

# CHAPTER 7

## DETERMINATION OF THE ANTIMYCOBACTERIAL ACTIVITY OF THE FRACTIONS AND ISOLATED COMPOUNDS FROM THE ROOTS OF *GALENIA AFRICANA* L. *VAR. AFRICANA*

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### 7.1. Introduction

The escalation of infections caused by *M. tuberculosis*, particularly those caused by MDR-TB strains is of considerable global concern. Since no new antituberculosis drugs have become available during the last forty years, there is obvious need for new and effective anti-tubercular drugs. There are reports of phytochemical analysis of essential oils, glycolipids, sesquiterpenoids, triterpenoids terpenes, steroids, tannins and triterpenoid as having potential antimycobacterial activity (Rivero-Cruz *et al.*, 2005, Redo *et al.*, 2006, Mann *et al.*, 2008,). Previous studies of isolated compounds such as naphthoquinones (diospyrin, isodiospyrin, mamegakinone, 7-methyljuglone, neodiospyrin and shinanolone) were found to have antimycobacterial properties (Lall, 2005). Prenylated xanthenes ( $\alpha$ - and  $\beta$ -Mangostins and garcinone $\beta$ ) isolated from *Garcinia mangostana*, were found to have strong antimycobacterial inhibitory effect (Suksamrarn *et al.*, 2003).

In this study, antimycobacterial activity of the isolated compounds from fractions of the ethanol extract of the roots of *G. africana* are evaluated using two *Mycobacterium* species.

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### **7.2. Materials and Methods**

Fractionation of fractions and isolation of compounds from *G. africana* are described in Chapter 6. Ciprofloxacin and Isoniazid (INH) were purchased from Sigma-Aldrich, Johannesburg, South Africa. The drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) and *M. smegmatis* (Mc<sup>2</sup> 155) were similar to the ones used previously (Chapter 4 and 7).

#### **7.2.1. Microplate susceptibility testing against *M. smegmatis***

To test for antimycobacterial activity, the ethanol extract, Fractions I – VI and the isolated compounds from *G. africana*, were tested against *M. smegmatis* using the microplate dilution method described in Chapter 4, section 4.2.3.2. Each sample was dissolved in 10% DMSO in sterile Middlebrook 7H9 broth base to obtain a stock concentration of 100.0 mg/mL and 0.5 mg/mL respectively. Dilution series of the sequential extracts, Fractions I – VI and the isolated compounds to be evaluated were made with 7H9 broth to yield volumes of 100.0 µL/well with the final concentrations ranging from 25.0 to 0.195 mg/mL. Ciprofloxacin at a final concentration of 0.156 mg/mL, served as the positive drug control. The MIC and MBC were determined as described in Chapter 4, section 4.2.3.2.

#### **7.2.2. Antitubercular rapid radiometric assay using *M. tuberculosis***

The radiometric respiratory system with the BACTEC apparatus was used for the susceptibility testing of *M. tuberculosis*. Bacterial cultures utilized in this study were grown from specimens received from the Medical Research Council (MRC) in Pretoria. A drug-susceptible strain of *M. tuberculosis*, H37Rv obtained from American Type, MD.USA Culture Collection (ATCC), 27294, was used in the screening procedure. Each sample was dissolved at 10.0 mg/mL in DMSO and added to 4.0 mL of BACTEC 12B broth to achieve the final concentrations ranging from 5.0 – 0.05 mg/mL (in triplicates, one with PANTA, and two without PANTA, an

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antimicrobial supplement). The BACTEC drug susceptibility testing was also done for the primary drug INH at concentration of 0.2 µg/mL against the H37Rv sensitive strain. Preparation of bacterial cultures and the testing procedures were the same as described in Chapter 4, section 4.2.3.4.

### **7.2.3. Direct bioassay**

Due to insufficient amounts of the isolated compounds, only one compound, (2*S*)-5,7,2'-trihydroxyflavanone (40.0 µg/mL), was evaluated on TLC plates by applying a small spot of 20.0 µL to silica gel 60 PF<sub>254</sub> plates. The plates were developed in CH<sub>3</sub>(CH<sub>2</sub>)<sub>4</sub>CH<sub>3</sub> : EtOAc (8:2) and dried carefully (Figure 7.2a). The 24 hour *M. smegmatis* (1.26 x 10<sup>8</sup> CFU/mL) in 7H9 broth was centrifuged at 1000 rpm for 15 minutes. The supernatant was discarded and the pellet was resuspended in fresh 7H9 broth. A fine spray was then used to apply the bacterial suspension onto the TLC plates (Dilika and Meyer, 1996). The plates were then incubated at 37°C for 24 hours in humid conditions. After incubating, the plates were sprayed with 2.0 mg/mL INT and the inhibition zones were noted.

## **7.3. Results and Discussion**

In the present study, the ethanol extract, fractions and the compounds were found to have inhibitory activity. The ethanol extract of *G. africana* was found to be active at 0.78 mg/mL and 1.2 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. *G. africana* had an MBC of 1.56 mg/mL against *M. smegmatis* (Table 7.1). Fractions II - V showed activity and Fraction IV was the most active fraction exhibiting an MIC of 0.12 and 0.5 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. Fraction IV also showed bactericidal properties with an MBC of 0.06 mg/mL. The antituberculosis activity of (2*S*)-5,7,2'-trihydroxyflavanone showed activity at 0.03 and 0.1 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively. (2*S*)-5,7,2'-trihydroxyflavanone showed bactericidal effects at 0.12 mg/mL against *M. smegmatis* (Table 7.1).

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**Table 7.1.** Antimycobacterial activity of the ethanol extract, fractions and isolated compounds from *G. africana* leaves against *M. smegmatis* and *M. tuberculosis*

Tested samples	<i>M. smegmatis</i>		<i>M. tuberculosis</i>	
	MIC <sup>a</sup> (mg/mL)	MBC <sup>b</sup> (mg/mL)	MIC (mg/mL)	ΔGI <sup>c</sup>
Ethanol extract of <i>G. africana</i>	0.78	1.56	1.20 (S <sup>d</sup> )	0.0 ± 0.0
Fraction I	na <sup>e</sup>	nt <sup>f</sup>	na <sup>g</sup>	227.5 ± 314.6
Fraction II	0.50	na	na	200.5 ± 122.3
Fraction III	0.25	na	1.00 (S)	18.5 ± 4.9
Fraction IV	0.12	0.06	0.50 (S)	20.0 ± 1.4
Fraction V	0.25	na	1.00 (S)	28.0 ± 5.6
Fraction VI	na	nt	na	35.5 ± 6.3
(2 <i>S</i> )-5,7,2'-trihydroxyflavanone	0.03	0.12	0.10 (S)	8.0 ± 2.8
( <i>E</i> )-2',4'-dihydroxychalcone	0.12	0.06	0.05 (S)	2.0 ± 1.4
( <i>E</i> )-3,2',4'-trihydroxychalcone	na <sup>h</sup>	nt	0.10 (S)	2.0 ± 1.4
( <i>E</i> )-3,2',4'-trihydroxy-3'-methoxychalcone (Novel)	na	nt	0.05 (S)	19.0 ± 2.6
Ciprofloxacin (positive drug control for <i>M. smegmatis</i> )	0.15	0.31	nd <sup>i</sup>	nd
Isoniazid (positive drug control for <i>M. tuberculosis</i> )	nd	nd	2 x 10 <sup>-4</sup> (S)	13.0 ± 0.7

<sup>a</sup>Minimum inhibitory concentration.

<sup>b</sup>Minimum bactericidal concentration.

<sup>c</sup>ΔGI value (mean ± SD) of the control vial (10<sup>-2</sup>), 38.0 ± 3.8 for the sensitive strain.

<sup>d</sup>Susceptible.

<sup>e</sup>Not active at the highest concentration tested (6.25 mg/mL).

<sup>f</sup>Not tested for MBC determination.

<sup>g</sup>Not active at highest concentration tested (200.0 mg/mL).

<sup>h</sup>Not active at the highest concentration tested (0.62 mg/mL).

<sup>i</sup>Not determined.

