Akselband *et al*., 2005; Pina-Vaz *et al*., 2005; Kirk *et al*., 1998 and Ryan *et al*., 1995). However, a disadvantage associated with the use of this stain, is the relatively high cost (R6 000 for 250 µL), which could be problematic if large numbers of anti-tuberculosis drugs need to be screened (Govender *et al*., 2010).

1.4.2 Screening for Activity against HIV

1.4.2.1 Reverse transcriptase inhibition

Bioassays to determine anti-HIV activity involve screening for presence of HIV-specific enzyme inhibitors. The Reverse Transcriptase (RT) assay is a colorimetric enzyme immunoassay for the quantitative determination of retroviral reverse trancriptase activity by incorporation of digoxigenin and biotin labelled dUTP into DNA (www.cssportal.roche.com/LFR_PublicDocs/ras/11468120910_en_13.pdf).

The detection and quantification of the synthesized DNA as a parameter for RT activity follows a sandwich ELISA protocol, where the biotin labelled DNA binds to the surface of streptavidin coated microplate components. An antibody to dig-oxigenin, conjugated to peroxidase (anti-DIG-POD), is then added and binds to the dig-oxigenin labelled nucleotides. A peroxidase substrate is then added so that the peroxidase enzyme can catalyze the cleavage of the substrate to produce a coloured reaction product. The absorbance of the samples is determined using a microplate reader and is directly correlated to the level of RT activity in the sample (www.cssportal.roche.com/LFR_PublicDocs/ras/11468120910_en_13.pdf).

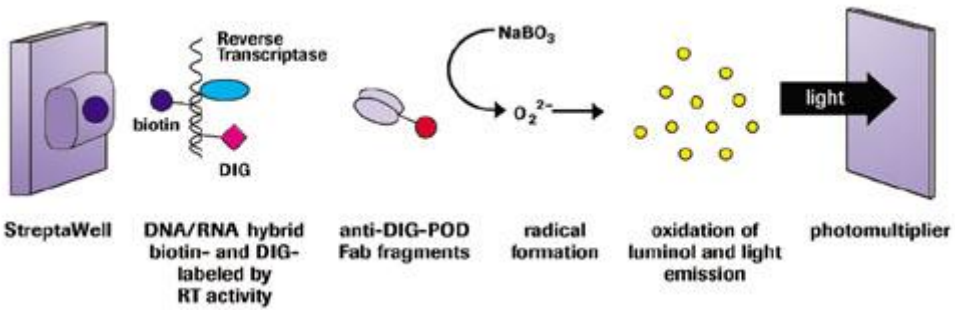

Figure 1.1: Principle of Reverse Transcriptase Assay (www.cssportal.roche.com/LFR_PublicDocs/ras/11468120910_en_13.pdf).

1.4.2.2 HIV-integrase inhibition

The HIV-1 integrase assay was developed for the screening of HIV-1 integrase inhibitors using a colorimetric enzyme immunoassay. This assay can be used to quantitatively measure the inhibition of HIV-1 strand transfer activity due to the presence of interacting agents (www.xpressbio.com/ebi/pdf/EZ-1700hiv_integrase_wildtype_kit%20v%203.0_pi_061411.pdf). Integrase catalyses two sequential, spatially distinct and metal-dependant reactions, namely 3'-end processing and strand transfer. Although inhibitors of both steps have been identified, only agents that inhibit the strand transfer reaction have been shown to be biologically active in cell based assays and *in vivo* (www.xpressbio.com/ebi/pdf/EZ-1700hiv_integrase_wildtype_kit%20v%203.0_pi_061411.pdf).

This assay functions by detecting the absence of strand transfer and thus if an inhibitor is not present, the HIV-1 integrase incorporates the target DNA into donor DNA and the products of the reaction can be determined through a colorimetric reaction. If a strand transfer inhibitor is present, this will reduce product formation and this inhibitory activity of the test agent can then be determined quantitatively (www.xpressbio.com/ebi/pdf/EZ-1700hiv_integrase_wildtype_kit%20v%203.0_pi_061411.pdf). Drugs which are inhibitory to integrase would therefore be valuable in antiviral therapy (Craigie, 2001).

1.4.2.3 HIV-1 protease inhibition

The HIV-1 protease assay was developed for the screening of HIV-1 protease inhibitors. This fluorescence resonance energy transfer (FRET) based assay could also be used for quantification of HIV-1 protease. The Human immunodeficiency virus type 1 (HIV-1) precursor poly-protein is generated during the virion maturation process. HIV-1 protease is responsible for cleaving up to 12 sites in the group specific antigen (Gag) and Gag-Pol precursor polypeptides (Klos *et al.,* 2009). The order of cleavage and the extent of precursor processing appear to be critical steps in the generation of fully infectious, appropriately assembled viral particles. Therefore, inhibition of HIV-1 protease represents an important aspect for antiviral therapy (Klos *et al.,* 2009).

Fluorescence resonance energy transfer (FRET) assays have become a popular and effective means for drug screening. The assay functions by utilizing a FRET peptide, where the sequence is derived from the native p17/p24 cleavage site on Pr-gag for HIV-1 protease. In the FRET peptide, the green fluorescence is quenched by an appropriate fluorescence quencher until this peptide is cleaved into two separate fragments by HIV-1 protease at the cleavage site. Upon cleavage, the green fluorescence is recovered and can be monitored at excitation/emission of 490 nm/530 nm (Roche, Germany).

Although the FRET assay has proven to be very effective, a novel method for HIV-1 protease detection was recently published by Esseghaier *et al.,* 2012. They designed a very simple and inexpensive sensing surface for impedimetric HIV-1 protease detection. The principle of the method is based on the utilization of a probe consisting of a specific HIV-1 protease substrate peptide which has a magnetic bead attached to its N-terminus. This probe is then attached onto a gold sensor surface at the C-terminus of the same peptide, resulting in a layer of magnetic beads close to the sensor surface. Upon cleavage of the probe peptide by the HIV-1 protease, the physical link between the magnetic beads and the sensor surface is broken. An externally applied magnetic field accelerates the dissociation of the magnetic beads from the sensor surface, resulting in a significant shift of the electrochemical signal due to the protease induced release of the magnetic beads from the sensor. This system showed very high specificity and sensitivity, capable of detecting HIV-1 protease at a concentration of 10 pg/mL in 25 min. The sensor was also capable of detecting the inhibitory activities of the anti-HIV drug Saquinavir mesylate with great accuracy, providing a novel approach to high throughput drug screening (Esseghaier *et al.,* 2012).

1.4.3 Cytotoxicity Assay (MTT Assay) and Synergistic effects

1.4.3.1 Cytotoxicity

The MTT assay is a colorimetric assay where the reduction of 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), which is a yellow colour, by succinate dehydrogenase is measured (Wilson, 2000; Cory *et al.,* 1991). MTT is reduced to an insoluble, dark purple, formazan product and this will only occur if the cells are viable. The total amount of formazan product is measured spectrophotometrically once the

formazan product has been dissolved in a suitable organic solvent such as isopropanol. MTT is reduced by metabolically active cells after exposure to a particular toxin or treatment (Wilson, 2000; Cory *et al.,* 1991). A main application allows one to assess the viability and the proliferation of cells. It can be used to determine cytotoxicity of potential medicinal agents and toxic materials, since those agents would either stimulate or inhibit cell viability and growth. When the amount of the purple formazan produced by cells that have been exposed to a treatment is compared with the amount of formazan produced by the untreated control cells, the effectiveness of that treatment in causing death or changing the metabolism of the cells can be deduced through the production of a dose response curve (Wilson, 2000; Cory *et al.,* 1991).

1.4.3.2 Synergistic effects

Treating bacterial infections with antibiotics is beneficial but their mis-use has led to an increase in resistance among microorganisms as well as to the re-emergence of old infectious diseases (Chanda and Rakholiya, 2011). One approach to treat infectious diseases is the use of plant extracts individually and, as an alternative approach, using them in combination with antibiotics. This latter approach is referred to as combination or synergistic therapy, and applying this new found approach against resistant microorganisms may lead to novel ways of treating infectious diseases and is found to be even more effective for patients with serious infections caused by drug resistant pathogens, such as TB and HIV (Chanda and Rakholiya, 2011). These synergistic effects may be due to certain complex formation which becomes more effective in the inhibition of a particular species of microorganisms either by inhibiting the cell wall synthesis or by causing its lysis or death (Chanda and Rakholiya, 2011).

1.5 PLANT MEDICINES FOR TREATMENT OF TB AND HIV

1.5.1 *Annona muricata L.*

Annona muricata L. (Annonaceae), commonly known as soursop, is found from Central America to South America, including the North, Northeast and Southeast regions of Brazil and Central Africa. It is a small, tropical evergreen tree, 5–6 m high, with large dark green leaves. It produces a large edible fruit which is yellow/green in colour. Traditionally, the leaves are used for headaches, insomnia, cystitis, liver

problems, diabetes, hypertension and as an anti-inflammatory, anti-spasmodic and anti-dysenteric (de Sousa *et al.,* 2010).

The extracts of the leaves have parasiticide, anti-rheumatic and anti-neuralgic effects when used internally, while the cooked leaves, applied topically, fight rheumatism and abscesses (de Sousa *et al.,* 2010). *A. muricata* has also been investigated for accessory substances present in the acetogenins, which is a unique class of secondary metabolites that have been isolated from different parts of the plant. Many of these acetogenins have been found in the leaves such as annomuricins A and B (de Sousa *et al.,* 2010; Aminimoghodamfarouj *et al.,* 2011). These acetogenins have cytotoxic properties against tumour cell lines, molluscicidal activity, anti-parasitic, insecticidal, immunosuppressive effects and anti-oxidant properties and is regarded as a likely source for the development of potential drugs (de souse *et al.,* 2010; Aminimoghodamfarouj *et al.,* 2011).

Annonacin is found in the fruits of *A. muricata*. Along with other acetogenins, annonacin is reported to block mitochondrial complex I (NADH dehydrogenase), which is responsible for the conversion of NADH–NAD^+ and the build up of a proton gradient over the mitochondrial inner membrane. This effectively disables a cell's ability to generate ATP via an oxidative pathway, ultimately forcing a cell into apoptosis or necrosis (Aminimoghodamfarouj *et al.,* 2011).

1.5.2 *Artemisia afra*

Artemisia afra (Asteraceae) is one of the most popular and commonly used herbal medicines in South Africa. *Artemisia afra* is a perennial woody shrub, which grows up to 2 m tall with a leafy, hairy and ridged stem and is very aromatic (Liu *et al.,* 2009). In the English language, it is referred to as African wormwood and is usually employed for treating various ailments such as coughs, colds, headaches, chills, dyspepsia, loss of appetite, gastric derangements, colic, croup, whooping cough, gout, asthma, malaria, diabetes, bladder and kidney disorders, influenza, convulsions, fever, heart inflammation, rheumatism and is also used as a purgative (Liu *et al.,* 2009 and van Wyk, 2008). These uses indicate that *A. afra* possesses antiviral, antibacterial and anti-inflammatory activities. Respiratory infections specifically, are treated through inhaling the vapour from boiling leaves.

It is widely distributed in southern Africa, such as South Africa, Namibia and Zimbabwe. In South Africa, it grows in the northern provinces of Gauteng and Limpopo along the eastern parts of South Africa, including Swaziland and Lesotho, to the Western Cape in the south (Liu *et al.,* 2009). *A. afra* is rich in terpenes and is therefore likely to have valuable biological activities. From past studies it can be noted that this plant has a broad spectrum of inhibitory activity against the organisms such as *Bacillus, Micrococcus, Streptococcus, Staphylococcus, Acinetobacter, Erwinia, Enterobacter, Escherichia coli, Proteus, Pseudomonas, Salmonella, Shigella and Yersinia* (Liu *et al.,* 2009).

1.5.3 Plants as sources of anti-tuberculosis and anti-HIV agents

Presently used drug regimens to combat TB infections comprise a combination of drugs administered over a period of several months, however patients frequently do not complete treatment when the symptoms begin to lessen and thus therapies that act faster may enhance patient compliance. In addition, lead structures, with novel or more effective mechanisms of action are required urgently to overcome the problems of drug resistance (McGaw *et al.,* 2008). Natural products continue to play a role in the drug discovery and development process and plants are becoming increasingly recognised as a useful source of highly active antimycobacterial metabolites (Pauli *et al.,* 2005; Gautam *et al.,* 2007). Many South African plants have ethnobotanical uses for the treatment of tuberculosis and its related symptoms such as coughing, respiratory ailments and fever.

The only treatment with proven efficacy for HIV is lifelong with the use of antiretroviral drugs (ARVs) (Palella *et al.,* 1998; Lamorde *et al.,* 2010). In South Africa it is evident that majority of the ARV treatment sites are located in towns and urban centres whereas access to treatment in rural areas remains incredibly low and thus access and continuation of treatment is difficult. Thus investigations on the biological activity of plant extracts against the crucial steps in the establishment of infection with regards to HIV Reverse Transcriptase and Integrase would widen the scope of the search for plant based anti-HIV molecules.

With the great diversity of plants in South Africa, screening of extracts of these plants for antimycobacterial and anti-HIV efficacy has much to offer in the search for novel

active metabolites that may be effective against *M. tuberculosis* and HIV (McGaw and Eloff, 2008; McGaw *et al.,* 2008). It is now even more important to further research *A. afra,* as it was recently found by Lubbe *et al.* (2012) to possess anti-HIV activity at the same level as that of its closely related, artemisinin producing relative, *Artemisia annua.* Lubbe *et al.* (2012) used *A. afra* as *a* tea infusion through a method using genetically modified HeLa cell lines, which were then infected with HIV. Therefore, further determining which enzymes are affected by the plant extract as well as which compounds are responsible for this activity would be highly useful in the search for novel anti-HIV therapies.

It has been documented that Oleanolic acid (OA), which is a triterpenoid compound that exists widely in the human diet, medicinal herbs and various other plants, has potent antimycobacterial properties against drug sensitive and drug resistant *M. tuberculosis* and that it has favourable synergistic activity with the first line drugs INH, RIF and EMB (Ge *et al.,* 2010). Recently, it has been shown that OA can be modified at the C-3 position to form cinnamate based esters which yields high antimycobacterial activity. This ability to modify OA may allow the creation of new compounds with more effective derivatives and less toxicity (Ge *et al.,* 2010). It might be beneficial to determine if *A. afra* and *A. muricata* possess OA.

1.6 POTENTIAL PLANT-DRUG INTERACTIONS

1.6.1 Interactions between plant medicines and drug metabolising pathways

Drug interactions are caused by four major mechanisms namely, altered drug absorption, altered renal elimination, additive effects or toxicities and altered hepatic metabolism of drugs (Patel *et al.,* 2011). Drugs are generally absorbed from the intestine and can be eliminated too quickly if herbal laxatives are taken. The most important pathway for drug metabolism is the family of liver enzymes known as the cytochrome P450 group, of which CYP3A4 is the most commonly known and is responsible for the metabolism of many drugs, including antiretrovirals (ARVs) (Zhou *et al*., 2007; Patel *et al.,* 2011). The activity of these enzymes may be induced or inhibited by the use of traditional medicines. The second most important drug interaction in HIV patients on ARVs is altered efflux mechanisms, such as P-glycoprotein, which is responsible for transporting a range of compounds, including protease inhibitors, out of the intestinal epithelial cells and back into the intestinal

lumen (Brown *et al.,* 2008; Balayssac *et al.,* 2005; Patel *et al.,* 2011). *Hypoxis hemerocallidea*, commonly used in Southern Africa to boost immunity in HIV patients, interacts with the efflux mechanism of nevirapine across intestinal epithelial cells, which can result in an increase in the bioavailability of this drug, thus an increase in drug toxicity, drug resistance and side effects (Mills *et al*., 2005; Brown *et al*., 2008; Patel *et al.,* 2011).

It is estimated that more than 80% of the Southern African population makes use of traditional medicines, often in combination with prescription drugs (Maduna, 2006). Components of medicinal plants can alter the absorption and metabolism of conventional drugs leading to the reduced efficacy of the specific drug or systemic drug toxicity (Meijerman *et al.,* 2006; Zhou *et al.,* 2007 and Willet *et al.,* 2004).

Traditional remedies are usually complex mixtures of different molecules that are able to interact with various drug metabolising pathways, such as the inhibition or transcriptional activation of drug metabolising enzymes (Pal and Mitra*,* 2006). A common drug metabolic pathway involves the oxidation of the parent molecule, referred to as phase I metabolism, which will then be followed by conjugation of the oxidised group with polar molecules such as glucose, sulphate or glutathione and this is referred to as phase II metabolism (Pal and Mitra*,* 2006).

The major enzymes involved in human phase I metabolism are the different isoforms of cytochrome P450, namely CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (Zhou *et al.,* 2007; Crespi and Stresser, 2000). The latter isoform is the most abundant CYP isozyme and is believed to metabolise between 50 and 70% of the currently marketed drugs including HIV protease inhibitors, non-nucleoside reverse transcriptase inhibitors, macrolide antibiotics and azole antifungals (Pal and Mitra, 2006). The key phase II enzymes include UDP-dependant glucuronosyl transferase, sulfotransferase and glutathione-S-transferase (Meijerman *et al.,* 2006; Triplitt, 2006; Mouly *et al.,* 2006).

Since HIV protease inhibitors, macrolide antibiotics and azole are recognised substrates or inducers of these drug metabolising proteins, it is clear that plant components can adversely affect the course of tuberculosis and HIV treatment (Zhou

et al., 2007; Venkataramanan *et al.,* 2006). If plant remedies are used in combination with conventional drugs, knowledge on the possible interactions that may occur will enable physicians to suggest safe drug regimens, dose adjustment or discontinuation of therapy if toxic drug-plant interactions could occur (Zhou *et al.,* 2007; Venkataramanan *et al.,* 2006).