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The other compound, (*E*)-2',4'-dihydroxychalcone showed inhibitory activity at 0.12 and 0.05 mg/mL against *M. smegmatis* and *M. tuberculosis* respectively, with MBC of 0.06 mg/mL against *M. smegmatis* (Table 7.1).

(2*S*)-5,7,2'-trihydroxyflavanone previously isolated by Miyaichi *et al.*, 2006 from the roots of *Scutellaria amabilis* has been reported to have anticancer and anti-inflammatory activity. Other researchers (Vries *et al.*, 2005; Zampini *et al.*, 2005; Svetaz *et al.*, 2007), isolated (*E*)-2',4'-dihydroxychalcone from crude extracts of *G. africana* and *Zuccagnia punctata* which has been reported to have antifungal and antibacterial activity. Su *et al.*, 2003 isolated (*E*)-2',4'-dihydroxychalcone from *Muntingia calabura* and tested for an *in vitro* quinone reductase induction assay. The other compounds, (*E*)-3,2',4'-trihydroxychalcone (not reported from natural sources but previously isolated by Severi *et al.*, 1998 and (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone (novel compound) are isolated from *G. africana* for the first time.

In another study, alpinumisoflavone, genistein (5,7,4'-trihydroxyisoflavone), laburnetin and luteolin (3',4',5,7-tetrahydroxyflavone), were valuated against *M. smegmatis* and *M. tuberculosis*. Laburnetin showed the best activity against *M. tuberculosis* exhibiting an MIC of 4.88 µg/mL (Kuetze *et al.*, 2008).

Lin *et al* (2002), evaluated a series of flavonols (3-hydroxyflavones), flavonones, chalcones (1,3-diarly-2-propen-1-ones) and chalcone-like compounds (1,3-heteroaromatic ring-substituted 2-propen-1-ones) for inhibitory activity against *M. tuberculosis*. Chalcones with a 2-hydroxyl group: 1-(2-hydroxyphenyl)-3-(3-chlorophenyl)-2-propen-1-one and 1-(2-hydroxyphenyl)-3-(3-iodophenyl)-2-propen-1-one, demonstrated 92% inhibition at 12.5 µg/mL. Chalcone-like compounds (heterocyclic ringsubstituted 2-propen-1-one): 1-(4-fluorophenyl)-3-(pyridin-3-yl)-2-propen-1-one, 1-(3-hydroxyphenyl)-3-(phenanthren-9-yl)-2-propen-1-one, 1-(pyridin-3-yl)-3-(phenanthren-9-yl)-2-propen-1-one and 1-(furan-2-yl)-3-phenyl-2-propen-1-one, exhibited 96 - 98% inhibition at 12.5 µg/mL. All tested flavones and flavanones were found to have moderate to weak antimycobacterial activity.

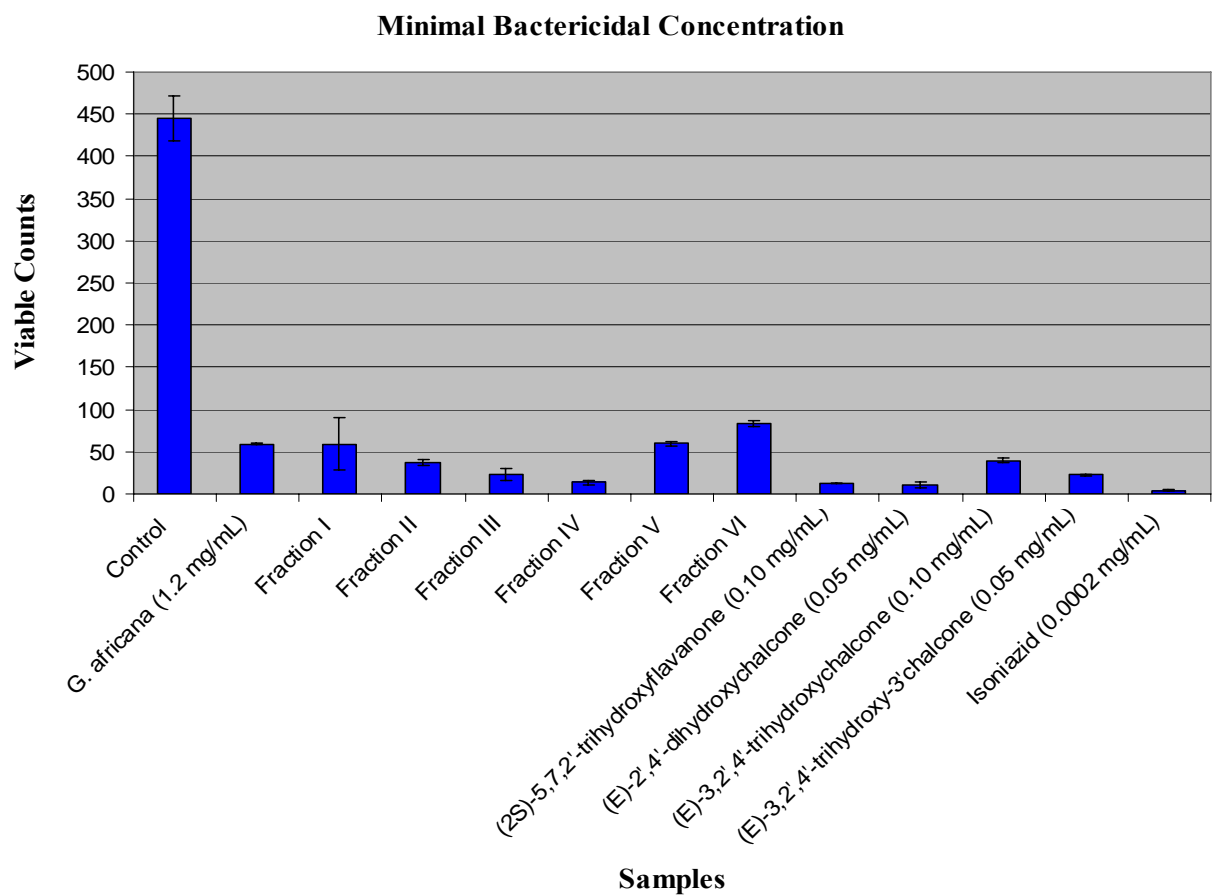
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*In vitro* studies of novel chalcones synthesised by condensing 2-(4-carboxyphenylazo) acetoacetate, were screened for their antimycobacterial activity against *M. tuberculosis* H37Rv using a BACTEC-460 radiometric system. The compounds exhibited >90% inhibition against *M. tuberculosis* at 6.25 µg/mL. This antimycobacterial activity indicated that the presence of 2-nitro, 3-nitro and 4-methoxy substitution on chalcone produced remarkable improvements in antitubercular activity (Trivedi *et al.*, 2008). A few compounds belonging to flavones, flavonones and chalcones showed comparatively better activity than the compounds tested in the present study.

The bactericidal effect of the ethanol extract, fractions, purified compounds and INH in the BACTEC system, was compared between the treated and untreated cultures. 100.0 µL of the bacterial suspensions from BACTEC vials (exhibiting MICs) at the end of the experiment were plated on 7H11 agar medium for viable count enumeration. Only selected results (expressed as mean viable counts ± standard error) in case of the treated and untreated vials are illustrated in figure 7.1. Fractions IV resulted in 1 log (90%) killing at 0.5 mg/mL. The four isolated compounds were more bactericidal than the crude ethanol extract and resulted in a 2 log (99.5%) killing at 0.1 to 0.05 mg/mL (Figure 7.1).

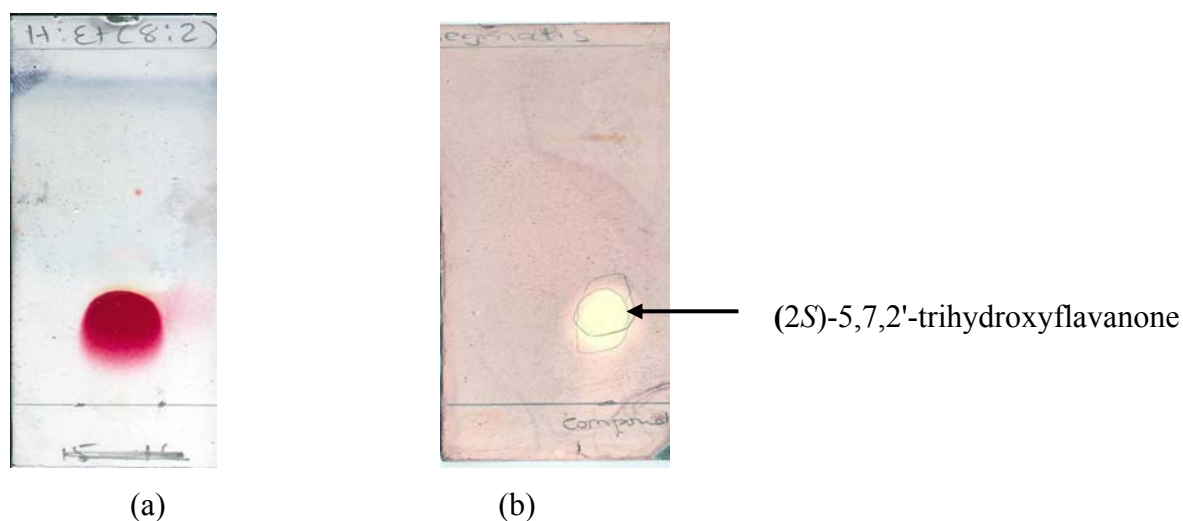
In a bioautographic assay, (2*S*)-5,7,2'-trihydroxyflavanone sprayed with INT and reincubated at 37°C for 4 hours, the plates changed colour to a reddish-pink where bacterial growth occurred and a zone of inhibition by the compound was observed (Dunigan *et al.*, 1995; Figure 7.2b).

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**Figure 7.1.** The comparative bactericidal effect of the ethanol extract, fractions and isolated compounds of *G. africana* against the drug-susceptibility strain of *M. tuberculosis*. Results illustrate the mean of the viable bacterial counts  $\pm$  standard error in the treated vials as compared to the untreated control vials.

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**Figure 7.2.** Zones of inhibition of *M. smegmatis* on TLC plates in a direct assay of (2S)-5,7,2'-trihydroxyflavanone isolated from the *G. africana* ethanol extract. Solvent systems: hexane: ethyl acetate (8:2)

(a) compound not sprayed with *M. smegmatis*

(b) compound sprayed with *M. smegmatis*

#### 7.4. Conclusion

This is the first report of the antituberculosis activity of ethanol extract of *G. africana* and its isolated compounds against *M. tuberculosis*.

Our findings indicated some correlation between the activities of the ethanol extract of *G. africana* with its constituents when screened against both *M. smegmatis* and *M. tuberculosis*. Selection of plants by ethnobotanical criteria offers a good probability of finding candidates which contain compounds active against mycobacteria (Lall and Meyer, 1999).



## CHAPTER 8

# SYNERGISTIC EFFECT, CYTOTOXICITY AND INTRACELLULAR ACTIVITY AGAINST *M. TUBERCULOSIS*

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### 8.1. Introduction

The recent upsurge in the incidence of TB with the significant emergence of multidrug-resistant cases has focused on the priority of discovering effective new drugs and on the strategies to augment the potential of existing drugs against *M. tuberculosis*. *M. tuberculosis* is a complex, resilient organism, and it is important to recognize that new, better TB treatments will still require drugs to be taken in combination, in order to reduce TB's six month treatment time, be effective against drug-resistant strains, and be compatible with anti-retroviral therapy used to treat patients with TB-HIV. One of the major components of the strategic plan to eliminate TB is the use of antimycobacterial drugs to destroy *M. tuberculosis* residing in the body. INH has been the drug choice for over 30 years in treating a quiescent condition and in prophylaxis therapy, however, long-term therapy results in hepatitis. This, coupled with the emergence of INH-resistant TB, has led to an increasing need for alternative preventive-therapy regimens (American Thoracic Society. 1986, Ferebee, 1970).

No new TB drugs have been discovered in the last 40 years and emerging strains of the bacterium, that are resistant to multiple drugs, are increasing. Current therapy for drug-sensitive TB recommended by WHO consists of a cocktail of four drugs (INH, RIF, PZA and EMB), taken for six to nine months. The first-line drugs are cheap and have few side effects. Second-line or third-line drugs are more expensive, less potent and some are as toxic as cancer chemotherapy and require hospitalization.

MDR-TB treatment using currently available second-line drugs may cure only 65% - 75% of patients (Mukherjee *et al.*, 2004). Drug-resistant TB is “humanmade”: it results from treatment with inadequate drugs or drug regimens, improper case management and preventable transmission. New drugs are badly needed that can outmatch mutated strains and shorten and simplify treatment.

Since the main route of entry of TB is the respiratory route, alveolar macrophages are the important cell types, which combat the pathogen (Alamelu, 2004). The initial step in the infection process of TB is inhaling the bacteria which is readily phagocytosed, processed and presented by alveolar macrophages to the T-lymphocytes (Hockings and Golde, 1979). *M. tuberculosis* is an obligatory aerobic, intracellular pathogen that resides predominantly within macrophages. Macrophages are leukocytes cells within the tissues that originate from specific white blood cells. Macrophages develop from bone marrow precursors which mature and enter the bloodstream as monocytes. When a monocyte enters a damaged tissue through a blood vessel, it undergoes a series of changes to become a macrophage. The main role of the macrophage is the removal of necrotic debris (unusual death of cells and living tissues) and dust in the lungs. The removal of dust and necrotic tissue is to a greater extent handled by fixed macrophages, which stay in strategic locations such as the lungs, liver, neural tissue, bone, spleen and connective tissue, ingesting foreign materials such as dust and pathogens, calling upon wandering macrophages if needed. When a macrophage ingests a pathogen, it will present an antigen of the pathogen to a corresponding helper T cell. The antigen presentation results in the production of antibodies that attach to the antigens of the pathogens, making it easier for the macrophages to adhere to their cell membrane and phagocytose. The pathogen becomes trapped in a food vacuole, which then fuses with a lysosome. Within the lysosome, enzymes and toxic peroxides digest the invader. However, some bacteria such as *M. tuberculosis* have become resistant to these methods of digestion (Fenton, 1998).

The entry of *M. tuberculosis* into the host macrophage is the key component of TB pathogenesis. Phagocytosis of *M. tuberculosis* by alveolar macrophages is the first event in the host-pathogen relationship that decides outcome of infection. Within 2 to

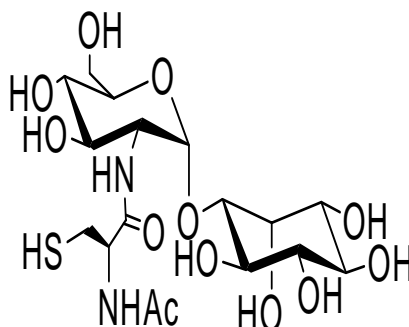
6 week of infection, cell-mediated immunity (CMI) develops, and there is an influx of lymphocytes and activated macrophages into the lesion resulting in granuloma formation. The exponential growth of the bacilli is checked and dead macrophages form a caseum. The bacilli are contained in the caseous centers of the granuloma. The bacilli may remain forever within the granuloma, or get re-activated later and may get discharged into the airways after enormous increases in numbers causing necrosis of bronchi and cavitation. Fibrosis represents the last-ditch defense mechanism of the host, and is the surrounding of the central area of necrosis to wall off the infection when all other mechanisms have failed (Alamelu, 2004). In order for *M. tuberculosis* to bind on monocytes macrophages, the complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptor molecules play an important role in the binding of the organisms to the phagocytes (Schlesinger, 1994). The interaction between MR on phagocytic cells and mycobacteria seems to be mediated through the mycobacterial surface glycoprotein lipoarabinomannan (LAM). Prostaglandin E2 (PGE2) and interleukin (IL)-4 (a Th2-type cytokine), regulate CR and MR receptor expression, and the interferon- $\hat{I}^3$  (IFN- $\hat{I}^3$ ) decreases the receptor expression, resulting in diminished ability of the mycobacteria to adhere to macrophages (Barnes *et al.*, 1994). Surfactant protein receptors, CD14 receptor and the scavenger receptors also have a role in mediating bacterial binding (Gaynor *et al.*, 1995). Novel approaches to therapy and new drugs are urgently needed in order to act within the host macrophages and to have direct access to the dormant organisms that presumably are within the macrophages (Quenelle *et al.*, 2001).