Therefore methods employed for the *in vitro* antimycobacterial and anti-HIV screening of natural compounds/ products (plant medicines) are important for validating the traditional use of herbal treatments.

1.6.2 Assays to investigate drug metabolising pathways

CYP450 screening kits are designed to assess the metabolism and inhibition of the major human P450 isozymes involved in hepatic drug metabolism, namely CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4 and CYP3A5. The kit will employ CYP450 substrates and CYP450 baculosomes reagents. The CYP450 baculosomes reagents are microsomes prepared from insect cells expressing a human P450 isozyme and rabbit NADPH-P450 reductase (www.tools.invitrogen.com/downloads/O-13873-r1_US_0405.pdf).

CYP450 baculosomes reagents offer a distinct advantage over human liver microsomes in that only one CYP450 enzyme is expressed, thereby preventing metabolism by other CYP450s. The substrates are metabolized by a specific CYP450 enzyme into products that are highly fluorescent in aqueous solutions. The fluorescent metabolites are excited in the visible light spectrum, which minimizes interference caused by the background fluorescence of UV excitable compounds and NADPH. Due to the functioning of the kit and the specific substrates that are used, even weak CYP450 inhibitors will be determined (www.tools.invitrogen.com/downloads/O-13873-r1_US_0405.pdf).

There are multiple drugs that can be utilized to help reduce the symptoms of HIV/AIDS. Such drugs include tenofovir, emtracitbine, efavirenz, nevirapine, zidovudine, lamivudine, lopinavir and ritonavir for the treatment of HIV/AIDS. Drugs utilized for TB include ofloxacin, streptomycin, ethambutol, rifampicin and isoniazid, all

of which are utilized in primary healthcare in South Africa. Of these drugs only a few have an effect on the CYP450 isozymes. These drugs include efavirenz, which is metabolized mostly by CYP3A4 and CYP2B6 but also inhibits CYP2C9 and CYP2C19; nevirapine, which is oxidatively metabolised by CYP3A4 and CYP2B6; lopinavir, which also undergoes oxidative metabolism by the CYP3A4 isozyme; ritonavir, which is metabolized by CYP3A4 and CYP2D6, however this may not be a good choice of drug as it both induces and inhibits the expression of CYP3A4; isoniazid, which inhibits CYP1A2, CYP2C9, CYP2C19 and CYP3A4 and rifampicin, which induces the isozyme CYP2C8 (Levien *et al.,* 2003).

Glutathione-S-transferases are thought to play a role in initiating the detoxification of potential alkylating agents including pharmacologically active compounds. These enzymes catalyze the reaction of such compounds with the -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water soluble (Habig *et al.,* 1974). Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid. The mercapturic acids are then excreted (Habig *et al.,* 1974). A widely used method for determining GST activity in living cells is by utilizing 1-Chloro-2,4-dinitrobenzene (CDNB) which is suitable for the broadest range of GST isozymes. Addition of CDNB to the cell culture medium and conjugation of the thiol group of glutathione to the CDNB substrate, results in an increase in the absorbance at 340 nm (www.sigmaaldrich.com/etc/medialib/docs/Sigma/Bulletin/cs0410bul.Par.0001.File.tmp/cs0410bul.pdf).

1.7 SCOPE AND OBJECTIVES

Current drug regimens to combat TB and HIV infections comprise multiple drugs administered over several months, hence patients frequently do not complete the treatment. New antimycobacterial and anti-HIV therapies that act faster may enhance patient compliance.

Many plant species are used in traditional South African medicine to alleviate symptoms of TB and HIV and several lead compounds have been reported for further research following *in vitro* antimycobacterial and antiretroviral activity evaluation (Klos *et al.,* 2009). *A. muricata* and *A. afra* are two plants which were investigated for inhibitory activity against *M. tuberculosis* and HIV. *A. afra* is one of the most popular and commonly used herbal medicines in southern Africa to treat diseases such as herpes simplex virus, asthma and several others. Despite its popularity, *A. afra* has been poorly researched and thus has limited ethnopharmalogical support (Liu *et al*., 2009 and Lubbe *et al.,* 2012).

There are few reports on *A. muricata,* which indicate that all parts of the tree have been used to treat multiple disorders (Boyom *et al.,* 2011). The leaves are especially used as an antispasmodic, sedative, also for coughs, grippe, asthma, catarrh and asthenia (Asprey and Thornton, 1955). As both plants have been shown to be used traditionally against chest infections, respiratory ailments and other viruses, it was relevant to determine if any activity was present against the two major diseases in South Africa namely TB and HIV.

1.7.1 Hypotheses to be tested

It was hypothesised that *A. muricata* and *A. afra* may have an inhibitory effect on *M. tuberculosis* and the Human Immunodeficiency Virus.

It was further hypothesized that possible drug interactions may occur with plant extracts and drugs used for treatment of TB and HIV which may interfere with drug metabolizing pathways involving the CYP3A4 isozyme and Glutathione-S-Transferases.

1.7.2 Objectives

The following objectives were established to test the above hypothesis:

- To screen two plant extracts (*A. muricata* and *A. afra*) for *in vitro* inhibitory activities against *M. tuberculosis* and Human Immunodeficiency Virus enzymes
- To determine cytotoxicity and possible synergistic effects of plant extracts, and
- To investigate drug interactions with plant extracts and drugs used for *M. tuberculosis* and HIV treatment.

CHAPTER TWO

MATERIALS AND METHODS

2.1. BACTERIAL STRAINS, GROWTH CONDITIONS AND MEDIA

M. tuberculosis H37Rv ATCC 27294 was used as a control culture for all assays as it has a drug susceptibility profile fairly representative of most drug susceptible clinical isolates. *M. tuberculosis* was grown in Middlebrooks 7H9 broth [88.95 mL ddH_2O; 0.470 g BD Difco™ Middlebrooks 7H9 broth base; 0.8 mL glycerol (Merck); 0.005 mL Tween® 80 (Sigma-Aldrich), 1 mL Middlebrook Albumin-dextrose-catalase (ADC) growth supplement (Sigma-Aldrich) added after autoclaving]. Cultures were incubated at 37^{o}C for 10 days after which the turbidity of the culture was assessed and adjusted to a McFarland 0.5 standard (1.5 x 10^8 cells/mL) [1.175% $BaCl_2$ (0.1175 g in 10 mL ddH_2O) and 1% H_2SO_4 (0.5 mL H_2SO_4 in 50 mL ddH_2O)].

2.2 CONTROL DRUGS: *M. TUBERCULOSIS*

The following antibiotics (Sigma-Aldrich) that are currently used for treatment of *M. tuberculosis* infections were tested and used as positive controls for optimization of all bioassays: ofloxacin (OFX), isoniazid (INH), rifampicin (RIF), streptomycin (ST) and ethambutol (EMB). All drugs were solubilized according to the manufacturer's instructions either in dimethyl sulfoxide (DMSO) (Merck), distilled water or 0.1 N NaOH (1 g NaOH in 250 mL ddH_2O) and stock solutions (1 mg/mL) were filter sterilized (0.2 µm filter) and stored in 1 mL aliquots at -20^{o}C until required.

2.3 PREPARATION OF PLANT EXTRACTS

Annona muricata L. was air dried for two days followed by drying in a hot air oven at 40°C, then ground into powder and stored. One hundred grams of powdered plant material was macerated with 80% ethanol at room temperature. The extract was then filtered and concentrated to dryness *in vacuo* at room temperature (Sowemimo *et al.,* 2009).

Fresh leaves of the *Artemisia afra* plant were blended and separated into two batches. The one batch was submerged in ddH_2O and the second batch submerged in 80%

absolute ethanol. These were then placed in the dark at room temperature overnight. After incubation, the two batches were filtered using a 0.1 µm Whatman filter paper, in order to obtain a sediment free liquid filtrate. The aqueous batch was collected in a 50 mL Falcon tube whereas the ethanol batch was placed in a 50 mL flask and placed over a water bath at 35°C where nitrogen gas was used to evaporate any excess ethanol. The remainder of the extract was then re-constituted in water so as to obtain an ethanol concentration of less than 5%. This was then placed into a 30 mL Falcon tube and frozen at -80°C overnight. These samples were then placed in freeze drying flasks and freeze dried for three days.

2.4 MICROPLATE ALAMAR BLUE ASSAY (MABA)

This assay was performed as described by Franzblau *et al.* (1998); Lougheed *et al.* (2009) with modifications. Clear 96 well plates (Corning, Scientific Group) were used initially, and for fluorimeter readings, contents were transferred to black plates. Outer perimeter wells were filled with sterile water to minimize evaporation of the medium in experimental wells during incubation. The wells in rows B to G in columns 3 to 11 received 100 µL of Middlebrooks 7H9 broth (prepared as described in 2.1, but without Tween® 80). One hundred microlitres of drug to be tested was added to wells in rows B to G in columns 2 and 3. This was followed by the transfer of 100 µL from column 3 to column 4 and the contents of the wells mixed well. Identical serial 1:2 dilutions were continued through to column 10 and 100 µL of excess medium was discarded from the wells in column 10. Final drug concentration ranges were as follows: OFX and RIF, 0.0625 to 8.0 µg/mL; INH, 0.125 to 16.0 µg/mL; EMB and SM, 0.25 to 32 µg/mL. *M. tuberculosis* inoculum (100 µL of $1x10^8$ cells/well) was added to the wells in rows B to G in columns 3 to 11. Wells in column 2 served as a drug only control to detect auto-fluorescence of compounds and to determine whether the compounds have any effect on the alamar blue dye. Wells in column 11 served as a bacterial growth control. Plates were sealed with clear microplate sealing tape (Scientific Group) and incubated at 37°C for 7 days. On day 7, 20 µL of alamar blue reagent (CellTiter-Blue, Promega) and 12.5 µL 20% Tween® 80 was added to each well. Plates were observed after 6 h and incubated for a further 18 h as the colour change was not sufficient. A blue colour in the well was interpreted as no growth and a pink colour was scored as growth. The MIC was defined as the lowest drug concentration which prevents a colour change from blue to pink.

Due to the biosafety hazard of *M. tuberculosis* aerosols, the *M. tuberculosis* cells were killed using 10% formaldehyde after addition of alamar blue dye reagent. The fluorescence (relative fluorescence units, RFU) was then measured using a plate reader at 535 nm excitation and 590nm emission (Reis *et al.,* 2004).

Percent inhibition was defined as:
Percent inhibition = 1 – (test well RFU/ mean RFU triplicate bacteria only well) x 100
MIC was taken to be the lowest concentration of a drug capable of causing ≥90% inhibition compared to the untreated bacteria only controls (Collins and Franzblau, 1997).

2.5 *P*-IODONITROTETRAZOLIUM CHLORIDE (INT) ASSAY

This assay was carried out as described by Eloff, (1998) using *p*-Iodonitrotetrazolium chloride (INT) dye. One hundred microliters of Middlebrooks 7H9 broth [supplemented with 10% albumin dextrose catalase (ADC), 1000 µg/mL of sodium nitrate ($NaNO_3$) and 0.05% Tween® 80] were added to wells of a sterile 96-well plate from columns 3 to 11. Drug concentration ranges were: RIF (0.01563 - 2.0 µg/mL); OFX (0.03125 - 4.0 µg/mL); INH (0.003125 - 0.4 µg/mL); EMB (0.1172 - 15 µg/mL) and SM (0.01563 - 2.0 µg/mL). Drug (100 µL) was added to wells in columns 2 and 3. This was followed by transfer of 100 µL from column 3 to column 4 and serially diluted (2-fold dilutions) up to column 10. Columns 11 and 12 contained 100 and 200 µL of medium without drugs, respectively and served as the growth and medium controls respectively. The bacterial suspension (100 µL) (1.5×10^8 cells/ well) was added to wells in columns 3 to 11 to give a final volume of 200 µL per well. All plates were then sealed with microplate sealing tape and incubated at 37°C for 7 days after which 40 µL of 0.2 mg/mL INT dye (Sigma) was added to each well and left to incubate for 30-60 minutes after which if no colour change was observed, incubated for a further 23 hours. Viable bacteria reduce the yellow dye to a purple/pink colour and no colour change indicated inhibition of bacterial growth.

2.6 FLOW CYTOMETRY

Flow cytometry analysis was conducted using fluorescein diacetate (FDA) according to the methods of Norden *et al*. (1995); Reis *et al*. (2004); Kirk *et al*. (1998) with modifications. *M. tuberculosis* culture [500 µL - equivalent to a McFarland 0.5 standard (1.5×10^8 bacteria)] was inoculated into freshly prepared Middlebrook 7H9 broth containing the following antibiotics and their concentration ranges respectively: OFX and RIF, 0.0625 to 8.0 µg/mL; INH, 0.125 to 16.0 µg/mL; EMB and SM, 0.25 to 32 µg/mL. Drug-free growth controls and all tubes with antibiotics and broth cultures were incubated at 37°C for 72 hours. After incubation, 500 µL of each assay suspension was incubated for 1 h at room temperature with 100 µL of a 10% solution of formaldehyde to inactivate the *M. tuberculosis* cells. Formaldehyde inactivated *M. tuberculosis* cells (500 µL) were then centrifuged for 60 sec at 12000x*g* to pellet the cells. The supernatant was removed and the pellet re-suspended in 250 µL Isoflow™ EPICS™ Sheath fluid (Beckman Coulter). Prior to analysis, the cell suspension was vortexed, passed through a Neomedic 25 gauge micro-emulsifying needle (Separations), and vortexed again to ensure complete separation of the cells, and to avoid clumping. The cells were re-suspended by repeated pipetting and transferred to a cell flow cytometry tube. This re-suspension step was repeated. The re-suspended mixture (200 µL) was then incubated with 200 µL FDA (freshly prepared) at 500 ng/mL in phosphate buffered saline at pH 7.4. The samples were then incubated at 37°C for 30 min before being analysed with a flow cytometer (Beckman Coulter FC500). Samples were analysed by histogram profiles of FDA fluorescence and 2 parameters were evaluated: events per minute (number of labelled mycobacteria) and mean channel fluorescence (intensity of fluorescence-labelled mycobacteria).

2.7 DETECTION OF ANTI-HIV REVERSE TRANSCRIPTASE ACTIVITY USING A NON-RADIOACTIVE ELISA KIT

The potential of plant extracts to inhibit reverse transcriptase was determined as outlined by Klos *et al*. (2009), with modifications, using a non-radioactive HIV-Reverse Transcriptase (RT) colorimetric ELISA kit from Roche Diagnostics. For the assay, reverse transcriptase was reconstituted in autoclaved re-distilled water to give a final concentration of 2 ng/µL stock. In a separate reaction tube, 1 µL of this stock solution was diluted with 19 µL of lysis buffer. Twenty microlitres of the extract samples and positive control, Nevirapine (Aspen), diluted to the required concentrations using lysis

buffer was then added to the respective tubes. The concentration range was the same for all extracts and for Nevirapine: 0.2 to 4 mg/mL. Twenty microlitres of prepared reaction mixture was then added to each tube. This was incubated for 1 h at 37°C. Controls included a background control containing lysis buffer alone, a negative control for inhibition which included HIV-1 RT with only lysis buffer and reaction mixture with no inhibitors, a solvent control which contained HIV-1 RT with only solvent (4% DMSO) in lysis buffer. After incubation, the total volume (60 µL) was transferred from each reaction tube to a well of a streptavidin coated module, covered with a cover slip and incubated for 1 h at 37°C. After incubation, the solution was removed from the wells and rinsed 5 times with 250 µL of washing buffer for 30 seconds each. Two hundred microlitres of freshly prepared anti-DIG-POD was then added to each well. The plate was covered with a cover slip and incubated for 1 h at 37°C. After incubation, the solution was removed and the rinsing step was repeated. Two hundred microlitres of ABTS (2, 2'-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) substrate solution was then added to each well and incubated at room temperature for 10-20 minutes. The absorbance was measured at 405 nm using a microplate reader. The HIV-RT inhibition by the plant extracts was measured as a percentage of the inhibition that occurred with HIV-1 RT in the absence of an inhibitor in the same solvent (4% DMSO) as the extracts.

2.8 DETECTION OF ANTI-HIV-1 INTEGRASE ACTIVITY

The potential of plant extracts to inhibit HIV-1 integrase was measured according to the manufacturer's instructions using a non-radioactive HIV-1 integrase colorimetric kit supplied by XpressBio. For the assay, 100 µL of donor substrate (DS) DNA was added to each well and subsequently incubated for 30 min at 37°C. The liquid was removed from the plates and washed 5 times with 300 µL of 1x wash buffer. Two hundred microlitres of blocking buffer was added to each well and incubated for 30 min at 37°C. The liquid was then aspirated from the wells and subsequently washed 3 times with 200 µL of reaction buffer. The integrase enzyme was diluted 1:300 with reaction buffer before use, after which 100 µL of the diluted integrase was added to each well and incubated for 30 min at 37°C. The liquid was then aspirated from the wells and washed 3 times with 200 µL of reaction buffer. Fifty microlitres of the extract samples and positive control, sodium azide, at 2x the required concentrations using reaction buffer, were then added to the respective wells and incubated for 5 min at

room temperature. The concentration range was the same for all extracts: 0.025-4 mg/mL. Controls included a background control, which contained reaction buffer alone, while the negative control for inhibition included HIV-1 integrase with only reaction buffer with no inhibitors, a solvent control which contained HIV-1 integrase with only solvent (4% DMSO) in reaction buffer. After incubation, 50 µL of target substrate (TS) DNA was added to each well and gently mixed. The plate was then incubated for 30 min at 37°C. The liquid was removed and the wells washed 5 times with 300 µL of wash buffer. One hundred microlitres of horseradish peroxidase (HRP) antibody solution was added to each well and incubated for 30 min at 37°C. The liquid was then removed and washed 5 times with 300 µL of wash buffer after which 100 µL of 3,3', 5,5'-tetramethylbenzidine (TMB) peroxidase substrate solution was added to each well and incubated for 10 min at room temperature. One hundred microlitres of TMB stop solution was then added to each well, which caused a colour change from blue to yellow at various intensities. The absorbance was measured at 450 nm using a microplate reader. The HIV integrase inhibition by the plant extracts was measured as a percentage of the inhibition that occurred with HIV-1 integrase in the absence of an inhibitor in the same solvent (4% DMSO) as the extracts.