The chemotherapy of pathogenic organisms relies on metabolic differences between pathogens and their mammalian hosts. A possible mode of action in *M. tuberculosis*, is the presence of thiols. The thiol compounds such as: cysteine, coenzyme A, glutathione, lipoamide, transglutaminase methanethiol, mycothione, play an important role in maintaining thiol groups, required for the activity of many enzymes in a reduced state and serve as cofactors for a number of enzymes involved in the detoxification and export of toxic compounds from cells. *M. tuberculosis* lacks glutathione, but instead maintains millimolar concentrations of the structurally distinct low molecular weight thiol mycothiol (MSH, Figure 8.1), found in actinomycetes,

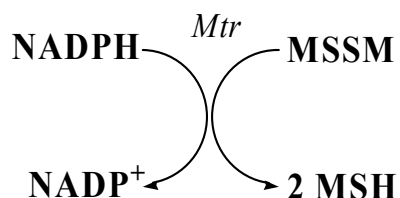
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mycobacteria and streptomycetes. MSH is comprised of N-acetylcysteine amide-linked to GlcN-alpha (1-1)-Ins (Newton and Fahey., 2002). In *M. tuberculosis*, MSH protects the bacteria from toxic oxidants and antibiotics. MSH-deficient mycobacteria exhibit increased sensitivity to oxidative stress, making this redox pathway a potential biological target for novel antitubercular chemotherapies (Patel and Blanchard, 1999). Many compounds like naphthoquinones are known to operate as subversive substrates with flavoprotein disulfide reductases such as glutathione reductase, trypanothione reductase and lipoamide dehydrogenase (Biot *et al.*, 2004., Salmon-Chemin *et al.*, 2001). The functions of these enzymes involve the NAD(P)H-dependent reduction of disulfide bonds in proteins or oxidised version of low molecular weight thiols such as glutathione. The NADPH-dependent enzyme mycothiol disulfide reductase (Mtr, Figure 8.2) helps to maintain an intracellular reducing environment by reducing MSSM back to MSH (Spies *et al.*, 1994). Since there is no mammalian counterpart to the pathway it should be possible to achieve a selective inhibition of mycothiol biosynthesis. Enzymes involved in the biosynthesis of mycothiol could make attractive drug targets and another possibility is to reduce the effect of endogenous mycothiol to inhibit enzymes involved in degrading the mycothiol-antibiotic complex (Newton *et al.*, 2000).

In the present study, we investigated the synergistic activity of only two isolated compounds from the roots of *G. africana* (same as in Chapter 7) in combination with INH against *M. tuberculosis*. The ethanol extract, and the two isolated compounds were also evaluated against the TB infected human macrophage cell line. The biological importance of Mtr, with its ability to turnover antimycobacterial compounds as subversive substrates and also to provide an insight into possible mode of action as properties with *M. tuberculosis* Mtr is included.



**Figure 8.1. Chemical structure of mycothiol (MSH)**



**Figure 8.2. Structure of mycothiol disulfide reductase (Mtr)**

## 8.2. Materials and Methods

The drug-susceptible strain of *M. tuberculosis*, H37Rv (ATCC 27264) was similar to the one used previously (Chapter 4 and 7). INH (Sigma-Aldrich, South Africa) was purchased in a powder form. Due to insufficient amounts of the compounds, only two compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone isolated from the roots of *G. africana*, as described in Chapter 6, were tested.

### 8.2.1. MIC determination: combination drug-action

MIC's of the test compounds and INH were determined by the radiometric method as described in Chapter 4 and 7. Final concentrations of each test compounds ranged in a two-fold dilution from 100.0 to 6.25 µg/mL and for INH from 0.2 to 0.0125 µg/mL.

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### **8.2.1.1. Combined drug action by the radiometric method**

The activity of the individual drugs and the two-drug combination were evaluated at sub-MIC levels so that each compound were present at concentrations corresponding to 1/2; 1/4; 1/8; 1/16 and 1/32 of the documented MIC. Analysis of the drug combination data was achieved by calculating the fractional inhibitory concentration (FIC) index as follows:  $FIC = (MIC_{a \text{ combination}} / MIC_{a \text{ alone}}) + (MIC_{b \text{ combination}} / MIC_{b \text{ alone}})$ . The FIC was interpreted as:  $FIC \leq 0.5$ , synergistic activity;  $FIC = 1$ , indifference / additive activity;  $FIC \geq 2$  or more, antagonistic activity. Subscripts a and b represent the two different compounds (Bapela *et al.*, 2006; Berenbaum 1978; De Logu *et al.*, 2002).

### **8.2.2. Cell line**

Culture of human monocytes, U937, displaying macrophage-like activity, were cultured in RPMI (developed at Roswell Park Memorial Institute) 1640 medium (pH 7.2 Sterilab, South Africa), supplemented with 10% fetal bovine serum and 2 mM<sub>L</sub>-glutamine and with antibiotics-penicillin, streptomycin and fungizone solution (0.1%). Cells were grown to a density of  $5 \times 10^8$  cells/mL (the cells were grown till confluent-thus till the concentration of the cells were very high), centrifuged and washed with phosphate buffered saline solution. The cells were then counted with a hemacytometer using a light microscope to determine the concentration of the cells and resuspended in the correct amount of supplemented RPMI 1640 medium as calculated and resuspended in supplemented RPMI 1640 medium. The concentration of cells was thus adjusted to  $10^5$  cells/mL. At first 1.0 uL of TPA (12-o-tetradecanoyl 13-acetate) DMSO solution was added to 10.0 mL of complete medium, then 20.0 uL of the solution was added to all of the wells receiving the cells and finally 180.0 uL of cell suspension was added to each well of the 96-well tissue culture plate and incubated for 24 hours to stimulate maturation of the monocytes (Passmore *et al.*, 2001; Hosoya and Marunouchi, 1992).

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### **8.2.2.1. Cytotoxicity: U937 cell line**

Cytotoxicity was measured by the 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-5-[(phenylamino)carbonyl]-2-*H*-tetrazolium hydroxide (XTT) method using the Cell Proliferation Kit II (Roche Diagnostics GmbH). 100.0 µL of U937 cell lines ( $1 \times 10^5$  cells/mL) were seeded into the inner wells of a microtiter plate, while in the outer wells 200.0 µl of incomplete medium was added. The plates were incubated for 24 hours to allow the cells to attach to the bottom of the plate. Dilution series were made of the ethanol extract of *G. africana* at the various concentrations (400.0 to 3.125 µg/mL) and for (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone, at 100.0 to 1.563 µg/mL. These dilutions were added to the inner wells of the microtiter plate and incubated for 72 hours. After 72 hours, 50.0 µL of XTT reagent (1.0 mg/mL XTT with 0.383 mg/mL PBS) was added to the wells and the plates were then incubated for 1-2 hours. The positive control, (Doxorubin), at final concentration ranging from 0.5 - 200.0 µg/mL, was included. After incubation, the absorbance of the colour complex was spectrophotometrically quantified using an ELISA plate reader, which measures the OD at 450 nm with a reference wavelength of 690 nm. DMSO was added to serve as the control for cell survival. The 'GraphPad Prism 4', statistical program was used to analyse the 50% inhibitory concentration (IC<sub>50</sub>) values.

### **8.2.2.2. MIC determination: U937 cell line**

The ethanol extract and each compound were dissolved at 20.0 mg/mL and 10.0 mg/mL in DMSO respectively, diluted further in complete RPMI 1640 medium to obtain concentrations that were 10 x higher than the final concentration required in the wells.