2.9 CYTOTOXICITY OF PLANT EXTRACTS

2.9.1 Cell culture conditions and Cytotoxicity testing

The adherent liver cell line, Chang Liver cells, and the adherent hepatocellular cell line, HepG2 were used for the experimental procedures. The cells were routinely maintained in 10 cm culture dishes without antibiotics in Eagle's Minimal Essential Medium (EMEM) supplemented with non-essential amino acids (NEAA) and 10% foetal bovine serum (FBS) and incubated in a humidified 5% CO_2 incubator at 37ºC.

Cytotoxicity was determined using the MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay. A 10 cm culture dish containing either Chang liver or HepG2 cells was removed from the incubator and the monolayer subsequently trypsinised. Cells were suspended in EMEM supplemented with 10% FBS and NEAA to give 150,000 cells/mL (30 000 cells/well) and then seeded into a 96 well plate, (200 µL suspension per well) and incubated overnight to allow the cells to attach. After incubation, the spent medium was removed and 200 µL of fresh EMEM supplemented

with 10% FBS and NEAA containing the plant extracts at concentrations ranging between 3.91 and 250 µg/mL was then added to the wells in triplicate. Both cell lines were incubated at 37°C in a humidified 5% CO_2 incubator for 24 and 48 hours. After the incubation, the medium was aspirated and replaced with 200 µL of fresh EMEM supplemented with 10% FBS and NEAA containing 0.5 mg/mL MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] and subsequently incubated for 3 hours. The medium was then removed and the MTT crystals solubilised by addition of 200 µL dimethyl sulfoxide (DMSO). The absorbance was then read at 540 nm using a BioTek® PowerWave XS spectrophotometer (Winooski, VT, USA).

2.9.2 Potential for Synergistic hepatotoxicity between Plant Extracts and Rifampicin

The adherent liver cell line, Chang liver, and the adherent hepatocellular cell line, HepG2, were used to investigate potential synergism. Cells were routinely maintained in 10 cm culture dishes, in Eagle's Minimal Essential Medium (EMEM), without antibiotics, supplemented with non-essential amino acids (NEAA) and 10% foetal bovine serum (FBS) and incubated in a humidified 5% CO_2 incubator at 37ºC.

Synergistic testing was conducted by calculating the combination index (CI) according to the method outlined by Chou (2010). A 10 cm culture dish containing either Chang liver cells or HepG2 cells was removed from the incubator and the monolayer subsequently trypsinised. An appropriate volume of cells was suspended in EMEM supplemented with 10% FBS and NEAA to give 150,000 cells/mL (30 000 cells/well) and these were then seeded into a 96 well plate, using 200 µL suspension per well and incubated overnight to allow the cells to attach.

After incubation, the spent medium was removed and 200 µL of fresh EMEM supplemented with 10% FBS and NEAA containing the combined plant extract and antibiotics at a fixed ratio, which was determined empirically using the following equation:

$$\mathrm{IC}_{50}(\mathrm{a}+\mathrm{b}) = 10^{\frac{\log \mathrm{IC}_{50}(\mathrm{a}) + \log \mathrm{IC}_{50}(\mathrm{b})}{2}}$$

Where (a) represents the plant extract and (b) represents the antibiotic, in this case rifampicin.

The above equation calculates the total concentration that was present in each well. This concentration was then divided by the fixed ratio, previously determined based on the respective IC_{50} values of the extracts and the antibiotic obtained from cytotoxicity tests, to determine the concentration of each component individually. Serial dilutions were made to obtain the appropriate concentration range. The plates were incubated for 24 hours and IC_{50} of the new combination was determined. The combination index was calculated using the following equation:

$$\mathrm{CI} = \frac{\mathrm{C_a}}{\mathrm{IC_{50}(a)}} + \frac{\mathrm{C_b}}{\mathrm{IC_{50}(b)}}$$

Where C_a is the IC_{50} of the combination mixture plotted on an axis using the respective antibiotic concentrations, C_b is the IC_{50} of the combination mixture plotted on an axis using the respective plant extract concentrations. A combination index greater than 1, indicated a potential antagonistic relationship, and a value less than 1 indicated a synergistic relationship. A value of 1 implies that the two components produced a purely additive effect.

In order to obtain a full spectrum of whether synergistic effects were present at all concentrations of the extracts and the antibiotic, the above experiments were repeated using an IC_{25} and IC_{70}.

2.10 INTERACTIONS OF PLANT EXTRACTS WITH DRUG METABOLIC PATHWAYS

2.10.1 CYP3A4 Inhibition Assay

The CYP3A4 inhibition assay was conducted according to the manufacturer's instructions (Life Technologies). The CYP3A4 baculosomes, regeneration system and $NADP^+$ was thawed at room temperature and kept on ice until ready to use. The CYP3A4 baculosomes and regeneration system was mixed gently after thawing. The assay conditions for screening was performed as indicated in Table 2.1.

Table 2.1: Assay Conditions (Life Technologies, USA).

Condition	Purpose	Dispensing
Test Compound	Screen for inhibition by compound of interest	40 µL 2.5X test compound 50 µL Master Pre-Mix 10 µL Substrate and $NADP^+$
Positive Inhibition Control	Inhibit the reaction with a known P450 inhibitor - Ketoconazole	40 µL 2.5X positive inhibition control 50 µL Master Pre-Mix 10 µL Substrate and $NADP^+$
Solvent Control (No Inhibitor)	Accounts for possible solvent inhibition caused by introduction of test compounds originally dissolved in an organic solvent such as DMSO	40 µL 2.5X solvent control 50 µL Master Pre-Mix 10 µL Substrate and $NADP^+$
Background	Enables subtraction of background fluorescence during data analysis	40 µL 2.5X solvent control 50 µL CYP450 Reaction Buffer 10 µL Substrate and NADP+

The substrate was reconstituted using anhydrous acetonitrile according to Table 2.2. The fluorescent standard was reconstituted using DMSO. These solutions, once prepared, were then stored at room temperature for immediate use or at -20°C for long term use.

Table 2.2: Reconstitution of the CYP3A4 substrates (Life Technologies, USA).

Isozyme Type	CYP450 Substrate	mg/ tube	µmol/ tube	µL acetonitrile added per tube	[stock solution] (mM)	[screening conc.] (µM)
3A4	BOMR (benzyloxymethylresorufin)	0.1	0.30	150	2	3

The 2X CYP3A4 reaction buffer was diluted with nanopure water to a desired concentration of 100 mM. This was used for the preparation of standards, inhibitors,

master pre-mix and substrate/$NADP^+$ solutions. The diluted reaction buffer was stored at room temperature.

In order to determine the IC_{50}, a 2.5X dilution of the test compounds was prepared as well as a 2.5X solution of a known CYP3A4 inhibitor, ketoconazole, which acted as the positive control. The solvent, which was used to dissolve the test compounds, and ketoconazole, was prepared at 2.5X the final concentration. Forty microliters of the 2.5X solutions prepared above was then added to desired wells of the 96 well plate and this was conducted in triplicate.

The master pre-mix was then prepared by diluting the CYP3A4 baculosomes reagent and regeneration system in 1X CYP3A4 Reaction Buffer according to the values seen in Table 2.3. The solutions were kept at room temperature for immediate use. After preparation of the master pre-mix, 50 µL was added to each well and mixed.

Table 2.3: Master pre-mix preparation for the CYP3A4 isozyme (Life Technologies, USA).

Isozyme Type	CYP450 Substrate	µL of CYP450 Reaction Buffer (2X) added	µL of Regeneration System (100X) added	µL of CYP450 Baculosomes added	Conc. Of CYP450 in Master pre-mix (2X), nM	Screening conc of CYP450, nM
3A4	BOMR	4850	100	50	10	5

The plates were then incubated for 10 minutes at room temperature to allow the compounds to interact with the CYP3A4 in the absence of enzyme turnover. During this pre-incubation, the pre-mixture of 10X substrate and $NADP^+$ was prepared according to Table 2.4.

Table 2.4: Preparation of the substrate and $NADP^+$ (Life Technologies, USA).

Isozyme Type	CYP450 Substrate	µL of CYP450 Reaction Buffer (1X) added	µL of Reconstituted Substrate added	µL of $NADP^+$ (100X) added
3A4	BOMR	885	15	100

The reaction was then started by adding 10 µL per well of the 10X substrate and $NADP^+$ mixture prepared above and the entire solution mixed. The plate was transferred into the fluorescent plate reader and the fluorescence monitored over time at excitation and emission wavelengths listed in Table 2.5. Readings were taken at 1 minute intervals over a period of 60 minutes.

Table 2.5: Excitation and emission wavelengths for the various fluorescent standards (Life Technologies, USA). The red fluorescent standard was used for this specific experiment.

		Fluorescent Standard							
		Red		Blue		Green		Cyan	
Fluorescence Plate Reader	Excitation/ Emission	Center (nm)	Band width	Center (nm)	Band width	Center (nm)	Band width	Center (nm)	Band width
With Filters	Excitation	535	25	415	20	485	20	415	20
	Emission	590	20	460	20	520	25	420	45

Red Standard was sodium salt or resorufin. Blue Standard was 3-cyano-7-hydroxycoumarin. Cyan Standard was 7-hydroxy-4-trifluoromethylcoumarin. Green Standard was fluorescein.

The reaction rates were then obtained by calculating the change in fluorescence per unit time and the percent inhibition due to presence of test compound or positive inhibition control were calculated using the equation below:

$$\%\, Inhibition = \left(\frac{(\mathrm{A} - \mathrm{X})}{\mathrm{A}}\right) \mathrm{x}\, 100$$

Where X is the rate observed in the presence of the test compound, A is the rate observed in the absence of inhibitor and extract.

2.10.2 Glutathione-S-Transferase Inhibition Assay

Glutathione-S-Transferase (GST) inhibition by the plant extracts was conducted using the Glutathione-S-Transferase assay kit, with modifications, supplied by Sigma. 1-

Chloro-2,4-dinitrobenzene (CDNB) was used as the substrate as it is suitable for a wider range of GST enzymes. Human placental GST (Sigma) was purchased separately to be used in the assay in order to determine inhibition or stimulation, as opposed to GST production. A substrate master mix was prepared according to the amount of assays that needed to be conducted. Generally, a 10 mL master mix was sufficient for 50 assays and contained 9.8 mL of Dulbecco's Phosphate Bufferred Saline (DPBS), 0.1 mL of 200 mM reduced L-Glutathione and 0.1 mL of 100 mM CDNB. Initial experiments were conducted to determine optimal GST activity and optimal extract concentrations that will not be too dark to be measured at an absorbance of 340 nm. The optimal amount of GST was found to be 55 units/mL. The optimal extract concentrations that gave reasonable readings were 0.0625 and 0.03125 mg/mL. One hundred and eighty six microlitres of the substrate master mix was added into the required wells of a 96 well microplate. Ten microlitres of the plant extracts at the required concentrations was then added. Lastly, 4 µL of GST at 55 units/mL was added and placed immediately in the plate reader and the absorbance was measured at 340 nm every minute for 30 minutes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in absorbance. Controls included a negative control, which contained only substrate master mix, a positive GST control, which contained GST with no inhibitors and then a positive control for GST inhibition, which was ethacrynic acid (2 µg/mL) as it is a known inhibitor of GST.

2.11 STATISTICAL ANALYSIS

Significance determinations were obtained by applying Tukey's HSD test. All results with $P < 0.05$ were considered significant. IC_{50} values were calculated using GraphPad Prism Version 5.0.

CHAPTER THREE

INHIBITORY ACTIVITIES AGAINST *Mycobacterium tuberculosis* AND HUMAN IMMUNODEFICIENCY VIRUS

3.1 INTRODUCTION

Tuberculosis (TB) is a contagious disease caused by *Mycobacterium tuberculosis*, which most commonly affects the lungs and is transmitted from person to person via droplets from the throat and lungs of people with the active respiratory disease (Tekwu *et al.,* 2012). In 2011, TB remained the second cause of death from infectious disease worldwide. In 2010, according to the World Health Organization (WHO), TB incidence and prevalence were estimated at 8.8 and 12 million cases respectively. A total of 1.1 million among HIV-negative people and 0.35 million among HIV-positive people died from TB in the same year (Villemagne *et al.,* 2012, WHO, 2011). The association with the HIV epidemic, the increasing emergence of multi-drug resistant TB (MDR-TB) and extensively drug resistant TB (XDR-TB) have worsened the situation and posed a serious health threat (Rattan *et al.,* 1998; Tekwu *et al.,* 2012). TB is treatable but curing multi-drug resistant TB (MDR-TB) is very difficult and often requires very long courses of toxic drugs, thereby, raising serious problems of compliance (WHO, 2009).

There is an urgent need to search for alternative antituberculosis drugs. Medicinal plants have become the focus of intense study recently in terms of conservation and as to whether their traditional uses are supported by actual pharmacological effects or merely based on folklore (Mohamad *et al.,* 2011; Rabe and van Staden, 1997). The medicinal plant *Annona muricata* has been reported to be effective when applied topically as well as internally, for insomnia, diabetes, abscesses, headaches (de Sousa *et al.,* 2010) while *Artemisia afra* has been employed in treating ailments ranging from coughs to kidney disorders (Liu *et al.,* 2009).

Bioassays for detecting activity against *M. tuberculosis* include: Microplate Alamar Blue assay (MABA), ρ-iodonitrotetrazolium chloride (INT) assay and flow cytomtery. MABA is a colorimetric method using an oxidation/reduction indicator dye, alamar blue

that changes colour from blue to pink to indicate bacterial growth and can be read visually without the need for instrumentation (Collins and Franzblau, 1997). The ρ-iodonitrotetrazolium chloride (INT) assay is similar to MABA, however it uses *p*-Iodonitrotetrazolium chloride (INT) dye which acts as an electron acceptor and is reduced to a purple/pink colour by biologically active organisms.

The flow cytometry method employing fluorescein diacetate (FDA) is based on the ability of mycobacteria to hydrolyse fluorescein diacetate (FDA) to free fluorescein via non-specific cellular esterases. Accumulation of fluorescein in metabolically active mycobacterial cells can then be easily detected by using a flow cytometer. By contrast, mycobacteria that are killed or inhibited by anti-mycobacterial agents hydrolyse significantly less FDA and therefore have reduced levels of fluorescence (Kirk *et al*., 1998).

Reports at the end of 2010 indicated that there was an estimated 34 million people currently living with HIV globally, 2.7 million new infections and 1.8 million deaths due to AIDS related illnesses worldwide (UNAIDS, 2011). South Africa remains the most severely affected country with the HIV/AIDS epidemic having almost half of the total AIDS related deaths in 2010 as well as an estimated 5.6 million people living with HIV, more than any other country in the world (UNAIDS, 2011).

As a result, an appreciable amount of research efforts have been devoted to the discovery of improved anti-retroviral agents especially through the screening of natural products. Only 25 drugs for the treatment of AIDS have been approved to date, of which none are a natural product (Sabde *et al.,* 2011). Due to their relatively low cost, plants have been increasingly explored for production of medicinal compounds and vaccines. Many plant derived substances including phenyl coumarins and plant proteins have shown good anti-HIV activity (Tshikalange *et al.,* 2008).

Some of the main targets for HIV treatment include specific enzymes which are vitally important in the HIV life cycle such as reverse transcriptase, integrase and protease enzymes. HIV-1 reverse transcriptase is a multifunctional enzyme which catalyzes the synthesis of proviral DNA, using viral RNA as a template (Menéndez-Arias, 2002). HIV-1 integrase is responsible for two of the most essential steps in the HIV life cycle,

3’-processing and DNA strand transfer (Craigie, 2001). Due to the fact that HIV integrase plays such an integral role in the replication of HIV and because no cellular homologues are found in humans, inhibitors targeted selectively at HIV integrase are expected to have low cytotoxicity (Xu *et al.,* 2009). HIV-1 protease functions by cleaving a number of specific sites on the precursor gag and pol polyproteins thus releasing other viral proteins. It is therefore essential for the production of infectious viral particles because if these polyproteins are not cleaved, no infectious viral particles are produced (Weber *et al.,* 2001). For that reason, HIV protease is a prime target for anti-viral agents but because of the rapid development of inhibitor resistant variants of the protease enzyme, the therapeutic effectiveness of potential inhibitors is limited (Weber *et al.,* 2001).

HIV-1 reverse transcriptase inhibition is detected using a non-radioactive colorimetric enzyme immunoassay, which determines the quantity of retroviral reverse trancriptase activity by incorporation of digoxigenin and biotin labelled dUTP into DNA, producing a coloured reaction product. HIV-1 integrase is also detected using a colorimetric enzyme immunoassay, which quantitatively measures the inhibition of HIV-1 strand transfer activity due to the presence of interacting agents.

The objective of this chapter was to investigate two South African medicinal plants: *A. muricata* and *A. afra* for potential anti-TB activity by preliminary bioassay screening using MABA, INT assays and flow cytometry. In addition the inhibition of important HIV enzymes in the virus lifecycle i.e. reverse transcriptase and integrase were also determined. The selection of plants for evaluation was based on traditional use and the presence of highly active compounds like acetogenins and terpenes, which have previously been shown to possess anti-TB and anti-HIV activity (Cantrell *et al.,* 2001; Vik *et al.,* 2007 and Sun *et al.,* 2003).

3.2 RESULTS

3.2.1 Anti-TB Screening

3.2.1.1 Microplate Alamar Blue Assay

The Microplate Alamar Blue Assay using *M. tuberculosis* H37Rv ATCC 27294 (7 tests) revealed that the ethanolic extract of *A. muricata* produced >81% inhibition of *M. tuberculosis* at an MIC of 125 µg/mL. From visual observation, MIC values of five control drugs were 0.3125 µg/mL (rifampicin), 2 µg/mL (ofloxacin), 0.4 µg/mL (isoniazid), 15 µg/mL (ethambutol) and 2 µg/mL streptomycin (Fig. 3.1). Percentage inhibition of *M. tuberculosis* calculated from MABA absorbance values was, 75.89% (rifampicin), 91.09% (ofloxacin), 91.04% (isoniazid), 92.13% (ethambutol), 91.76% (streptomycin), and 81.63% for *A. muricata* (Fig. 3.2, Appendix Table A1). *A. afra* (aqueous and ethanolic extract) did not show any anti-TB activity using MABA at the highest concentration tested.

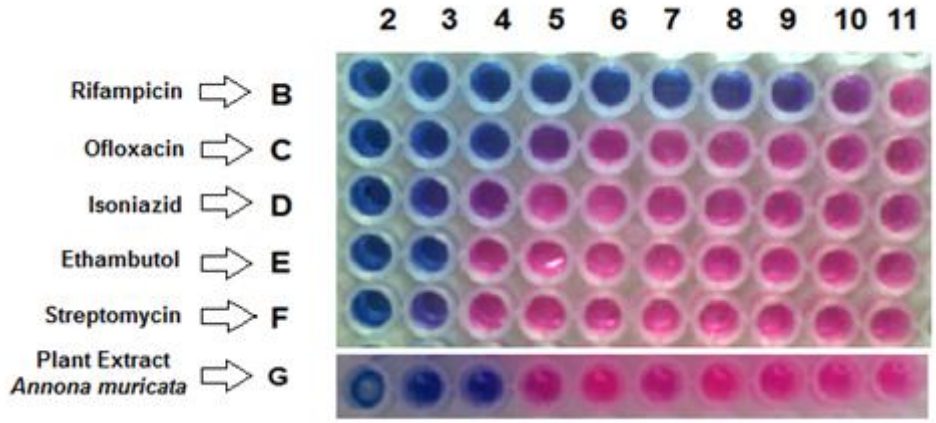

Figure 3.1: Anti-TB activity of *A. muricata* using MABA. Well 2 (all rows): Antibiotic Control. Well 11 (all rows): Bacterial Growth Control. Concentrations of antibiotics: Rifampicin wells 3-10 (0.016 – 2.0 µg/mL), Ofloxacin wells 3-10 (0.031 – 4.0 µg/mL), Isoniazid wells 3-10 (0.003 – 0.4 µg/mL), Ethambutol wells 3-10 (0.117 – 15.0 µg/mL), Streptomycin wells 3-10 (0.016 – 2.0 µg/mL). Concentrations of *A. muricata* wells 3-10 (3.906 – 500 µg/mL).

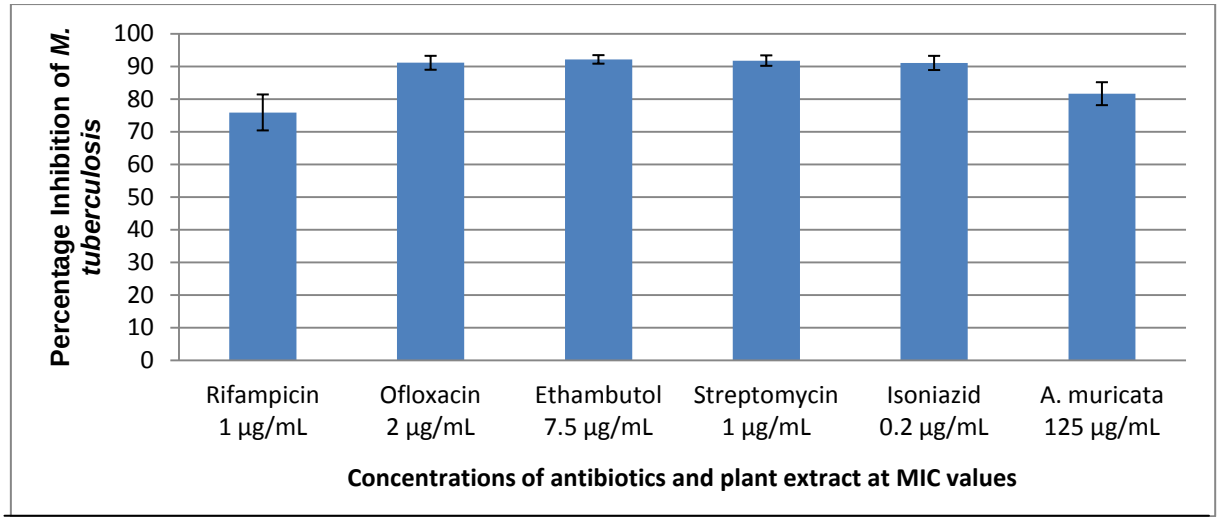

Figure 3.2: Percentage inhibition of *M. tuberculosis* determined using MABA for the five control drugs and for *A. muricata*. Data points represent the mean ± SD of several determinations, representative of seven independent experiments.

3.2.1.2 ρ-Iodonitrotetrazolium Chloride Assay

The ρ-iodonitrotetrazolium chloride assay using *M. tuberculosis* H37Rv ATCC 27294 (7 tests) indicated that the ethanolic extract of *A. muricata* showed inhibition of *M. tuberculosis* with MIC 125 µg/mL which corresponds to the MIC obtained by MABA (Fig. 3.3). *A. afra* (aqueous and ethanolic extract) did not show any anti-TB activity using INT assay, which confirmed the results obtained using MABA.

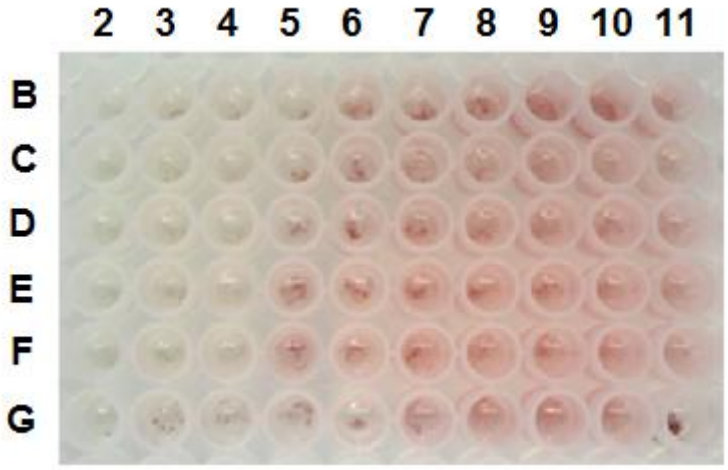

Figure 3.3: Anti-TB activity of *A. muricata* using INT assay. Well 11 (all rows): Bacterial Growth Control. Wells B-G: replicates of *Annona muricata.* Concentrations of *Annona muricata:* wells 2 – 10: 500 µg/mL - 3.906 µg/mL.

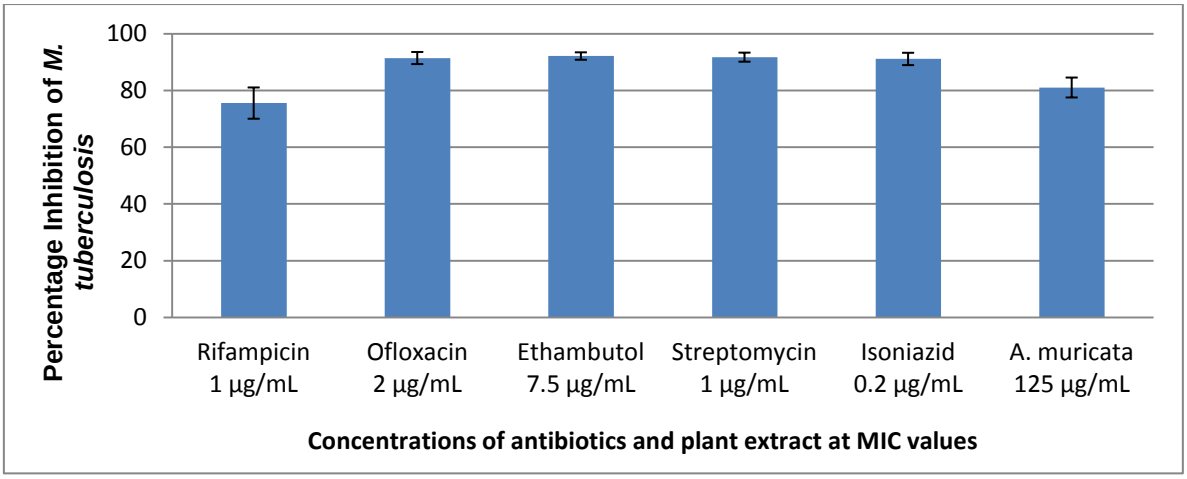

Figure 3.4: Percentage inhibition of *M. tuberculosis* determined using INT for the five control drugs and for *A. muricata*. Data points represent the mean ± SD of several determinations, representative of seven independent experiments.

3.2.1.3 Flow Cytometry

Susceptibility of *Mycobacterium tuberculosis* H37Rv ATCC 27294 was determined by comparing the mean channel fluorescence of the drug free control with those that were exposed to antibiotics and plant extracts. For the seven tests, there was a decrease in the mean channel fluorescence of the antibiotic treated samples, at various concentrations, compared to the drug free control, indicating susceptibility of *M. tuberculosis* cells after exposure to ethambutol, which resulted in 74.1% inhibition (Figure 3.5A). A similar effect was observed for the *M. tuberculosis* and extract treated samples compared to the extract free control (Figure 3.5B), with the ethanolic extract of *A. muricata* producing 57% inhibition at a concentration of 500 µg/mL. Of the five control drugs previously used for MABA and INT, only ethambutol was used for flow cytometry because of discrepancies when the other antibiotics were used. Due to negative inhibitory results obtained for both the aqueous and ethanol extracts of *A. afra* in both the MABA and INT assays, they were not tested utilising flow cytometry due to the financial implications of the technique.

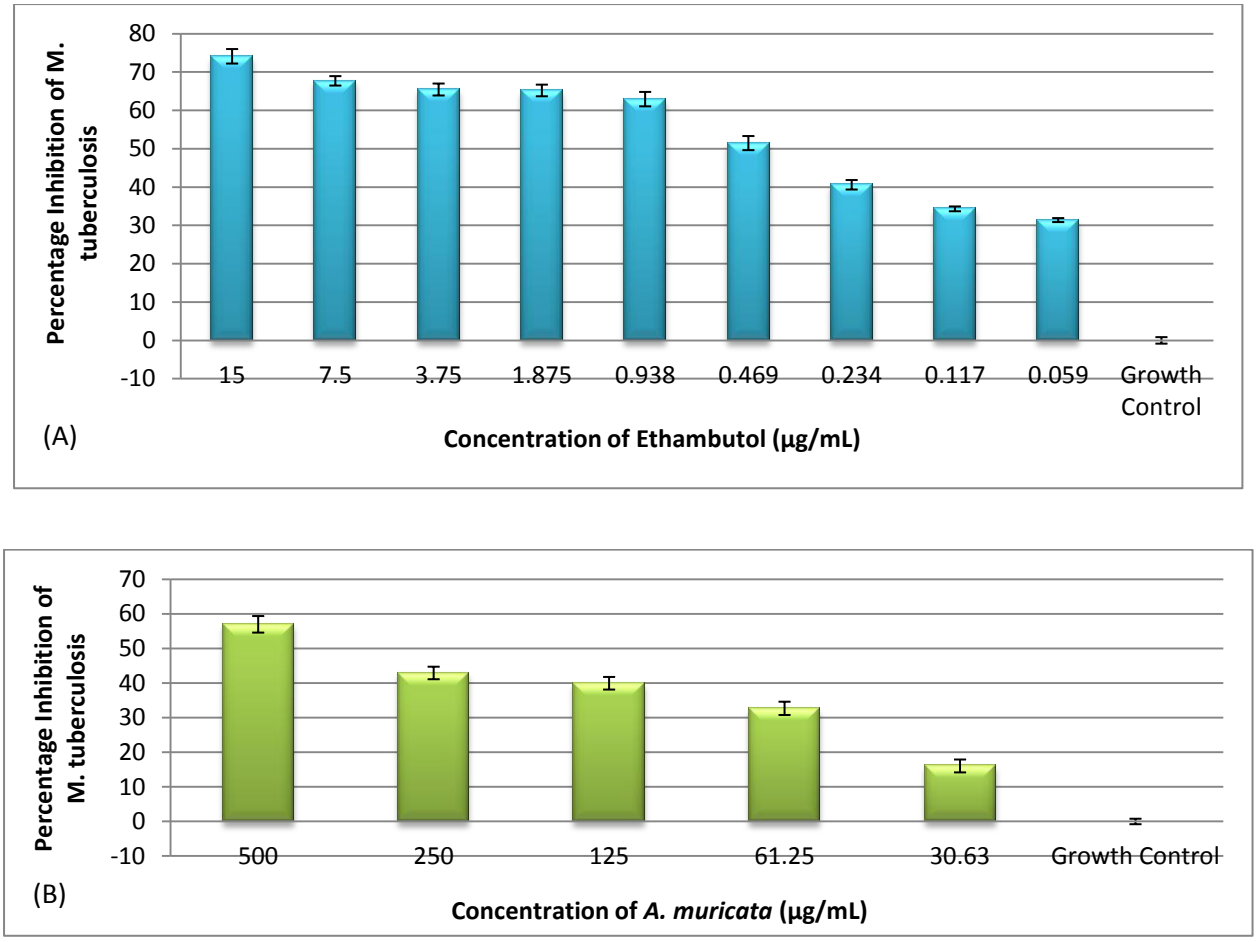

Figure 3.5: Flow cytometry data of the *M. tuberculosis* culture H37Rv ATCC 27294 exposed to various concentrations of **(A)** ethambutol **(B)** *Annona muricata* versus the untreated growth control. Percentage inhibition was calculated using the mean fluorescence intensities of metabolised FDA. Data points represent the mean ± SD of several determinations, representative of seven independent experiments.

3.2.1.4 Comparison of bioassays used for detection of anti-TB activity of *A. muricata*

Comparison of bioassays revealed that MABA exhibited 100% sensitivity and is considered to be the best assay when compared with INT and flow cytometry, which exhibited sensitivities of 85.7% and 100% respectively (Table 3.1). However, only one antibiotic was used for flow cytometry and therefore the 100% sensitivity is not directly comparable to the other methods utilised. There was a strong correlation between MABA and INT as both assays indicated an MIC of 125 µg/mL. However, flow cytometry is a more sensitive technique and detected small amounts of inhibition occurring at low concentrations of antibiotic and extract and therefore the MIC was inconclusive.

Table 3.1: Comparison of the assays used for detection of anti-tuberculosis activity.

	***M. tuberculosis* H37Rv ATCC 27294**							
	Antibiotics (A)		**Plant Extracts (P)**		**Total Tests for antibiotics**	**Total Tests for plant extracts**	**Sensitivity (%)**	
Assay	**Reproducible Results**	**Discrepancies**	**Reproducible Results**	**Discrepancies**			A	P
MABA	7	0	7	0	7	7	100	100
INT	6	1	6	1	7	7	85.7	85.7
Flow Cytometry	7	0	7	0	7[a]	7	100	100

*Sensitivity = [(no. True positives)/(no. True positives + no. False negatives)] * 100
[a]Only one antibiotic was utilised – Ethambutol, as discrepancies occurred with other antibiotics

Direct comparison of the results obtained with the different methods revealed the following: MABA and flow cytometry produced statistically significant differences with regards to the average percentage inhibition obtained ($P < 0.05$). MABA and INT did not show statistically significant differences, indicating that they produced very similar average percent inhibitions, whereas INT and flow cytometry also produced statistically significant differences (Appendix Table A3).

3.2.2 Anti-HIV Screening

3.2.2.1 HIV-1 Reverse Transcriptase Assay

The non-radioactive ELISA assay used to determine reverse transcriptase inhibition (3 tests in triplicate) revealed that the only extract exhibiting significant reverse transcriptase inhibition was the aqueous extract of *A. afra* with >78% inhibition at 0.5 mg/mL. The ethanolic extracts of *A. muricata* and *A. afra* showed no inhibitory activity against reverse transcriptase at very high concentrations of the extracts. Nevirapine, supplied by Aspen, was used as the positive control and exhibited 100% inhibition of reverse transcriptase at concentrations higher than 2 mg/mL (Fig. 3.6 and Fig. 3.7).

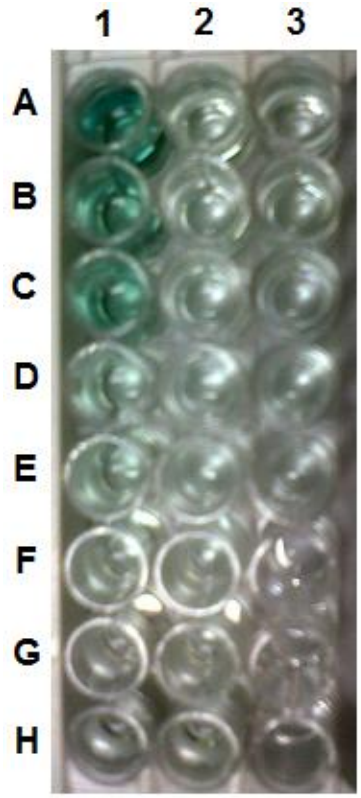

Figure 3.6: Visual representation of colour change observed for five concentrations (in triplicate) of the aqueous extract of *Artemisia afra*. A1, B1, C1: 0.2 mg/mL; D1, E1, F1: 0.5mg/mL; A2, B2, C2: 1 mg/mL; D2, E2, F2: 2 mg/mL; A3, B3, C3: 4 mg/mL; G1, G2, D3: Nevirapine (0.5 mg/mL); H1, H2, E3: Negative Control.