The final concentrations of the ethanol extract ranged from 0.1 to 0.025 mg/mL and for the test compounds from 0.4 to 0.010 mg/mL. The primary drug INH at concentrations of 0.6 and 0.4 µg/mL was included. The highest concentration of DMSO in the wells for testing against *M. tuberculosis* was 1% (Bapela, 2005).

### **8.2.2.3. Infection of cells**

The cells were first washed with PBS three times, to remove extra-cellular bacteria and then infected with tubercle bacilli at a ratio of 10 to 20 bacilli per cell. The macrophages were allowed to phagocytize the bacteria for 4 hours at 37<sup>0</sup>C. Then the number of intracellular organisms was determined by lysing the macrophages with 0.25% (w/v) sodium dodecyl sulfate (SDS), doing serial dilutions and plating the lysate on 7H11 agar medium for viable count determinations. After phagocytosis, 100.0 µL of the fresh medium containing the desired antimycobacterial agents (extract and compounds) was added to each macrophage containing well, and the culture was enumerated after lysing the macrophages on day 5. After 5 days of incubation, the contents of each well were resuspended with a needle and syringe, and 100.0 µL was transferred to a degassed 4.0 mL BACTEC 12B vial. The vials were incubated at 37<sup>0</sup>C overnight. The change in GI was recorded daily until the vials containing the 1:100 dilution of the untreated control suspension reached a GI of 30 or more. Interpretation of the results was the same as in Chapter 4, section 4.2.3.4. The MBC of the tested samples was assessed by plating the bacterial suspensions from the BACTEC vials which exhibited MIC, at the end of the experiment, on 7H11 agar medium for viable count enumeration (Rastogi *et al.*, 1991).

### **8.2.3. Subversive substrate properties with mycothiol reductase**

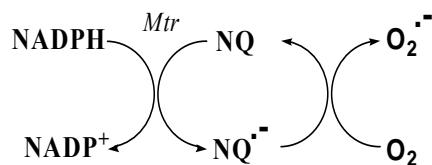
It was decided to investigate the effect of one compound, (2S)-5,7,2'-trihydroxyflavanone on mycothiol reductase. Other isolated compounds were insufficient. At our department a group of naphthoquinones have been identified with potent antituberculosis activity (Lall and Meyer., 2001). Since naphthoquinones are known to operate as subversive substrates with flavoprotein disulfide reductase such as glutathione reductase, trypanothione reductase and lipoamide dehydrogenase (Biot *et al.*, 2004., Salmon-Chemin *et al.*, 2001), it was therefore, decided to include these naphthoquinones also in this study in order to compare with the compound '(2S)-5,7,2'-trihydroxyflavanon' isolated from *G. africana*. The derivatives of naphthoquinones used in this study were kindly donated by Dr Anita Mahapatra and



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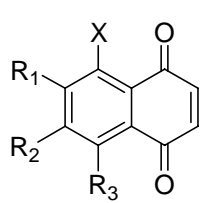
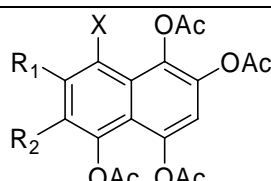
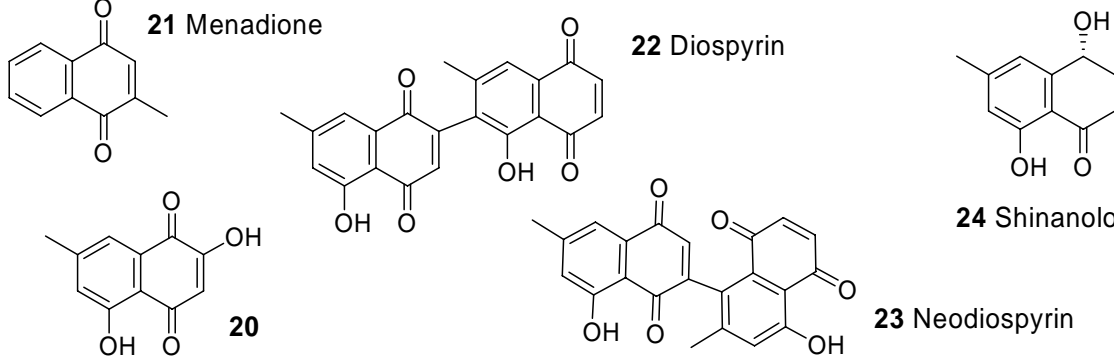
Dr Frank van der Kooy (Table 8.1). The enzyme-mediated toxicity of quinones or naphthoquinones is a consequence of their enzymatic reduction to semiquinone radicals. The naphthoquinone is then regenerated via the concomitant reduction of oxygen to toxic superoxide anion radicals. In this manner the naphthoquinone substrate is regenerated and the futile redox cycle continues (Figure 8.3). It seems plausible that some of these naphthoquinones could be exerting their biological activity as subversive substrates with similar disulfide reductases found in *M. tuberculosis*. Due to the availability of this '(2*S*)-5,7,2'-trihydroxyflavanone' only, one compound was included in this study.

Recombinant *M. tuberculosis* mycothione reductase (Mtr) was purified from *M. smegmatis* MC<sup>2</sup>155 transformant (a generous gift from J. Blanchard) as previously described (Patel *et al.*, 1999). Only (2*S*)-5,7,2'-trihydroxyflavanone was tested. Subversive substrate assays of (2*S*)-5,7,2'-trihydroxyflavanone with Mtr were carried out at 30°C in 1 cm<sup>3</sup> of 50 mM HEPES (pH 7.6), 0.1 mM EDTA containing Mtr (30.0 µg), NADPH (70.0 µM), and varying concentrations of substrate. Mtr was pre-incubated with NADPH for 5 min at 30°C before initiating the reaction by addition of a DMSO solution of (2*S*)-5,7,2'-trihydroxyflavanone. The final DMSO concentration in the assays was (2 % (v/v)). Enzyme activity was monitored by the decrease in absorbance at 340 nm due to NADPH consumption.



**Figure 8.3. Enzymatic reduction of naphthoquinone to semiquinone**

**Table 8.1** List of naphthoquinones studied for disulfide reductase activity

Structure	NQ	X	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>
	<b>1</b>	H	H	H	OH
	<b>2</b>	F	Me	H	OH
	<b>3</b>	Cl	Me	H	OH
	<b>4</b>	Br	Me	H	OH
	<b>5</b>	Cl	H	Me	OH
	<b>6</b>	H	Me	H	OH
	<b>7</b>	H	H	Me	OH
	<b>8</b>	H	Me	H	OAc
	<b>9</b>	Cl	Me	H	OAc
	<b>10</b>	Cl	H	Me	OAc
	<b>11</b>	H	Me	H	OMe
	<b>12</b>	Cl	Me	H	OMe
	<b>13</b>	Cl	H	Me	OMe
	<b>14</b>	H	Me	H	OEt
	<b>15</b>	Cl	Me	H	OEt
	<b>16</b>	Cl	H	Me	OEt
	<b>17</b>	H	Me	H	
	<b>18</b>	Cl	Me	H	
	<b>19</b>	Cl	H	Me	
					

## 8.3. Results and Discussion

### 8.3.1. Combination drug action

The development of antibiotic resistance can be natural or acquired and this can be transmitted within same or different species of bacteria. Natural resistance is

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achieved by spontaneous gene mutation and the acquired resistance is through the transfer of DNA fragments like transposons from one bacterium to another. Bacteria gains antibiotic resistance due to three reasons namely: (i) modification of active site of the target resulting in reduction in the efficiency of binding of the drug, (ii) direct destruction or modification of the antibiotic by enzymes produced by the organism or, (iii) efflux of antibiotic from the cell (Sheldon, 2005). One strategy employed to overcome these resistance mechanisms is the use of combination of drugs. Few new agents are in development today for treating TB, and none has been designed specifically to shorten the treatment regimen. Drug design targeting the latency stage and synergistic interaction between the various drug candidates might prove to be good alternatives (Shanmugam *et al.*,2008).