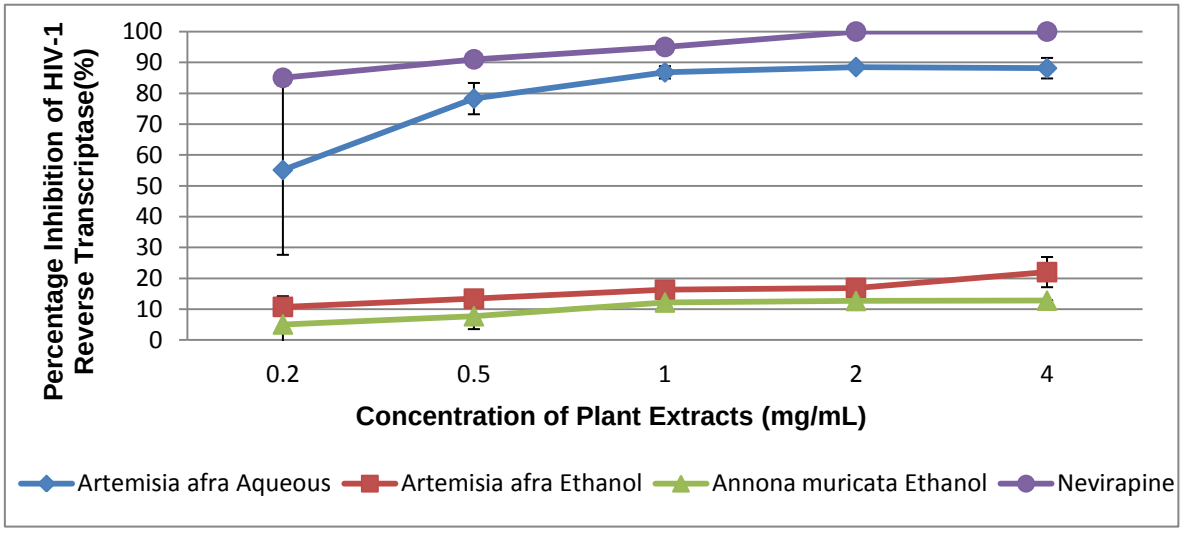

Figure 3.7: Percentage inhibition of HIV-1 reverse transcriptase obtained for various concentrations of ethanolic extract of *A. muricata* and aqueous and ethanolic extracts of *Artemisia afra.* Positive control [Nevirapine (Aspen) – 2 mg/mL, 100% inhibition]. Data points represent the mean ± SD of three determinations, representative of three independent experiments.

3.2.2.2 HIV-1 Integrase Assay

The non-radioactive enzyme immunoassay used to determine integrase inhibition (2 tests in triplicate) indicated that all extracts possessed activity against HIV-1 integrase (Fig. 3.8). The ethanolic extracts of *A. afra* and *A. muricata* produced similar results, with extensive inhibition >86.8% and >88.54% respectively at concentrations >0.5 mg/mL to 4 mg/mL. The aqueous extract of *A. afra* produced less activity, but still noticeable with inhibition of HIV-1 integrase >52.16% at 0.5 mg/mL increasing to 72.89% at 4 mg/mL of the extract. It was possible to determine IC_{50} values for *A. muricata,* the ethanolic extract of *A. afra* and the aqueous extract of *A.afra.* They were 0.125 mg/mL, 0.082 mg/mL and 0.450 mg/mL respectively.

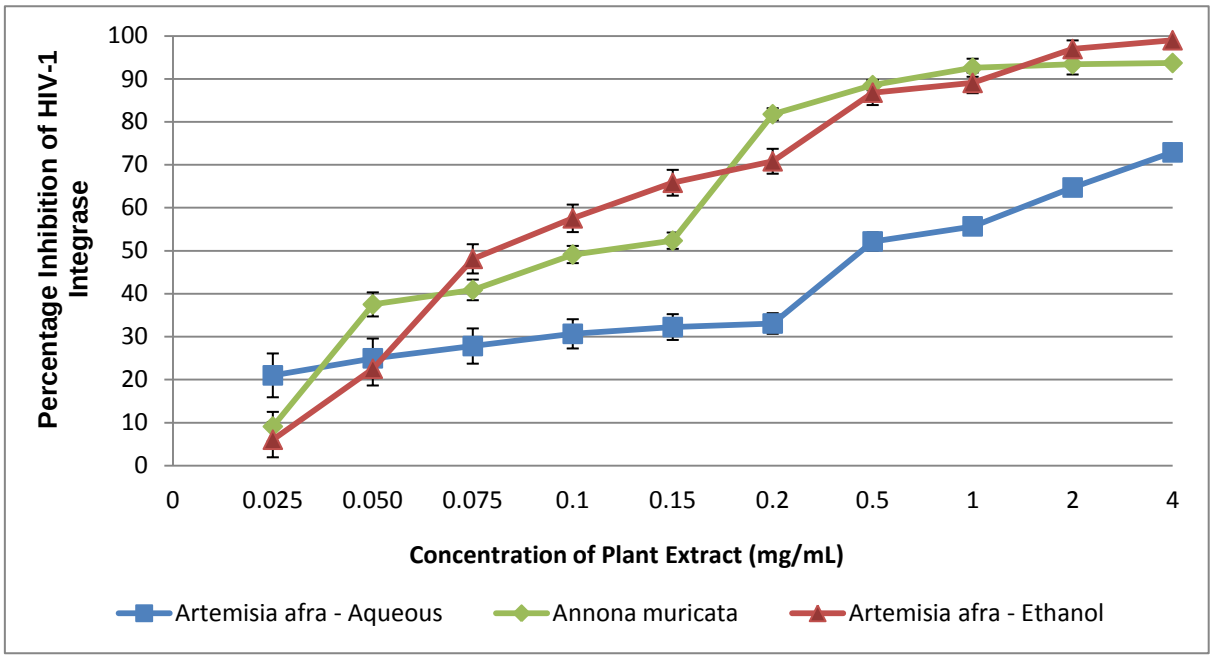

Figure 3.8: Percentage inhibition of HIV-1 integrase obtained for various concentrations of ethanolic extract of *A. muricata* and aqueous and ethanolic extracts of *Artemisia afra.* Data points represent the mean ± SD of three determinations, representative of three independent experiments.

The positive control used for HIV-1 integrase inhibition was sodium azide at concentrations ranging from 0.25 to 2% (v/v) (Fig. 3.9). Sodium azide, at concentrations 1% and above, achieved HIV-1 integrase inhibition >97%.

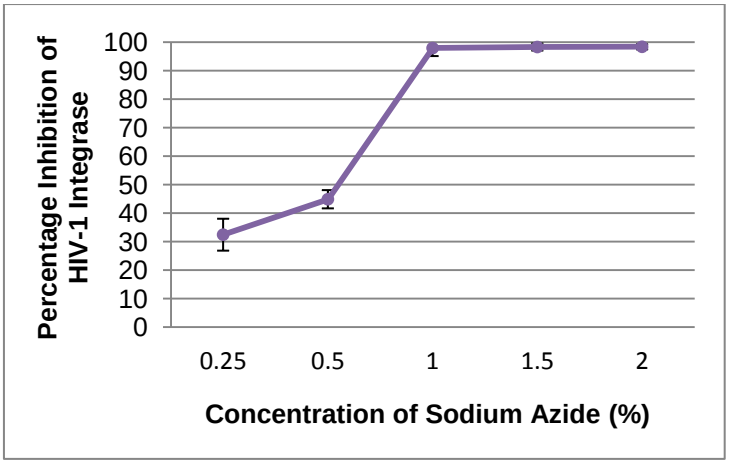

Figure 3.9: Percentage inhibition of HIV-1 integrase obtained for various concentrations of sodium azide (a known inhibitor of HIV-1 integrase). Data points represent the mean ± SD of three determinations, representative of three independent experiments.

3.3 DISCUSSION

The increase of resistance to conventional antibiotics by *M*ycobacterium *tuberculosis* has necessitated the search for new, efficient and cost effective ways for the control of this disease as well as a more rapid approach in novel drug discovery which would also aid in the control of MDR-TB (Green *et al.,* 2010).

A. muricata ethanolic extract exhibited anti-TB activity while *A. afra* showed no activity. In traditional medicine *A. muricata* extracts are used to alleviate coughs, asthma and chest pains and thus this anti-TB activity is relevant. It has also been known to be effective against *Staphylococcus aureus, Enterococcus faecalis, Escherchia coli* and *Plasmodium falciparum* (Yasunaka *et al.,* 2005 and Boyom *et al.,* 2011). This is the first report of an *A. muricata* extract showing activity against *M. tuberculosis.*

Inhibitory activity of *A. afra*, has been reported against *M. tuberculosis* but only in certain chemical extracts. Ntutela *et al.* (2009) used aqueous, methanol and dichloromethane extracts of *A. afra* and showed, that only the dichloromethane extract inhibited *M. aurum* and *M. tuberculosis* cultures in a dose dependant manner, with the methanolic and aqueous extracts having no inhibitory effects. However, it was found that treatment of *M. tuberculosis* infected mice with the aqueous extract of *A. afra* regulated pulmonary inflammation during early infection (Ntutela *et al.,* 2009 and Patil *et al.,* 2011). These reports were confirmed by Liu *et al.* (2009) and Mativandlela *et al.*

(2008) who also found no anti-TB activity present in the methanolic extract of *A. afra*, but when *Mycobacterium smegmatis* was tested, inhibitory activity was detected. This corresponds to our findings, with no activity in the ethanolic extract of *A. afra*. This lack of activity may be as a result of the solvents used as the different phytochemicals present in the plant extract into different solvents due to the differences in polarity. *A. afra* has a wide array of phytochemicals present ranging from monoterpenoids to sesquiterpenes (Liu *et al.,* 2009). The solvent used to extract certain phytochemicals would differ, for example, some plants contain alkaloids that will only extract into alcohol, whereas others contain an alkaloid that will extract into water, but will be destroyed in alcohol. Therefore the most active phytochemicals may extract best into dichloromethane (Ntutela *et al.,* 2009).

The overall performance of MABA in this study was good indicating MIC's of the antibiotics by visual detection. This is in agreement with reports of MABA for susceptibility testing demonstrated by other investigators, including a concordance rate of 93.6% with the BACTEC 460 results observed by Franzblau *et al.* (1998); Bastian *et al.* (2001); Chauca *et al.* (2007); Leonard *et al.* (2008); Estrada-Soto *et al.* (2009); Alba-Romero *et al.* (2011); Lawal *et al.* (2011) and Tekwu *et al.* (2012). The INT assay, which also performed adequately, exhibited identical results to that of MABA, showing anti-TB activity at MIC of 125 µg/mL for *A. muricata.* INT also confirmed that no anti-TB activity was present in *A. afra.* The INT assay has been applied to *E. coli*, *S. aureus, P. aeruginosa* and *M. smegmatis* for the detection of antibacterial activity (Eloff, 1998; Kuete *et al.,* 2008; Smith and McFeters, 1997).

Due to the biohazard risk of *M. tuberculosis,* exposure to 10% formaldehyde for one hour was required in order to kill the TB cells. The addition of the formaldehyde decreased the colour intensity of the wells, consistently including the control. There was a strong correlation with the visual MIC's and the fluorimetric readings obtained, which is similar to the findings of Collins and Franzblau (1997), who reported a high correlation between MIC obtained visually and fluorimetrically using MABA. MABA has many advantages by being a non-radiometric, inexpensive, rapid, high-throughput assay, which could prove very useful for large scale screening of plant extracts against *M. tuberculosis.*

Flow cytometry makes use of fluoroscein diacetate (FDA) for the detection of *M. tuberculosis*, FDA diffuses across cell walls and membranes by active transport and is rapidly hydrolyzed by mycobacterial esterases. Metabolically inactive cells have decreased quantities of esterases and thus exhibit less fluorescence. Mycobacteria are susceptible to various anti-mycobacterial agents and the effect of these on the cells causes decreased capacity to hydrolyze FDA. Susceptibility was determined by comparing the mean channel fluorescence of the drug free control with those that were exposed to the antibiotic, ethambutol, and the plant extract *A. muricata*. The decrease in mean channel fluorescence for those cells that were treated with ethambutol, were similar to findings of Kirk *et al.* (1998); Reis *et al.* (2004) and Norden *et al.* (1995). The *A. muricata* exposed samples also exhibited a decrease in the mean channel fluorescence similar to the drug control.

It was also possible to determine which assay was the cheapest and fastest to conduct, however when observing the price, there was no substantial difference between the preparation and starting materials of each assay, provided that the necessary equipment was already available. In terms of duration, MABA and INT took longer as they required 10 days growth period and 7 days assay incubation whilst flow cytometry only requires 3 days assay incubation in addition to the 10 day growth period. This 5 day difference favours flow cytometry with a shorter duration for obtaining results, however flow cytometry did not provide accurate MIC values and is more technically complicated to conduct as opposed to MABA and INT.

MABA was the most sensitive and simplest with regards to antibiotic and plant extract testing, and was 100% reproducible for both. Flow cytometry can only be used for the detection of susceptibility of *M. tuberculosis* to antibiotics, as accurate MIC values could not be ascertained. INT showed 85.7% reproducibility, possibly because of the presence of chloride in the dye, which may have interfered with the results obtained.

This is the first report of the plant extract *A. muricata* showing anti-TB activity. There is paucity of information and scientific validation on the use of plant extracts to cure tuberculosis and its related symptoms. Further experiments need to be conducted to confirm this finding and to determine the active compound of the *A. muricata* extract which is causing inhibition of *M. tuberculosis*.

Despite advances in anti-retroviral therapy which has transformed HIV/AIDS from a fatal to a manageable chronic disease, there remain considerable challenges. Highly active anti-retroviral therapy (HAART) is limited by its cost, the requirement of lifelong adherence, toxicity and side effects. In addition, the poor results of vaccine development along with the emergence of drug resistant HIV strains makes it impossible to rely on a few standard drug regimens (Leteane *et al.,* 2012; Bessong *et al.,* 2005; Klos *et al.,* 2009; Mukhtar *et al.,* 2008).

The HIV enzymes, such as reverse transcriptase, integrase and protease, have been specific targets for many therapeutic drugs. The aqueous extract of *A. afra* exhibited substantial inhibition of HIV-1 reverse transcriptase while the ethanolic extracts of *A. afra* and *A. muricata* showed slight inhibition but at very high concentrations of the extracts. It was recently reported by Lubbe *et al.* (2012), that *A. afra* possesses anti-HIV activity through the utilisation of tea infusions, however the method of detection was different to this study. Lubbe *et al.* (2012) made use of two independent approaches, namely the Infection format of Fusion Induced Gene Stimulation (iFIGS) and the dual enhancement of Cell Infection to Phenotype Resistance (deCIPhR), which both used genetically modified human HeLa cell lines, which were infected with HIV-1. However, inhibition of HIV-1 reverse transcriptase activity has not been previously documented for *Artemisia afra* aqueous extract. The anti-HIV activity reported by Lubbe *et al.* (2012) could possibly be a result of the reverse transcriptase inhibition observed in the present study.

Liu *et al.* (2009) reported that the combination of *A. afra* with standard forms of HIV treatment yielded positive results and suggested that *A. afra* may exhibit some antiviral activity or immune boosting properties. The ethanolic extract of *A. afra* exhibited considerably lower activity when compared to the aqueous extract. This may be due to solvent compatibility to phytochemical extraction, the ethanol may destroy the active compound(s) responsible for the HIV reverse transcriptase inhibition.

The ethanolic extracts of *A. afra* and *A. muricata* showed greater activity against HIV-1 integrase than that of the aqueous extract of *A. afra.* This is interesting and suggests that there is a specific mechanism and a specific compound acting in each mode of inhibition and not just a compound which has a general affect on the envelope or the

receptors of the virus. The activity observed for the ethanolic extracts of *A. afra and A. muricata* was very similar, which might suggest that the ethanol extracted a compound which is structurally similar to both plants. The activity exhibited from *A. muricata* may be due to the group of compounds known as acetogenins, as they have previously displayed anti-HIV activities at very low concentrations (Aminimoghadamfarouj *et al.,* 2011). The inhibition of HIV-1 integrase by *A. afra* and *A. muricata* has not been previously documented. It would be beneficial to determine which phytochemical(s) are being produced from each of these plants, that are responsible for this activity against HIV-1 integrase and to establish whether the active chemicals are similar.

The determination of anti-HIV-1 protease activity exhibited by these two extracts would have provided more evidence to the efficacy of these two extracts against HIV, however due the availability of reagents being limited and the cost for an appropriate kit being out of budget, anti-HIV-1 protease activity was unfortunately not determined.

These findings provide evidence that natural sources, such as plants, can provide new, inexpensive means of treatment. However, further experiments should be conducted in order to verify the anti-HIV activities exhibited by *A. muricata* and *A. afra* as well as to identify the active compounds present.

CHAPTER FOUR

PLANT EXTRACT CYTOTOXICITY AND SYNERGISTIC EFFECTS

4.1 INTRODUCTION

Tuberculosis (TB) and Human Immunodeficiency Virus (HIV) have a very high prevalence in South Africa and it is known that the current treatment therapies used to combat TB and HIV can lead to adverse reactions such as hepatotoxicity. The incidence of hepatotoxicity may vary from 2 to 28% in different populations and can occur even when the drug has been given at the recommended doses (Tostmann *et al.,* 2008; Singh *et al.,* 2011). This side effect often results in patients discontinuing treatment, which inevitably leads to morbidity, mortality and most likely the emergence of drug resistant strains (Singh *et al.,* 2011).

As a result of this high hepatotoxicity, many individuals have redirected their interest to natural products such as plants. The secondary metabolites produced by some plants possess many biological activities, either serving as protective agents against various pathogens or as specific growth regulatory molecules, which can stimulate or inhibit cell division (Mativandlele, 2008). The popular perception is that, because the products from plants are natural, they are safe and that they have been used for centuries without harmful effects (Willet *et al.,* 2004). The history of a product's use is, however, not a guarantee of safety, particularly with respect to long term use, at relatively high doses or with other medications (Willet *et al.,* 2004). One of the most serious safety concerns for natural plant product usage, is the potential for liver injury, as many have documented that herbal products such as kava, germander, chaparral, Ephedra, and comfrey have been associated with rare but severe cases of liver failure (Estes *et al.*, 2003; Favreau *et al.*, 2002; Stedman, 2002; Teschke *et al.*, 2003; Willet *et al.,* 2004). As a result of these reactions, the cytotoxic evaluation of plant extracts is essential before they can be considered for new drug development.

Cytotoxicity tests are part of developing a potential pharmaceutical product into a clinically acceptable drug. These tests provide a screening method to determine that the compounds being tested are not more harmful to the normal biological processes

than the effects they are being tested for (Mativandlele, 2008). There are various methods to screen for possible cytotoxic effects of plant extracts, however the simplest and most commonly used is that of the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. It provides a quantitative approach of determining mammalian cell survival and proliferation *in vitro*. There are many other methods such as the sulforhodamine B (SRB) assay and the chlonogenic assay.

As a result of plant extracts being used as alternative medicine to alleviate the symptoms of TB and HIV, it is important to determine if any synergistic or antagonistic relationships could develop between the newly found plant extracts and existing drugs which the patient may be using. Synergism is defined as a positive interaction created when two agents are combined and together they exert an inhibitory effect on the targeted organisms that is greater than the sum of their individual effects (Chanda and Rakholiya, 2011). Antagonism occurs when the effect of two drugs together is less than the effect of either alone and indifference is when no effect is exhibited (Chanda and Rakholiya, 2011).

Altered pharmacokinetics almost inevitably leads to a significant change in the response to drugs that have narrow therapeutic indices; however, given that a single herbal preparation may contain more than 100 components, all of which may have unknown biological activities, a herb has the potential to mimic, increase, or reduce the effects of co-administered drugs through simultaneous effects on the same drug targets (Zhou *et al.,* 2007). If the effect of the drug in combination with the herbal medicine is enhanced, i.e. synergistic or additive effect, unfavourable toxicity may occur. By contrast, some herbal remedies may contain compounds with antagonistic properties, which are likely to reduce drug efficacy and produce therapeutic failure (Zhou *et al.,* 2007). The synergistic or antagonistic effects between herbs and drugs often result from the competitive or complementary effect of the drug and the co-administered herbal constituents at the same drug targets (Zhou *et al.,* 2007).

In phytotherapy, there are significant advantages that can be associated with synergistic interactions, such as increased efficiency, reduction of undesirable effects, increase in stability or bioavailability of the free agents and obtaining an adequate therapeutic effect with relatively small doses, when compared with synthetic

medication (Chanda and Rakholiya, 2011). Some examples where synergism has been observed include the experiments conducted by Souto de Oliveira *et al.* (2011) who showed synergistic activity of norfloxacin, tetracycline and erythromycin with the ethanol extract of *Mangifera indica* L. peel against *S. aureus* strains; Aiyegoro *et al.* (2009) showed that acetone, chloroform, ethyl acetate and methanol extracts of *Helichrysum longifolium* in combination with six antibiotics comprising of penicillin G sodium, amoxicillin, chloramphenicol, oxytetracycline, erythromycin and ciprofloxacin improved the bactericidal effects of the antibiotics against a panel of bacterial isolates; Chatterjee *et al.* (2009) showed the *in vitro* synergistic effect of doxycycline and ofloxacin in combination with ethanolic leaf extracts of *Vangueria spinosa* against four pathogenic bacteria. This substantiates the need for understanding the molecular mechanisms of synergy, which could provide a new strategy for the treatment of infectious diseases, overcome drug resistant pathogens and decrease the use of antibiotics and hence the side effects created by them.

Therefore, the ethanolic and aqueous extracts of *Artemisia afra* and the ethanolic extract of *Annona muricata* were subjected to cytotoxicity testing with MTT and synergistic testing by the combination index method, performed using the Chang Liver cell line and the Human hepatoma cell line, HepG2. Previously it was shown that extracts with high antioxidant activity could convert the MTT dye to the purple formazan product, therefore it should be stated that the MTT assay was performed in such a way that the extracts did not interfere with the result, by firstly removing the extract before addition of the MTT, in fresh medium, and secondly the medium containing the MTT was removed after incubation and only the purple formazan crystals in the cells were solubilised with DMSO.

4.2 RESULTS

4.2.1 Cytotoxicity

4.2.1.1 Chang Liver Cell Line

The cytotoxic effects of the aqueous and ethanolic extracts of *A. afra* and the ethanolic extract of *A. muricata* were determined using the MTT assay for Chang liver cells. The aqueous and ethanolic extracts of *A. afra* yielded IC_{50} values greater than 250 µg/mL and thus were considered to be nontoxic to confluent Chang liver cells (results not shown). The IC_{50} value obtained for the ethanolic extract of *A. muricata* against the confluent Chang liver cell line was 29.38 µg/mL (Fig. 4.1). From these results, the concentration of the ethanolic extract of *A. muricata* to be used for further experiments was fixed at 30 µg/mL for Chang liver cells.

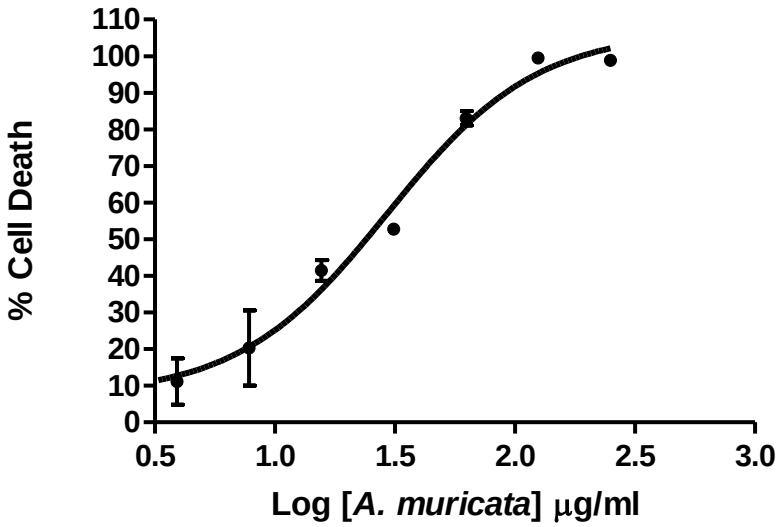

Figure 4.1: Cytotoxic effect of *A. muricata* ethanol extract on confluent Chang liver cells after 24 hours of exposure. Cell viability was determined by using MTT assay. Error bars indicate Standard deviation (SD) of ten replicate values.

The cytotoxic effects for rifampicin, one of the first line drugs used to treat TB, was determined using the MTT assay for confluent Chang Liver cells. A concentration range between 0 and 150 µM was tested and the IC_{50} value obtained was 72.40 µg/mL (Fig. 4.2). From these results, the concentration of rifampicin to be used for synergy experiments was fixed at 70 µg/mL for Chang liver cells.

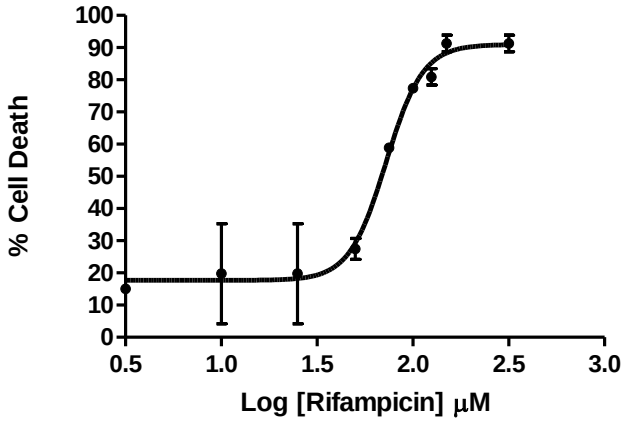

Figure 4.2: Cytotoxic effect of Rifampicin on confluent Chang liver cells after 24 hours of exposure. Cell viability was determined by using MTT assay. Error bars indicate SD of four replicate values.

4.2.1.2 HepG2 Cell Line

The cytotoxic effects of the aqueous and ethanolic extracts of *A. afra* and the ethanolic extract of *A. muricata* were determined using the MTT assay for HepG2 cells. The aqueous and ethanolic extracts of *A. afra* yielded IC_{50} values greater than 250 µg/mL, similar to that of the Chang liver cells, and thus were considered to be nontoxic to confluent HepG2 cells (results not shown). The IC_{50} value obtained for the ethanolic extract of *A. muricata* against the HepG2 cell line was 76.68 µg/mL (Fig. 4.3). From these results, the concentration of the ethanolic extract of *A. muricata* to be used for further experiments was fixed at 77 µg/mL for HepG2 cells.

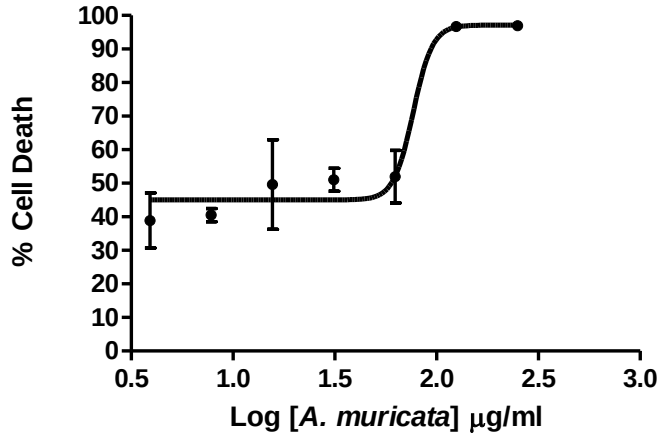

Figure 4.3: Cytotoxic effect of *A. muricata* ethanol extract on confluent HepG2 cells after 24 hours of exposure. Cell viability was determined by using MTT assay. Error bars indicate SD of four replicate values.

The cytotoxic effects for rifampicin were determined using the MTT assay for HepG2 cells. A concentration range between 0 and 150 µM was tested and the IC_{50} value obtained was 134.80 µg/mL (Fig. 4.4). From these results, the concentration of rifampicin to be used for synergy experiments was fixed at 135 µg/mL for HepG2 cells.

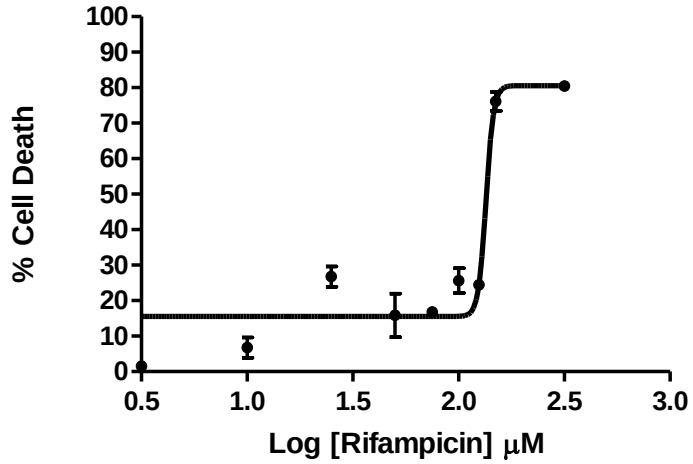

Figure 4.4: Cytotoxic effect of Rifampicin on confluent HepG2 cells after 24 hours of exposure. Cell viability was determined by using MTT assay. Error bars indicate SD of four replicate values.

4.2.2 Synergy Experiments using the CI method

Combination studies were conducted using the IC_{50} values obtained in section 4.1, to determine whether there was a synergistic, antagonistic or purely additive effect when the ethanolic extract of *A. muricata* and the anti-TB drug, rifampicin, are used simultaneously. The combination index (CI) is the natural law based general expression of pharmacologic drug interactions (Chou, 2010). It is shown to be the simplest possible way for quantifying synergism or antagonism (Chou, 2010).

Using the IC values, C_a and C_b values in Table 4.1, the combination index was determined using Equation below:

$$CI = \frac{C_a}{IC_{25,50,70}\,(Extract)} + \frac{C_b}{IC_{25,50,70}\,(Rif)}$$

Example using the IC_{50} for *A. muricata,* rifampicin and the combination of both for Chang Liver cells:

$$CI_{50} = \frac{22.43}{30} + \frac{24.03}{70} = 1.10$$

The CI obtained in the example above was very close to 1, indicating a purely additive effect was achieved when the extract and rifampicin were used in combination. This equation was applied to all IC values obtained for both cell lines and CI results obtained are indicated in Table 4.1.

Table 4.1: Summary of the IC_{25}, IC_{50} and IC_{70} values obtained for the ethanolic extract, *A. muricata* alone, rifampicin alone as well as the IC_{25}, IC_{50} and IC_{70} values of each when used in combination for both the Chang liver and HepG2 cell lines. Combination index also provided.

	(A) Chang liver Cells (µg/mL)	**(B) HepG2 Cells (µg/mL)**
(a) *Annona muricata*		
IC_{25}	9	ND
IC_{50}	30	77
IC_{70}	80	90
(C_a) Combination IC_{25}	5.322	ND
(C_a) Combination IC_{50}	22.43	27.78
(C_a) Combination IC_{70}	19.43	3.454
(b) Rifampicin	**(A) Chang liver Cells (µM)**	**(B) HepG2 Cells (µM)**
IC_{25}	35	ND
IC_{50}	70	135
IC_{70}	47	145
(C_b) Combination IC_{25}	191.2	ND
(C_b) Combination IC_{50}	24.03	46.72
(C_b) Combination IC_{70}	26.04	5.819
CI – IC_{25}	6.05	ND
CI – IC_{50}	1.10	0.71
CI – IC_{70}	0.74	0.079

*ND – Not determined

From Table 4.1, when focussing on Chang liver cells, it is evident that using *A. muricata* and rifampicin in combination only at their IC_{70} values produces a synergistic effect, where as an antagonistic effect is observed when used at their IC_{25} values and a purely additive effect when used at their IC_{50} values. For HepG2 cells, it seems that a synergistic effect is produced when the extract and rifampicin are used in combination at both the IC_{50} and IC_{70} values. This effect was more pronounced at the IC_{70}. No IC_{25} value was observed for HepG2 cells.

4.3 DISCUSSION

Cell based assays are frequently used for drug discovery using high throughput screening, environmental assessment of chemicals and biosensors for monitoring cellular behaviour (Vahdati-Mashhadian *et al.,* 2007). Some biochemical methods, such as the MTT assay are widely used in toxicity screening assays. Due to the rapid spread of TB and HIV and the desperate need for novel, less toxic forms of treatment, natural products have become a major source of potential anti-TB and anti-HIV compounds. However, before these compounds can be considered for human use, their cytotoxic properties need to be determined as well as any synergistic or antagonistic properties that might arise when used in combination with regular prescription drugs that are used for the treatment of TB and HIV.

Chang liver and HepG2 cells were used for the cytotoxic experiments as they are both representative liver cell lines and as majority of drugs are detoxified by the liver, any cytotoxic effects produced from these extracts would thus have an effect on the course of TB and HIV treatment and thus would not be beneficial. Results of the MTT assay show that the ethanolic extract of *A. muricata* possesses significant toxicity at concentrations of 30 µg/mL and 77 µg/mL for Chang liver cells and HepG2 cells respectively. *A. muricata* has been reported to have cytotoxic effects on human melanoma cells, while the aqueous, ethanolic and pentane extracts had IC_{50} values greater than 500 µg/mL, 20 µg/mL and 120 µg/mL respectively (Menan *et al.,* 2006). These cytotoxic concentrations produced by *A. muricata* are reasonably low, indicating that any dose greater than these concentrations will cause cell death, which is undesirable especially because *A. muricata* inhibited *M. tuberculosis* at an MIC of 125 µg/mL. *A. muricata* posesses over 50 mono-THF acetogenins, which have been

proven to be cytotoxic against other cell lines such as U937's, however further experiments need to be conducted in order to determine whether acetogenins are causing this cytotoxicity on Chang liver and HepG2 cells.

The ethanolic and aqueous extracts of *A. afra* showed toxicity at concentrations greater than 250 µg/mL for both Chang liver and HepG2 cells. This finding is supported by Spies *et al.* (2013), who also reported an IC_{50} greater than 250 µg/mL. Spies *et al.* (2013) also reported the cytotoxic effects of the ethanolic and aqueous extracts of *A. afra* on HeLa and U937 cancer cell lines, with the aqueous extract showing no toxicity while the ethanolic extract had IC_{50} values of 31.88±1.09 µg/mL and 18.21±0.9 µg/mL against HeLa and U937 cells, respectively. The absence of cytotoxic effects from the ethanolic and aqueous extracts of *A. afra* on both the liver and hepatocyte cell lines is favourable as a dose of 0.2 mg/mL of the aqueous extract of *A. afra* causes >50% inhibition of HIV-1 reverse transcriptase, whereas a dose of 0.075 mg/mL of the ethanolic extract of *A. afra* exhibits ≈50% inhibition of HIV-1 integrase.

Rifampicin is an important drug in the treatment of human mycobacterial and other infections. It is widely used as an essential drug in the treatment of tuberculosis and leprosy, in combination with other drugs (Vahdati-Mashhadian *et al.,* 2007). The drug has been shown to produce hepatic toxicity in animal studies and tubulo-interstitial nephritis and acute renal failure is another adverse effect of the drug (Vahdati-Mashhadian *et al.,* 2007). The drug is also known to inhibit protein synthesis and induces chromosomal aberration (Vahdati-Mashhadian *et al.,* 2007). In order to determine possible synergistic effects of these extracts, cytotoxicity of rifampicin on Chang liver and HepG2 cells was determined. Rifampicin yielded IC_{50} values of 57.7 µg/mL (70 µM) and 111.1 µg/mL (135 µM) for Chang liver and HepG2 cells respectively. These results are similar to those presented by Vahdati-Mashhadian *et al.* 2007, where the toxic effect was evident from the concentration of 8.23 µg/mL (10 µM) and increased in a concentration dependent manner. However, the maximum therapeutic plasma concentrations of rifampicin are 7-10 µg/mL, which overlaps the lowest toxic concentration in those experiments. IC_{50} values vary between experiments possibly due to the culture conditions or due to experimental procedure. Rifampicin was the only drug tested because the concentrations of nevirapine, ofloxacin,

streptomycin and ethambutol were too high in order to obtain cell death and required a high quantity of DMSO to dissolve.