The MICs and FICs of compounds alone and the combination effect against *M. tuberculosis* are shown in Table 8.2. The MICs of the ethanol extract, compounds and INH, which were previously reported in Chapter 7 were still the same (Table 7.1). The combination drug action showed that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone have synergistic action. Combination of (2*S*)-5,7,2' trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone reduced their original MICs four-fold resulting in an FIC of 0.5 indicating synergistic activity. The most pronounced effect of the two-drug action was demonstrated by both compounds with INH. This combination reduced their MICs sixteen-fold. The FIC index of (2*S*)-5,7,2'-trihydroxyflavanone and INH was 0.125 and for (*E*)-2',4'-dihydroxychalcone with INH was 0.18 indicating synergistic activity. The crude ethanol extracts from which the compounds were isolated, showed inhibitory activity at MIC of 1200.0 µg/mL, which suggests that there are other components within the extract which might have antimycobacterial effect. There have been no reports of similar flavonoids for combination action against *M. tuberculosis*. This is the first report on the synergistic effects of these compounds.

In another study, flavonoids (epicatechin, isorhamnetin, kaempferol, luteolin, myricetin, rutin, quercetin and taxifolin) were screened in combination with INH

**Table 8.2. Synergistic effect and intracellular activity of the ethanol extract and isolated compounds against *M. tuberculosis***

Tested samples	<i>M. tuberculosis</i>				
	Combination drug action			Intracellular activity	
	MIC <sup>a</sup> (µg/mL)	ΔGI <sup>b</sup>	FIC <sup>c</sup>	MIC (µg/mL)	ΔGI <sup>d</sup>
Ethanol extract	1200.00 (S <sup>e</sup> )	0.0 ± 0.0	nd <sup>f</sup>	50.0(S)	23.0 ± 16.2
(2 <i>S</i> )-5,7,2'-trihydroxyflavanone	100.00 (S)	5.0 ± 3.5	nd	100.0 (S)	09.0 ± 06.3
( <i>E</i> )-2',4'-dihydroxychalcone	50.00 (S)	6.0 ± 4.2	nd	50.0 (S)	02.0 ± 01.4
INH	0.20 (S)	0.0 ± 0.0	nd	0.4 (S)	13.0 ± 09.4
(2 <i>S</i> )-5,7,2'-trihydroxyflavanone + INH	1/16 + 1/16 (S)	0.0 ± 0.0	0.1250	nd	nd
( <i>E</i> )-2',4'-dihydroxychalcone + INH	1/8 + 1/8 (S)	0.0 ± 0.0	0.1875	nd	nd
(2 <i>S</i> )-5,7,2'-trihydroxyflavanone + ( <i>E</i> )-2',4'-dihydroxychalcone	¼ + ¼ (S)	16.0 ± 11.3	0.5000	nd	nd

<sup>a</sup>Minimum inhibitory concentration.

<sup>b</sup>ΔGI value (mean ± SD) of the control vial (10<sup>-2</sup>) was 32.0 ± 22.6 for the sensitive strain.

<sup>c</sup>Fractional inhibitory concentration.

<sup>d</sup>ΔGI value (mean ± SD) of the control vial (10<sup>-2</sup>) was 42.0 ± 29.6 for the sensitive strain within macrophages.

<sup>e</sup>Susceptible.

<sup>f</sup>Not determined.

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against different mycobacterial strains. The best synergistic activity was observed on myricetin which exhibited a synergistic and not additive antibiotic effect with INH (FIC of 0.2) by decreasing the MIC of INH 16-fold at a concentration of 8 µg/mL and 64-fold at a concentration of 16 µg/mL against *M. smegmatis* (Lechner *et al.*, 2008). Bapela *et al* (2006), reported combination studies of 7-methyljuglone with INH and RIF. FIC indexes obtained were 0.2 and 0.5 for RIF and INH respectively, suggesting a synergistic interaction between 7-methyljuglone and these anti-TB drugs.

### **8.3.2. Intracellular activity**

Effective doses of antimycobacterial drug should also be evaluated in a macrophage model to ensure intracellular drug effectiveness (Chanwong *et al.*, 2007). *M. tuberculosis* infection is a complex process that initiates with aerosol inhalation to the lung, wherein; the mycobacteria are phagocytosed by alveolar macrophages. Upon entry into macrophage, the TB bacilli interfere with normal phagosomal maturation, preventing fusion with lysosomes. In response to this infection, macrophages produce pro-inflammatory signals (cytokines and chemokines) to recruit T-cells to the infected lung tissue which induces coughing and provides an exit strategy for the bacteria to spread to another host (Bhave *et al.*, 2007).

Broad ranges of the biological activities of compounds have been reported using *in vitro* studies. The antimycobacterial activity of the ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone against *M. tuberculosis* residing within U937 macrophage cells were investigated. The ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone inhibited the growth of *M. tuberculosis* residing within macrophages at concentrations of 50.0; 100.0 and 50.0 µg/mL respectively (Table 8.2). This study indicates that the activity of the ethanol extract is more active in macrophages and that of the isolated compounds is still at their MIC values obtained in extracellular experiments (Chapter 7, Table 7.1). This might be an indication that the ethanol extract could be more taken up by macrophages or other molecules in the cell, leading to the increased interaction with the bacteria. In human macrophage

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cultures, antituberculosis drugs have been found to have inhibitory effects on tubercle bacilli residing within cultured human macrophages. INH was found to have an MIC of 0.05 µg/mL against tubercle bacilli in macrophages. PZA was found to have a bacteriostatic effect, at MIC of 64.0 µg/mL (Crowle *et al.*, 1988).

It has been hypothesized that PZA inhibits tubercle bacilli through its metabolite, pyrazinoic acid (POA). Tubercle bacilli are able to convert PZA to POA by the production of pyrazinamidase (Crowle *et al.*, 1986). STR was found to be effective on macrophages at concentrations between 5.0 and 50.0 µg/mL (Crowle *et al.*, 1984). EMB was found to inhibit the tubercle bacilli within the cells at the same concentrations as it did in bacteriologic culture medium. This occurs because macrophages enhance EMB effectiveness by killing tubercle bacilli, which have defective cell walls due to the effects of the drug (Crowle *et al.*, 1985). RIF was found to be effective in macrophages at concentrations of 0.5 and 2.5 µg/mL (Duman *et al.*, 2004).

### **8.3.3. Cytotoxicity activity**

The cytotoxicity results indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone demonstrated less toxicity, showing IC<sub>50</sub> of 110.3 and 80.2 µg/mL respectively against the U937 cells. The ethanol extract of *G. africana* had an IC<sub>50</sub> value at 120.0 µg/mL (Table 8.3).

The ratio of the IC<sub>50</sub> values to MIC values of the ethanol extract, 2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone was less than 10, which indicates less activity and samples are not considered to be lead anti-TB samples. The significance of these values depends on factors such as components of the crude extract, compound structures and potential mechanism of action.

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**Table 8.3.** Cytotoxicity of the ethanol extract and the isolated compounds against U937 cells

Plant species	MIC <sup>a</sup> (µg/mL)	IC <sub>50</sub> <sup>b</sup> (µg/mL ± SD)	SI <sup>c</sup>
<i>G. africana</i>	1.20 x 10 <sup>3</sup>	120.0 ± 2.31	0.100
(2 <i>S</i> )-5,7,2'-trihydroxyflavanone	100.0	110.3 ± 2.16	1.103
( <i>E</i> )-2',4'-dihydroxychalcone	50.0	080.2± 1.15	1.604
Doxorubin (positive drug)	nd <sup>d</sup>	1.153 ± 0.15	nd

<sup>a</sup>Minimum inhibitory concentration.

<sup>b</sup>IC<sub>50</sub>: 50% inhibitory concentration of samples on U937 cell line.

<sup>c</sup>SI, selectivity index (*in vitro*): IC<sub>50</sub> in U937 cells/MIC against *M. tuberculosis*.

(If SI > 10, the compound is evaluated further).

<sup>d</sup>Not determined.

#### 8.3.4. Subversive properties

In order to replicate and persist in its human host, *M. tuberculosis* must survive within the hostile environment of the macrophage, where bacterial oxidants, superoxide (O<sub>2</sub><sup>-</sup>) and nitric oxide (NO<sup>·</sup>) are generated in response to infection (Nathan and Shiloh, 2000). Two enzymes, NADPH oxidase and inducible nitric oxide synthase (NOS2), are largely responsible for production of O<sub>2</sub><sup>-</sup> and NO respectively (Huang *et al.*, 1993, Pollock *et al.*, 1995). The O<sub>2</sub><sup>-</sup> is reduced by superoxide dismutase (SOD) to form hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). A consequence of NADPH oxidative activity is that the phagocytosed bacteria are killed by oxidative damage to protein and DNA targets (Bhave *et al.*, 2007). *M. tuberculosis* relies upon MSH for protection against toxic oxidants, for growth in an oxygen-rich environment and for establishing the pattern of resistance to TB drugs (INH and RIF). The metabolism of MSH involves Mtr (containing FAD as a cofactor), a member of the NADPH, which reduces FAD to the redox-active disulfide in Mtr (Bhave *et al.*, 2007).

Our investigation on the NADPH oxidase activity of (2*S*)-5,7,2'-trihydroxyflavanone

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with Mtr, found that this compound failed to exhibit any NADPH oxidase activity at 800.0  $\mu\text{M}$  concentrations. Mtr is evidently not the target for the antitubercular activity of (2*S*)-5,7,2'-trihydroxyflavanone. The subversive substrate properties of naphthoquinones (**1-3**, **5-6**, **12** and **22-24**) with Mtr and the comparisons of their MIC values (expressed as  $\mu\text{M}$  concentrations) in whole cell assays, are summarised in Table 8.4. Whilst  $K_m$  values show the substrate binding affinities and the  $k_{cat}$  values express the maximum turnover rates, the overall catalytic efficiency ( $k_{cat}/K_m$ ) best expresses the futile substrate efficiency of these substrates. It is the latter value that was used to look for a direct correlation between futile substrate properties (*in vitro*) and the whole cell antibacterial activities of these compounds. The  $K_m$  values of compounds **1**, **2**, **3**, **6**, **8** and **12** are all in the 200.0 – 500.0  $\mu\text{M}$  range whereas **5**, **22** and **23** are significantly lower (30.0 – 60.0  $\mu\text{M}$ ). The previously reported  $K_m$  value of **2** with Mtr is 540.0  $\mu\text{M}$  (Patel and Blanchard, 1999), which is comparable to the value determined herein. All of the naphthoquinones in Table 8.1 can be viewed as structural elaborations of the basic juglone scaffold **1** and in this context, it appears that methylation of the C-2 position or the 5-hydroxyl group are the most detrimental to substrate binding with Mtr.

Depending on which naphthoquinone motif acts as the electron acceptor during enzymatic turnover, diospyrin can be viewed as either a 2 - or a 6-substituted derivative of 7-methyljuglone. Similar observations can be made for neodiospyrin. These dimeric versions of 7-methyljuglone exhibit a 5 to 10-fold reduction in  $K_m$ . The turnover rates ( $k_{cat}$ ) of the compounds in Table 8.4 are also reported. 7-methyljuglone and its 5-acetoxy derivative **8** have the fastest turnover rate at substrate saturation followed by **1** and **2**. The addition of the 8-chloro substituent (compounds **3**, **5** and **12**) is notably detrimental to the turnover rate at a level which is comparable to that observed for the naphthoquinone dimers **22** and **23**. In terms of substrate efficiency ( $k_{cat}/K_m$ ) **5** is the most efficient subversive substrate with Mtr followed by **6** and **8**. There is no direct correlation between the antituberculosis activity in whole cell assays (MIC) and the  $k_{cat}/K_m$  values of these compounds with Mtr. Shinanolone lacks the conjugated benzoquinone motif that is required for subversive substrate activity



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hence it is not a substrate for Mtr. It also displays significantly weaker antibacterial activity than any of the other naphthoquinones in whole cell assays as do compounds **17-19** which also lack the quinone motif (Weigenand *et al.*, 2004). Comparing the Mtr substrate properties of the naphthoquinones (Table 8.4) with those observed for MSSM (Patel and Blanchard, 1999), it is evident that MSSM is turned over more efficiently. The absence of a direct correlation between the subversive substrate efficiency of these naphthoquinones and their MIC values is probably because their antituberculosis activity in whole cells is the accumulative consequence of their non-specific reactivity with multiple biological targets. In *M. tuberculosis* one of these targets could plausibly be Mtr. Additional targets could include other flavoprotein oxidoreductases such as lipoamide dehydrogenase and thioredoxin reductase, which unlike Mtr are also found in eukaryotes. It has also been suggested that naphthoquinone structures such as **6** could behave as non-functional ubiquinone and/or menaquinone surrogates, which may perturb electron transfer in respiratory chain processes (Patel and Blanchard, 2001).

**Table 8.4. Substrate properties of substituted naphthoquinones with *M. tuberculosis* Mtr**

Compound	$K_m$ ( $\mu\text{M}$ )	$k_{cat}$ $\text{s}^{-1}$	$k_{cat}/K_m$ ( $\times 10^{-5} \text{s}/\mu\text{M}$ )	MIC ( $\mu\text{M}$ )
<b>1</b>	324 ( $\pm 50$ )	1.088 ( $\pm 0.079$ )	336	6
<b>2</b>	483 ( $\pm 146$ )	0.732 ( $\pm 0.140$ )	152	29
<b>3</b>	233 ( $\pm 45$ )	0.212 ( $\pm 0.021$ )	91	45
<b>5</b>	36 ( $\pm 5$ )	0.592 ( $\pm 0.017$ )	1644	22
<b>6</b>	254 ( $\pm 23$ )	2.365 ( $\pm 0.214$ )	931	3
<b>8</b>	389 ( $\pm 59$ )	3.169 ( $\pm 0.168$ )	815	11
<b>12</b>	435 ( $\pm 112$ )	0.142 ( $\pm 0.018$ )	33	85
<b>22</b>	33 ( $\pm 4$ )	0.118 ( $\pm 0.005$ )	357	21
<b>23</b>	63 ( $\pm 8$ )	0.308 ( $\pm 0.010$ )	488	27
<b>24</b>	----not active-up to 800 $\mu\text{M}$ ----			>530
<b>MSSM</b>	70 <sup>a</sup>	6.667 <sup>b</sup>	9524	-----
Isoniazid				0.2

<sup>a</sup>As reported in Patel and Blanchard, 1999

<sup>b</sup>Derived from the  $k_{cat}$  value (400/min) as reported in Patel and Blanchard, 1999

## **8.4. Conclusion**

The resurgence of TB worldwide coupled with the increase in MDR *M. tuberculosis* has renewed an interest in the development of new antituberculosis treatment regimens (Cavalieri *et al.*, 1995). A key target for antimycobacterial chemotherapy is cell wall biosynthesis. The complex lipoglycan calyx on the mycobacterial cell surface provides a significant physical barrier to intracellular-acting drugs. The lack of penetration is thought to be a reason why many antibiotics show no activity against *M. tuberculosis* (Gao *et al.*, 2003). Since INH inhibits the biosynthesis of mycolic acids present in the cell wall of *M. tuberculosis*, this first-line drug is used in direct treatment programmes because of its activity against *M. tuberculosis*.

The identification of new antimycobacterial targets is essential to address MDR and latent TB infections. Numerous studies have validated amino acid biosynthetic pathways and downstream metabolites as antimicrobial targets and sulphur metabolic pathway are required for the expression of virulence in many pathogenic bacteria (Bhave *et al.*, 2007). Any drug that could enhance the activity with other standard drugs like INH, EMB, PZA and STR would therefore be valuable in the treatment of TB.

Since (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone from *G. africana* showed synergistic activity with INH, it is speculated that the compounds might have similar mechanisms as that of INH. However, mechanistic studies on these compounds should be done in order to get an indication of 'flavonoids and chalcones' interference on mycolic acid synthesis, membrane synthesis and enzyme inhibition. The successful use of combinations of plant extracts is not only observed in anti-infective therapy, but also seen in the treatment of several disorders including cancer, HIV, inflammatory, stress-induced insomnia, osteoarthritis and hypertension.