Using the IC_{25}, IC_{50} and IC_{70} values for the ethanolic extract of *A. muricata* on the different cell lines and the IC_{25}, IC_{50} and IC_{70} values obtained for rifampicin, synergistic and antagonistic effects could be determined. *A. muricata* was the only extract tested for synergistic effects, as it was found to be the only extract that possessed cytotoxicity, however the possibility of synergistic effects of the ethanolic and aqueous extracts of *A. afra* should not be disregarded. Using the combination index (CI) method of Chou-Talalay, as proposed by Chou, (2010), it was found that the CI for IC_{25} was 6.05 for Chang liver cells, which indicates a possible antagonistic effect when using rifampicin and *A. muricata* in combination, which could prove desirable in terms of toxicity as it may be possible that the extract could protect the patient from the toxic side effects of the drug. The IC_{25} for HepG2 cells was not determined. The CI for IC_{50} was 1.10 and 0.71 for Chang liver and HepG2 cells respectively, which indicates a purely additive effect on Chang liver cells and a synergistic effect on HepG2 cells. The synergistic effect observed indicates that the extract could possibly enhance the toxic side effects of the drug, which could prove harmful to the patient, however these results are not an indication of the therapeutic effects that may occur. The purely additive effect observed could even prove to be harmful because the patient's liver could be challenged with the toxicity of the drug as well as that of the extract. The CI for IC_{70} was 0.74 and 0.079 for Chang liver and HepG2 cells respectively, which indicates a possible synergistic effect for both cell lines.

When analysing these results, it looks promising that natural products can definitely play a major role in the search for novel drugs to fight TB and HIV. The ethanolic extract of *A. muricata* does prove to be cytotoxic and under certain conditions could be synergistic when used with rifampicin, however further testing needs to be conducted as there is no evidence as to what the extract is subject to *in vivo* and what changes may occur, which could lead to a less cytotoxic compound or an inactive compound all together. The same applies to the ethanolic and aqueous extracts of *A. afra.* It would also be beneficial to test the new found synergistic and antagonistic values on the actual microorganisms of *M. tuberculosis* and HIV in order to verify these results and determine whether or not the therapeutic effect will be enhanced or antagonised by

the combination of the extract and the drug. Isolation of the active compound is also important to ascertain whether the same compound is responsible for anti TB or anti-HIV activities, cytotoxicity and synergy.

CHAPTER FIVE

INTERACTIONS OF PLANT EXTRACTS WITH DRUG METABOLIC PATHWAYS

5.1 INTRODUCTION

There are more than 11 000 species of herbal plants that are used medicinally worldwide, most of which are often co-administered with therapeutic drugs raising the potential for drug herb interactions (Zhou *et al.,* 2007). The use of medicinal plants against a variety of diseases is gaining popularity because of several advantages such as fewer side effects, better patient compliance, relatively low cost and high accessibility (Brown *et al.,* 2008). Traditional herbal medicines that were formed and used thousands of years ago are still widely used today, especially in developing countries, where there is an abundance of indigenous knowledge (Brown *et al.,* 2008).

HIV/AIDS patients who make use of traditional herbal medicines may also simultaneously take prescription drug therapies, provided by clinics and hospitals, which could potentially cause drug herbal pharmacokinetic and/or pharmacodynamic interactions, depending on whether the drug has a narrow therapeutic index (Brown *et al.,* 2008). Although the high risks associated with potential drug interactions is well known, 14–31% of prescription drug users combine herbal products with traditional medicines (Calalto *et al.,* 2010). Due to this increased popularity and growth in sales of herbal medicinal products over recent years, many researchers have turned their focus to the pharmacological mechanisms underlying herb drug interactions (Calalto *et al.,* 2010).

Since the components of herbal products consumed must also be eliminated from the body by the same mechanism that removes drugs, there is a potential for interaction between herbal components and drugs (Ventkataramanan *et al.,* 2006). Chemical constituents in herbal products, similar to prescription drugs, are eliminated by various metabolic enzymes in the body and may be substrates for various transporters. Possible herb drug interactions may alter drug bioavailability through altered

absorption, metabolism and distribution. Primary mechanisms of drug herb interactions involve either induction or inhibition of intestinal drug efflux pumps, such as P-glycoprotein (P-gp) and multiple resistance proteins (MRPs) as well as intestinal and hepatic metabolism by cytochrome P450s (CYPs) (Ventkataramanan *et al.,* 2006). Many drug substances along with a variety of naturally occurring dietary or herbal components are capable of interacting with the CYP enzyme system and P-gp efflux pump in several ways:

1. A herbal component can be a substrate of one or several isoforms of CYP enzymes and/or efflux systems. Therefore, one substrate can compete with another substrate for either metabolism by the same CYP isozyme and/or efflux system resulting in higher plasma concentrations due to competitive inhibition (Ventkataramanan *et al.,* 2006).

2. A herbal constituent can also be an inducer of one or several CYP isoforms and/or efflux systems, thereby lowering plasma concentrations due to either higher metabolism and/or higher efflux. Such interactions may produce sub-therapeutic plasma drug concentrations (Ventkataramanan *et al.,* 2006).

3. A compound can also be an inhibitor of CYP450 enzymes resulting in reduced activity of one or several isoforms of CYPs. If a compound is an inhibitor of efflux system, it will reduce drug efflux resulting in improved absorption (Ventkataramanan *et al.,* 2006).

Absorption is a complex phase in pharmacokinetics and is a major determinant in drug efficacy and treatment outcome. The herbal interactions affecting it need to be considered, particularly in the case of drugs that have a narrow therapeutic index, such as digoxin or anti-cancer agents (Calalto, 2010). Given the increasing number of patients receiving multiple therapies, the FDA and European Medicines Agency (EMA) have recommended that pharmaceutical companies develop drug interaction investigations using cytochrome P450 and transporter enzymes because identifying possible factors and mechanisms involved with interactions related to drug absorption early in the development process helps to eliminate molecules with unwanted metabolic properties and guides medicinal chemists to produce better clinical

candidates and will inevitably aid the drug discovery process (Calalto, 2010). One major obstacle of drug absorption is intestinal metabolism. The cytochrome P450 superfamily of enzymes on enterocytes metabolise, detoxify and bioactivate xenobiotics (Calalto, 2010). The most studied and abundant cytochrome is CYP3A4, which is located on the top of mature enterocytes and metabolises many drugs, (Calalto, 2010).

Cytochrome activity can be drug modulated or change as a result of various polymorphisms. Some xenobiotics can enhance or reduce metabolism by inducing or inhibiting CYP activity, respectively, thereby increasing the range of variability up to 400 fold (Calalto, 2010). Induction of these metabolic enzymes by xenobiotics decreases the amount of drug absorbed, leading to loss of clinical efficacy. On the other hand, inhibition of intestinal metabolism raises the amount of drug available increasing the possibility of toxicity and adverse drug reactions (Calalto, 2010). The P450 hydrophilic conversion of xenobiotics is followed by the chemical attachment of endogenous molecules, such as glucuronide, glycine, glutamine and glutathione; these secondary processes are mediated by phase II conjugating enzymes such as Glutathione-S-transferases (GST's) and Sulfotransferases (SULT's) (Calalto, 2010).

GST's are thought to play a physiological role in initiating the detoxification of potential alkylating agents including pharmacologically active compounds. These enzymes catalyze the reaction of such compounds with the -SH group of glutathione, thereby neutralizing their electrophilic sites and rendering the products more water soluble (Habig *et al*., 1974). Glutathione conjugates are thought to be metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid. The mercapturic acids are then easily excreted (Habig *et al*., 1974).

Sulfotransferase enzymes catalyze the conjugation of sulfate groups onto a variety of xenobiotic and endogenous substrates that possess acceptor regions such as hydroxyl and amine groups (Chapman *et al.,* 2002). The cofactor 3'-phosphoadenosine 5'-phosphosulfate (PAPS) is required for sulfonation by these enzymes. Although sulfonation generally causes molecules to lose their biological activity, several documented examples indicate that the addition of sulfate can lead to

the formation of highly reactive metabolic intermediates (Chapman *et al.,* 2002). Several sulfotransferase enzymes with different biochemical properties have been characterized in animal and human tissue. Two general classes exist in tissue fractions: the cytosolic enzymes, which are considered important in drug metabolism; and the membrane bound enzymes, which are involved in the sulfonation of glycosaminoglycans and glycoproteins (Chapman *et al.,* 2002). There are currently 10 known sulfotransferases in humans, five of which are known to be expressed in adult liver, namely SULT1A1, SULT1A2, SULT1A3, SULT1E and SULT2A1 (Chapman *et al.,* 2002).

Because the use of complementary and alternative medicines, including herbal medicines, is common among individuals with HIV, the possible herb drug interactions that may occur are relevant to this study. The use of herbal remedies complementary to antiretroviral (ARV) medicine may cause clinically significant interactions, especially because two important groups of ARVs, namely the non-nucleoside reverse transcriptase inhibitors and protease inhibitors are CYP substrates and have a small therapeutic range (Mills *et al.,* 2005). It is expected that in Africa, where almost 80% of the inhabitants use traditional medicine and where the access to antiretroviral agents is growing, the risk for herb ARV interactions is higher (Snyman *et al.,* 2005).

Both protease inhibitors (PIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs), are mainly metabolized by CYP3A4 and, to a lesser extent, CYP2B6, 2C9, 2C19, and 2D6 (van den Bout-van den Beukel *et al.,* 2006). Multiple studies showed the existence of a number of important interaction risks of herbal medicines with antiretroviral agents and because many HIV patients use herbal medication in combination with antiretroviral therapy worldwide, more awareness and further research on the possible side effects and interactions that may occur are necessary (van den Bout-van den Beukel *et al.,* 2006).

Therefore it is of importance that potential drug herb interactions be identified in order to prevent adverse outcomes in patients taking combinations of drugs and herbal supplements (Ventkataramanan *et al.,* 2006). Therefore, the ethanolic and aqueous extracts of *Artemisia afra* and the ethanolic extract of *Annona muricata* were analysed for Glutathione-S-Transferase inhibition as well as CYP3A4 inhibition.

5.2 RESULTS

5.2.1 Detection of Glutathione-S-Transferase (GST) Interference

Interruption of Glutathione-S-transferase activity was determined using the GST assay kit (Sigma), where the change in absorbance per minute of the extract treated GST samples was compared to an inhibitor and extract free control (Appendix Fig. A1). All experiments made use of 55 units/mg of GST, as this was found to be the optimum level of GST activity in order to observe a substantial interruption, if any occurred. All extracts were tested at 0.03125 mg/mL and 0.0625 mg/mL, because higher concentrations of the extracts interfered with the absorbance readings and produced inaccurate results. It was found, for the 0.0625 mg/mL test samples, that the ethanolic extract of *A. muricata* and the aqueous extract of *A. afra* had no significant effect on GST activity, with only 12.83% and 26.79% GST inhibition respectively. The ethanolic extract of *A. afra* however, was found to inhibit GST activity extensively, with 75.67% [Fig. 5.1 (B)]. When observing the tests conducted with the 0.03125 mg/mL concentration, the same trend was observed however with less GST disruption, where the ethanolic extract of *A. muricata*, the aqueous extract of *A. afra* and the ethanolic extract of *A. afra* yielded 11.16%, 19.21% and 61.44% inhibition respectively [Fig. 5.1 (A)]. This concentration indicated that GST activity recovers in a dose dependant manner.

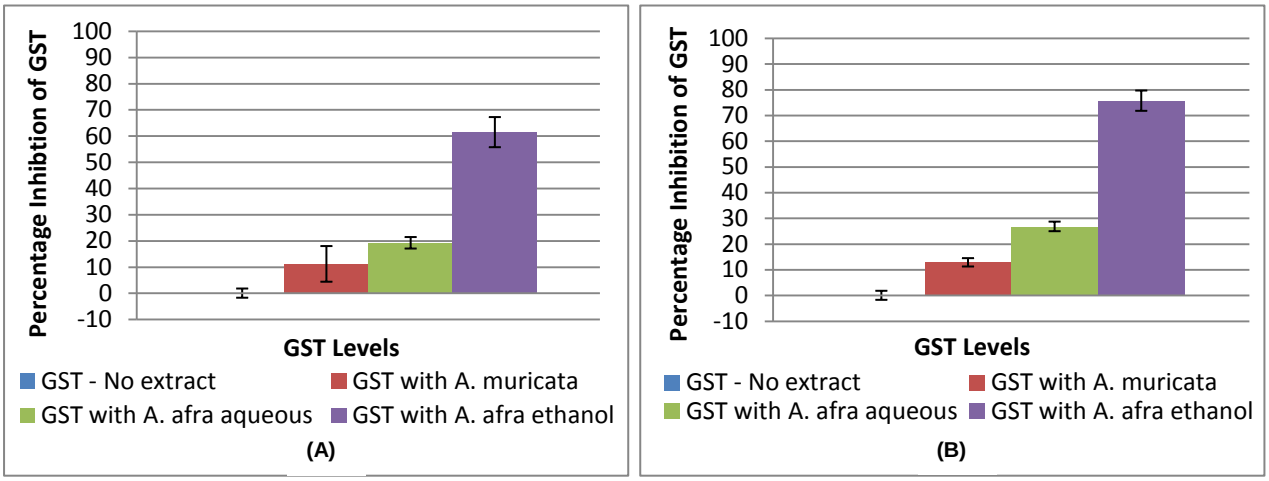

Figure 5.1: Percentage inhibition of GST activity of the extract and inhibitor free control compared to the test samples where the various plant extracts are present. The concentration of the extracts used was (A) 0.03125 mg/mL and (B) 0.0625 mg/mL. Data points represent the mean ± SD of three determinations, representative of three independent experiments.

5.2.2 Detection of Cytochrome 3A4 (CYP3A4) Interference

Susceptibility of CYP3A4 due to the presence of the ethanolic and aqueous extracts of *A. afra* and the ethanolic extract of *A. muricata* was determined by plotting the change in fluorescence per minute and determining the percentage inhibition obtained by the extract treated samples compared to an inhibitor and extract free control (Fig. 5.2). For the three tests conducted, it was found that all extracts exhibited CYP3A4 inhibition to some extent. The ethanolic extract of *A. muricata* had the highest range of CYP3A4 inhibition, with 50% inhibition being achieved at 4.5 µg/mL and 97% inhibition being achieved at 500 µg/mL. The ethanolic extract of *A. afra* exhibited 50% inhibition of CYP3A4 at 25 µg/mL and 98% inhibition at 500 µg/mL. The aqueous extract of *A. afra* produced the lowest amount of CYP3A4 inhibition, however the inhibition was still noteworthy as 200 µg/mL exhibited 50% inhibition of CYP3A4 and 500 µg/mL exhibited 78% inhibition.

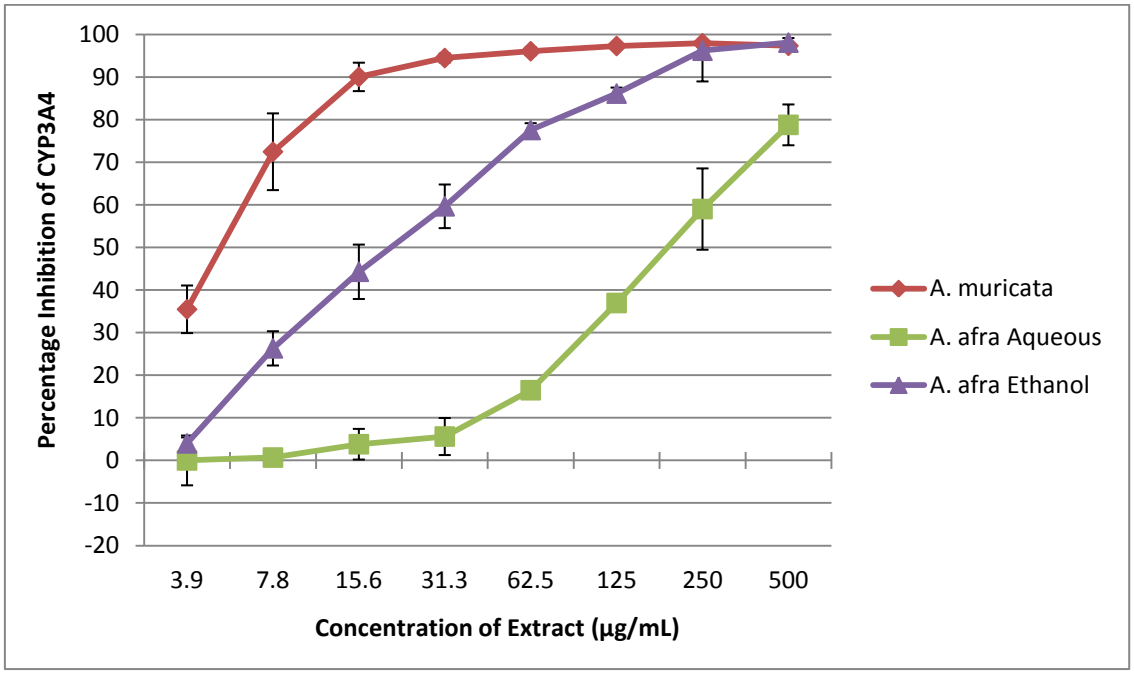

Figure 5.2: Percentage inhibition of cytochrome 3A4 due to the presence of various concentrations of the ethanolic extract of *A. muricata* and the ethanolic and aqueous extracts of *A. afra.* Data points represent the mean ± SD of three determinations, representative of three independent experiments.