The intracellular MIC of the ethanol extract of *G. africana* was higher than the extracellular MIC in this study. The activity might be due to *M. tuberculosis* being

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unable to avoid macrophage killing and its survival during phagocytosis. This includes inhibition of phagosome-lysosome fusion, inhibition of the acidification of phagosomes, resistance to killing by reactive oxygen intermediates and modification of the lipid composition of the mycobacterial cell membrane, thereby altering its capacity to interact with immune or inflammatory cells. However, other mechanisms responsible for these properties should be identified, and this will represent potentially interesting targets for novel drugs and vaccines.

When a drug is used therapeutically, it is important to understand the margin of safety that exists between the dose needed for the desired effect and the dose that produces unwanted and possibly dangerous side effects (Labuschagne, 2008). The cytotoxicity results indicated that less toxicity of the ethanol extract of *G. africana*, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone against the U937 cells. This makes the ethanol extract of *G. africana* to be a candidate for herbal formulation and will have to be constituted for further investigation. Considering that a SI value > 10 is required for a compound to be selected for further testing, the results in this study indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone are not antituberculosis leads due to their high MIC values.

In terms of substrate efficiency, the inactivity of (2*S*)-5,7,2'-trihydroxyflavanone is possibly due to its inactivity with Mtr which is found in *M. tuberculosis*. However, the mycothiol disulfide redox pathway is unique to actinomycetes (e.g. *M. tuberculosis*) and it substitutes the glutathione reductase pathway utilized in mammals. There is scope for narrowing the target specificity of (2*S*)-5,7,2'-trihydroxyflavanone and the naphthoquinones by appropriate structural modifications (e.g. carbohydrate/polyol motifs) so as to tailor their specificity for Mtr. Such an approach has previously been demonstrated by modification of simple naphthoquinone scaffolds with polyamine motifs to enhance their specificity for trypanothione reductase versus mammalian glutathione reductase and LADH enzymes (Salmon-Chemin *et al.*, 2001). It will be interesting to investigate if the compounds have subversive properties with other enzymes.

## CHAPTER 9

# OVERALL DISCUSSION AND CONCLUSION

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### OVERALL DISCUSSION AND CONCLUSION

In South Africa, TB is the most commonly notified disease and the fifth largest cause of mortality, with one in ten cases of TB resistant to treatment in some areas. Many plants are used locally in traditional medicine to treat TB-related symptoms (McGaw *et al.*, 2008). In this study, traditional healers and local people in areas of Venda region of Limpopo province, South Africa were interviewed, about medicinal plants species used traditionally to treat TB. The antimycobacterial activity tests of ethanol extracts and chemical constituents of selected medicinal plants (*A. afra*, *Dodonaea angustifolia*, *Drosera capensis*, *G. africana*, *P. africana*, *S. cordatum* and *Z. mucronata*), using different methods were investigated against selected two *Mycobacterium* species (*Mycobacterium smegmatis* and *M. tuberculosis*), which cause TB and infect the respiratory tract. Some of the selected plants showed antituberculosis activity *in vitro*. Ethanol extracts of *Dodonaea angustifolia*, *Drosera capensis* and *S. cordatum* showed inhibitory effects against *M. smegmatis*. The most potent plant was *G. africana* which showed good activity against both *Mycobacterium* species.

Due to the good inhibitory activity of the ethanol extract of *G. africana* amongst the other selected plants, the ethanol extract was selected for the isolation of compounds. Four compounds namely: (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-2',4'-dihydroxychalcone, (*E*)-3,2',4'-trihydroxychalcone and (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone, were isolated. Two compounds, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone were previously isolated by other researchers and have been reported to have anticancer, antifungal, antibacterial and anti-inflammatory activity. Compound, (*E*)-3,2',4'-

trihydroxychalcone has not been reported from natural sources but previously isolated by Severi *et al.*, 1998 and a novel compound, (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone is isolated from *G. africana* for the first time.

The compounds isolated were tested for antimycobacterial activity against *M. smegmatis* and *M. tuberculosis* using different methods. (2*S*)-5,7,2'-trihydroxyflavanone showed good inhibitory activity against *M. smegmatis* at 0.03 mg/mL. (*E*)-2',4'-dihydroxychalcone and the novel compound, (*E*)-3,2',4'-trihydroxy-3'-methoxychalcone, both showed antimycobacterial activity against *M. tuberculosis* at 0.10 and 0.05 mg/mL respectively.

In terms of synergistic activity, (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone reduced their original MICs four-fold resulting in an FIC of 0.5. The most pronounced effect of the two-drug action was demonstrated by both compounds with INH. The FIC index of (2*S*)-5,7,2'-trihydroxyflavanone and INH was 0.125 and for (*E*)-2',4'-dihydroxychalcone with INH was 0.1875 indicating synergistic activity.

The intracellular antimycobacterial activity of (2*S*)-5,7,2'-trihydroxyflavanone, (*E*)-2',4'-dihydroxychalcone and the ethanol extract of *G. africana* was good and MICs obtained were 50.0; 100.0 and 50.0 µg/mL respectively. This indicates that the ethanol extract could be more taken up by macrophages or other molecules in the cell, leading to the increased interaction with the bacteria.

The cytotoxicity results indicated that (2*S*)-5,7,2'-trihydroxyflavanone and (*E*)-2',4'-dihydroxychalcone have less toxicity. During our investigation on the NADPH oxidase activity of only one compound, (2*S*)-5,7,2'-trihydroxyflavanone with Mtr, it was found that this compound failed to exhibit any NADPH oxidase activity at 800.0 µM concentrations. The mycothione reductase pathway is evidently not the target for the antitubercular activity of (2*S*)-5,7,2'-trihydroxyflavanone.

This is the first report of selected medicinal plants for their antimycobacterial activity

against *M. smegmatis* and *M. tuberculosis*. A good correlation between the susceptibility test results of the radiometric assay and microtitre dilution method was observed. Initial susceptibility testing of *M. smegmatis* is fast, economical and reproducible. The advantage of BACTEC radiometric assay is that it is rapid and the liquid medium used has more cell to drug contact and hence, the results are more accurate.

This study gives some scientific basis to the traditional use of these medicinal plants for TB as potential antimycobacterial agents and warrant further investigation. Many plant species are used traditionally in South Africa to alleviate symptoms of TB, and several interesting leads have originated for further inquiry following *in vitro* antimycobacterial activity evaluation. However, much work remains to be done on the systematic assessment of anti-TB efficacy of local plants against pathogenic *Mycobacterium* species, both *in vitro* and *in vivo*.

Finding new TB drugs represents a challenge and it is hoped that new compounds can be found that will reduce the treatment period. The methods of antimycobacterial screening and the way in which results are reported need to be standardized to enable comparison to be made between different researchers, as some authors report activity of extracts at 3.0 or 10.0 mg/mL while others including McGaw *et al* (2008), believe that only MIC values less than 0.1 mg/mL are worthy of labeling active. A screening program which was established by the National Institutes of Allergy and Infectious Diseases, screened over 50 000 compounds and an initial cutoff of 12.5 µg/mL was amended to 6.25 µg/mL to reduce the number of compounds to be screened further (Orme, 2001). From both the biological and the natural products chemical perspective, the various aspects of the collaborative challenge faced in TB drug discovery are applicable to other infectious agents.

In the present study it was found that the ethanol extract of *G. africana* showed moderate antituberculosis activity. However, the isolated compounds from the extract exhibited good MICs against *M. tuberculosis*. During synergistic studies, the findings were significant as combination of (2*S*)-5,7,2' trihydroxyflavanone and (*E*)-2',4'-

dihydroxychalcone reduced their original MICs four-fold. Hence the use of the combination of anti-TB compounds identified in this study / herbal extract together with conventional medicine can be recommended after evaluating the efficiency of the aforesaid extracts / compounds in the pre-clinical and clinical studies.