5.3 DISCUSSION

It has been estimated that more than 80% of the Southern African population make use of traditional medicines, often in combination with prescription drugs and it is possible for the components of these medicinal plants to alter absorption and metabolism of conventional drugs leading to reduced efficacy of specific drug or systemic drug toxicity. Glutathione-S-transferases are thought to play a role in initiating detoxification of potential alkylating agents including pharmacologically active compounds (Habig *et al.*, 1974). In the experiments where *A. muricata* and the aqueous extract of *A. afra* were present, the GST levels are reasonably similar to the GST control, therefore indicating that they have little effect on its activity. However, the ethanolic extract of *A. afra,* caused a drastic decrease of GST activity, indicating inhibition of GST activity by this extract. In all experiments, 55 units/mg of GST were used and the concentration of all extracts tested was 0.0625 mg/mL and 0.03125 mg/mL, this was due to the fact that higher concentrations of the extracts interfered with the absorbance readings.

Extracts of *A. muricata* and *A. afra* aqueous have little to no effect on GST indicating that their concurrent use with conventional medicines will have no effect on drug metabolism. This is favourable as these two extracts have shown activity against TB and HIV respectively (Chapter 3). The ethanolic extract of *A. afra* has a drastic effect on GST activity interfering with phase II metabolism by inhibiting glutathione conjugation. There is an extensive amount of phytochemicals present in *A. afra* and it is possible they are causing this disruption of GST, however further experiments need to be conducted to determine which compounds are responsible for this inhibition.

The most important pathway for drug metabolism is the family of liver enzymes known as cytochrome P450, particularly 3A4, which is responsible for the metabolism of multiple drugs, including two important groups of ARVs, non-nucleoside reverse transcriptase inhibitors (NNRTI) and protease inhibitors (PI), which are also CYP substrates (Mills *et al.,* 2005; Zhou *et al*., 2007; Patel *et al.,* 2011). The activity of these enzymes may be induced or inhibited by the use of plant medicines (Patel *et al.,* 2011). The consequences of inhibition of these CYPs by plant medicines would be higher antiretroviral plasma levels, thus putting patients at greater risk of serious side effects, whereas induction of CYP would lead to sub-therapeutic plasma levels,

leading to therapeutic failure and an enhanced risk of developing antiviral drug resistance (van den Bout-van den Beukel *et al.,* 2006).

These results indicate that all extracts, the ethanolic extract of *A. muricata* and the ethanolic and aqueous extracts of *A. afra* inhibit CYP3A4 activity in some way. The concentrations at which 50% of the enzyme was inhibited were 4.5 µg/mL, 25 µg/mL and 200 µg/mL respectively. These concentrations are extremely low when comparing them to effective dose concentrations that need to be used in order to treat TB or HIV. It is well known that *A. muricata* and *A. afra* possess many compounds that could bring about this CYP3A4 inhibition, such as certain sesquiterpenoids like chrysanthenyl acetate, several sesquiterpene lactones as well as non-volatile constituents like triterpenes, alkanes and flavonoids, and the highly active acetogenins like annonacin (Van Wyk, 2008; Aminimoghadamfarouj *et al.,* 2011). However, the method and nature of CYP3A4 inhibition is unknown and further experiments need to be conducted to determine if the inhibition is due to substrate competition, substrate alteration or if the extracts change the surface of the actual CYP3A4 enzyme and affect binding in some way.

These effects on GST and CYP3A4 are unfavourable due to the fact that therapeutic treatment will be affected in some way if these extracts are taken simultaneously with antiretroviral drugs and could in turn have a negative effect on the patient as opposed to the desired effect. However, it should be kept in mind that it is difficult to state that these will be the definite effects that will occur in the human body, as *in vivo* experiments may have a different outcome compared to *in vitro* experiments. It was suggested by Zhou *et al.* (2007) that a possible approach to overcoming unfavourable drug interactions with herbal remedies is to design new drugs that are so called ‘hard drugs’ which are not metabolized by CYPs and/or not transported by P-glycoprotein. These drugs cannot be metabolised and are excreted through either the bile or kidney with simple kinetics (Zhou *et al.,* 2007). Thus, their pharmacokinetics will be simplified and usually predictable. When these drugs are administered, the potential for interactions with combined herbal remedies will be greatly reduced (Zhou *et al.,* 2007).

CHAPTER SIX

CONCLUSIONS

6.1 THE RESEARCH IN PERSPECTIVE

As a result of the increase in the spread of multidrug and extensively drug resistant strains of TB and the ineffectiveness of the current treatment against TB and HIV, the implementation of novel treatments is necessary. Due to the rich traditional values that Africa possesses, especially with respect to their traditional medicine and knowledge, many natural compounds can be identified and used to treat diseases like TB and HIV. The speed and accuracy of the method used to identify infection is important. At the start of this project, the effectiveness of two plant extracts, namely *Annona muricata* and *Artemisia afra* against TB and HIV was not known, and due to claims from traditional medicine reports which indicate effectiveness of these two plants against chest infections, coughing and certain antiviral properties, their potential activity against TB and HIV was investigated.

Minimal information regarding methods for anti-TB detection have been suggested, thus this study set out to compare the effectiveness and sensitivity of three *in vitro* assays, namely MABA, INT and flow cytometry, for the detection of anti-TB activity of the plant extracts. MABA was the most sensitive and simplest assay with regards to plant extract testing, and was reproducible, followed by INT and flow cytometry. This is plausible based on the fact that MABA has been used specifically for *M. tuberculosis* testing previously whereas INT and flow cytometry have not. The results indicated anti-TB activity of the *A. muricata* ethanolic extract while both extracts of *A. afra* showed no activity. This is the first report of an *A. muricata* extract showing activity against *M. tuberculosis.*

Due to the co-infection of many HIV infected patients with TB, it was appropriate to investigate whether the extracts that possessed anti-TB activity also had an effect on certain HIV-1 enzymes. Many publications, such as Bedoya *et al.,* 2001; Bessong and Obi, 2006; Lamorde *et al.,* 2010 and Lubbe *et al.,* 2012 explain the effect of many plant extracts on HIV however the majority only focus on general HIV inhibition and

not on target HIV enzyme inhibition. The extract of *A. muricata,* which was the extract which possessed the highest anti-TB activity, had the least effect on HIV-1 reverse transcriptase but was the second most effective against HIV-1 integrase. This provides evidence that using this extract could possibly target HIV and TB simultaneously in one patient. *A. afra* extracts retained the highest activity against HIV, which unfortunately showed no activity against *M. tuberculosis* and thus may not combat both diseases however there is no interpretation of what may occur whilst the extract is *in vivo*.

The objective of chapter 4 was to determine if these extracts, which now possess anti-TB and anti-HIV activity, if administered to patients, will have any toxic effect on confluent Chang liver and HepG2 cells, which represent the liver in the human body and would be responsible for the extract detoxification process as well as the identification of any synergistic, antagonistic or additive effects between the extract and current therapeutic drugs. The MTT assay that was performed allowed for dose response curves to be conducted and IC_{50} values determined. Substantial toxicity from the *A. muricata* extract on both cell lines were found, which is unfavourable due to its anti-TB activity, which indicated that the liver may become damaged if the extract is taken in high doses. The extracts of *A. afra* exhibited no toxicity on the respective cell lines, which indicates high levels of the extract may possibly be taken and should not have any negative effect on the liver, however it should be noted that there is no interpretation of what may occur *in vivo* in a human host. In terms of combination experiments, only the extract of *A. muricata* was examined, but the *A. afra* extracts should not be excluded and it is encouraged that they should also be tested to determine if any synergistic, antagonistic or additive effects may occur. The results revealed that the IC values at which the extracts and the antibiotics are used are very important as there was a large difference in the combination effect at the various IC values. Only the combination index for the IC_{25} for Chang liver cells yielded an antagonistic result, while the remaining yielded synergistic effects on both Chang liver and HepG2 cells. These synergistic effects are unfavourable and indicate that the extract may cause unwanted enhanced toxicity of the rifampicin, the specific drug in this case, whereas the antagonistic result proves positive for possibly protecting the patient in respect to the drug's toxic side effects. This is an interesting finding which may impact on future *in vitro* and *in vivo* experiments.

The observation of synergistic effects that may take place between the extract and the therapeutic drugs, implied that it was plausible to investigate whether any drug interactions could occur, based on the major drug metabolizing enzymes, such as the CYP3A4 and GST enzymes. These drug interactions aid in understanding whether the extracts had an effect on the vital enzymes responsible for detoxifying therapeutic drugs and thus giving insight into whether the extracts may affect the course of drug metabolism in some way and thus the treatment of these diseases. Only the ethanolic extract of *A. afra* had a significant effect on GST, while the other extracts showed no major effect. Investigation of the most important enzymes involved in metabolizing the majority of drugs, CYP3A4, showed interference from all extracts. This implies that the extracts may affect the drug metabolizing pathway where CYP3A4 is dominant, which may impede treatment of patients.

6.2 POTENTIAL FOR FUTURE DEVELPOMENT OF THE WORK

As natural sources are becoming increasingly popular as sources of anti-TB and anti-HIV agents, the need for determining whether they are safe and whether they can be used in conjunction with modern day therapeutic drugs also increases. There are various aspects in this project that can be reviewed and built on for future work. Anti-TB research is fundamental in South Africa, thus it could be useful to compare other methods of detection with the methods mentioned in this project in order to provide a broader comparison with regards to the most sensitive, cheapest and effective method for determination of anti-TB activity. Due to the presence of anti-TB activity in the ethanolic extract of *A. muricata* it might be valuable to ascertain which compound is responsible for inhibition of *M. tuberculosis* and the mode of action, whether it might target the mycolic acids present in the cell wall or target specific enzymes or inhibit DNA or protein synthesis of *M. tuberculosis*. It may also be interesting to determine whether the compound identified in *A. muricata* has the same effect on different species of mycobacteria, such *M. aurum, M. avium* and *M. smegmatis* as well as on resistant strains such as multi drug resistant (MDR) and extensively drug resistant (XDR) *M. tuberculosis*.

For the detection of anti-HIV activity, the focus was on the enzymes reverse transcriptase and integrase because they are involved in the process of replication of HIV, therefore inhibition of either or both enzymes would be beneficial. For the

detection of integrase inhibition, a larger range of concentrations were used for each extract when compared to the range used to detect reverse transcriptase inhibition. Lower concentrations of the various extracts, mainly the aqueous extract of *A. afra* should be used to determine at which concentration the inhibition of HIV-1 reverse transcriptase is halted. The inhibition of HIV-1 integrase by *A. afra* and *A. muricata* has not been previously documented. It would be beneficial to investigate which phytochemical(s) are being produced from each of these plants, that are responsible for this activity against HIV-1 integrase and to establish whether the active chemicals are similar. Testing these extracts on other enzymes which are active in the HIV-1 life cycle would also prove valuable, such as that of the protease enzyme and determine if phytochemicals are acting in the same manner with each different enzyme or if each mode of inhibition varies.

The cytotoxicity testing was conducted on two different cell lines, Chang liver and HepG2 cells, therefore it would be valuable if the extracts were tested on other cell lines, such as the Vero and RAW cell lines, which are representative kidney cells and macrophage cells respectively. Testing the extracts on these particular cells could provide insight into whether they are toxic to the kidney cells, which could prove detrimental as some of the extract will be excreted through the urine, or toxic to the macrophage cells, which may aid anti-TB detection as the macrophage cells would lyse, exposing the latent TB cells and make them vulnerable to treatment. Different cell viability assays could be used, however MTT has been proven to be the easiest, effective and the most sensitive. The synergistic experiments were only conducted with rifampicin as this was the only drug that could be dissolved in a low concentration of DMSO and still have an IC_{50} value on both cell lines, thus testing other anti-TB and anti-HIV drugs would be beneficial to determine if these specific extracts have any combinational effect when used with those therapeutic drugs. It would also be interesting to test the new found synergistic and antagonistic values on the actual microorganisms of *M. tuberculosis* and HIV in order to verify these results and determine whether or not the therapeutic effect will be enhanced or antagonised by the combination of the extract and the drug. It would also be interesting to ascertain whether the same compound is responsible for inhibitory activity, cytotoxicity and synergistic effects.

With regards to drug interactions, there are many enzymes involved in phase one and phase two metabolism, such as sulfotransferases, Uridine diphosphate glucuronosyltransferases and P-glycoproteins, and other cytochrome P450's. It would be valuable to understand all aspects of drug interactions that may occur with these extracts, as they play a vital role in establishing whether a novel drug is accepted for trials or not. It would be important to test the effect of these extracts on sulfotransferase activity especially because sulfotransferases have been implicated in numerous detoxification and bioactivation pathways, however, little is known regarding its endogenous function (Frame *et al.,* 2000).

It should be noted that although these studies look promising, the human system is complicated and it is difficult to ascertain whether observations made *in vitro* will be the same as *in vivo*, therefore *in vivo* studies would be necessary to conclude as to whether the extracts undergo some kind of change when entering the stomach, passing through the colon and inevitably whether they would have the same effect on the TB and HIV diseases in the human host.

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APPENDIX

Table A1: Average absorbance values for the growth control and test samples with the corresponding percentage inhibition for each of the drugs tested and plant extract, *A. muricata.*

Antibiotic	Growth Control Average (nm)	Test Average (nm)	Percentage Inhibition
Rifampicin	23398.86	5642.14	75.89
Ofloxacin	25593.86	2148.14	91.61
Isoniazid	25965.43	2325.29	91.04
Ethambutol	24986.71	1966.00	92.13
Streptomycin	23795.57	1960.14	91.76
Plant - *Annona muricata*	23593.71	4334.29	81.63

Table A2: Various concentrations of ethambutol and *Annona muricata* with the corresponding X – Mean Fluorescence and percentage inhibition values obtained for the antibiotic tested *M. tuberculosis* cells and for the extract treated cells.

Concentration of Ethambutol (µg/mL)	Ethambutol X - Mean Fluorescence	Percentage Inhibition
0	23.9	0.00
15	6.19	74.10
7.5	7.72	67.70
3.75	8.26	65.44
1.875	8.32	65.19
0.938	8.86	62.93
0.469	11.6	51.46
0.234	14.2	40.59
0.117	15.7	34.31
0.059	16.4	31.38

Concentration of *Annona muricata* (µg/mL)	*Annona muricata* X - Mean Fluorescence	Percentage Inhibition
0	23.1	0.00
500	9.9	56.97
250	13.1	42.91
125	13.9	39.94
62.5	15.5	32.69
31.25	19.4	16.04

Table A3: Two way analysis of variance (ANOVA) followed by Tukey's HSD test was applied for statistical analysis with the level of significance set at P < 0.05 was conducted utilising MABA, Flow Cytometry and INT results to determine if the results obtained are significantly different from one another.

	{1}	{2}	{3}	{4}	{5}	{6}
Assay	**M=92.12**	**M=81.03**	**M=71.49**	**M=84.51**	**M=87.28**	**M=84.82**
MABA Control {1}		0.0004	0.0001	0.0229	0.3026	0.0321
MABA Plant {2}	0.0004		0.0024	0.6527	0.0924	0.5663
Flow Cytometry Control {3}	0.0001	0.0024		0.0001	0.0001	0.0001
Flow Cytometry Plant {4}	0.0229	0.6527	0.0001		0.8266	1.0000
INT Control {5}	0.3026	0.0924	0.0001	0.8266		0.8869
INT Plant {6}	0.0321	0.5663	0.0001	1.0000	0.8869	

*M = Mean
*Red indicates statistical significance at the 5% level (p<0.05)

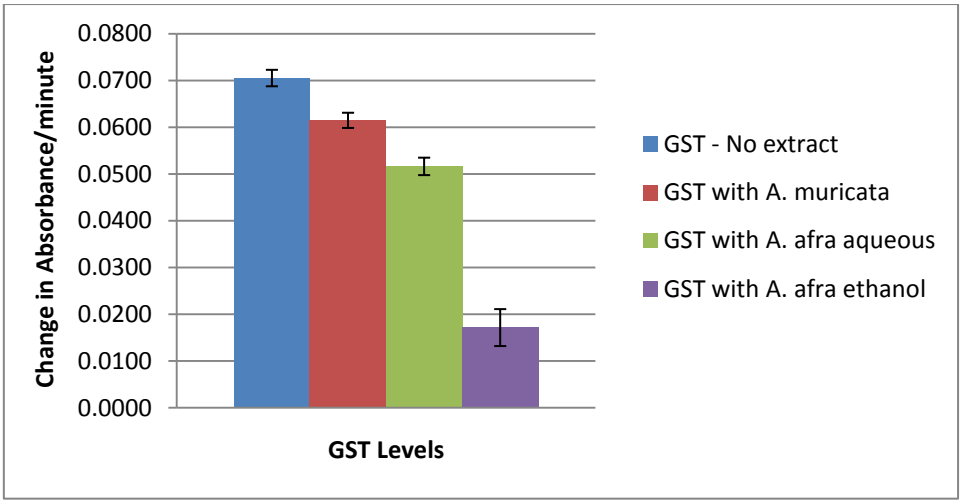

Figure A1: GST activity, expressed in change in absorbance per minute, with no extracts or inhibitor present compared to GST activity with the various plant extracts present. Data points represent the mean ± SD of three determinations, representative of three independent experiments.

LIST OF CONFERENCE PRESENTATIONS

Paper presentation

Pruissen, MC, M. van de Venter and S. Govender. 2012. Inhibitory activities of *Artemisia afra* and *Annona muricata* against *Mycobacterium tuberculosis* and Human Immunodeficiency virus. Indigenous Plant Use Forum (IPUF), University of Limpopo, 2nd – 5th July. (This presentation received 2nd best paper presentation by a young scientist).