THE ANTIMYCOBACTERIAL MICs, SARs AND QSARs OF SOME ETHNOBOTANICALLY SELECTED PHYTOCOMPOUNDS

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DECLARATION

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

Signature:…… Date:…….
Benson Muriuki

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

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DEDICATION

This work is dedicated to my immediate and extended family members.

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ABBREVIATIONS

CRL - Central Reference Laboratory for TB

DSTs - drug sensitivity tests

DOTs – directly observed therapy, short-course

GATB - the Global Alliance for TB Drug Research and Development

H37Rv - a standard strain of mycobacterium tuberculosis that is susceptible to common TB drugs

LJ medium - the solid egg based Lowenstein-Jensen medium

McFarland (mF) - a unit for defining *Mycobacterium tuberculosis* inoculum concentration. The cloudiness of a $BaSO_4$ suspension in water is compared to the bacterial suspension to be used for inoculation.

MDR - Multi-Drug Resistant Tuberculosis Isolates resistant to both Isoniazid and Rifampicin with or without resistance to other drugs

MIC - Minimum inhibitory concentration of a drug or compound in media that inhibit growth of organisms to a defined level.

MGIT - Mycobacterial Growth Indicator Tube. It is commonly used in automated instruments such as BACTEC MGIT 960 system.

MTB - *Mycobacterium tuberculosis*

QSARs - Quantitative Structure Activity Relationships

SARs - Structure Activity Relationships

QSARs – Quantitative Structure Activity Relationships

XDR TB - extensively drug-resistant tuberculosis.

ABSTRACT

The rise in cases of tuberculosis worldwide fuelled by the HIV/Aids pandemic highlights the need for new drugs that can shorten the duration of treatment, effectively treat MDR and XDR TB and be non interactive with available HIV/Aids treatment drugs. Fourty ethnobotanically selected phytocompounds and four conventional TB treatment drugs were screened for their *in vitro* inhibitory activity against two strains of *Mycobacterium tuberculosis* at the Central Reference Laboratory for TB (CRL) in Nairobi using the absolute concentration method at concentrations ranging from 0.05 to 120µg/ml. Compounds with better minimum inhibitory concentrations (MIC), than that of any of the four conventional TB treatment drugs, were identified as promising Leads. The study identified six promising Leads; three benzoquinones (114, 121 and 116), a flavone (141) an isoflavone (145) and an alkaloid (151). Structure activity relationships (SARs) and Quantitative structure activity relationships (QSARs) were generated for the flavonoid and the benzoquinone series members. QSARs were used in the prediction of structural modifications necessary for activity improvement and in the prediction of activity of untested compounds.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

1.1.1 Background

Tuberculosis (TB) is thought to have evolved before the fifth millennium BC (Manchester, 1984). The first drugs for TB treatment were introduced between 1940 and 1960 (Duncan, 2003). This caused a rapid decline of the disease especially in the industrialized countries leading to high optimism that it was no longer a threat. There was no longer any urgency for new TB drugs. This situation started changing in the 1980's leading to a large increase in TB incidence. This was attributed to an increase in immuno-suppressed individuals as a result of Aids, cancer chemotherapy, organ transplant practices and poor primary health care structures especially in developing countries. (Snider,1994).

Among infectious diseases, TB infection is currently the leading cause of death in the world, with approximately three million patients dying every year (WHO, 2006). TB is also the leading cause of mortality among persons infected with HIV (Churchyard and Wardell, 2007). Nearly one-third of the world's population is infected with *Mycobacterium tuberculosis*. The World Health Organization declared tuberculosis a global health emergency in 1993 (WHO, 2005a).

1.1.2 Epidemiology of Tuberculosis

TB cases emerge from the approximately 2 billion people infected with *Mycobacterium tuberculosis*. Only 3-4% of infected individuals usually develop active disease upon initial infection. These percentages are much higher in people living with HIV. While 8% of global

TB cases are attributable to HIV, in sub-Saharan Africa the figure is 40%. The trend in incidence of TB is upwards in developing countries but it is generally downwards elsewhere. Eight African countries are among the top twenty-two countries that account for 80% of the world's tuberculosis cases. These countries in order of prevalence are Nigeria, South Africa, Ethiopia, the Democratic Republic of Congo, Tanzania, Kenya, Uganda, and Zimbabwe (WHO/TDR, 2002; Fun *et al.*, 2006). About 75% of TB cases occur in people between 15-60 years of age, and the male: female ratio is 1.2-1.5:1 globally (WHO, 2005a).

The estimate of new TB cases in 2006 was 9.2 million Out of these 7.4 million were in Asia and sub-Saharan Africa. In the same year a total of 1.2 million people died of TB, including 200,000 patients infected with HIV (WHO, 2008). The global proportion of Multi-Drug resistant (MDR)-TB is around 1-2% of all cases, but the problem is greater in MDR 'hot-spots' around the world. Poverty, overcrowding, malnutrition, civil wars, natural disasters and refugees have been said to contribute to higher TB prevalence rates (USAID, 2001; WHO, 2005a). In a study conducted in Kenya, TB prevalence was found to decrease with altitude (Mansoer *et al.*, 1999).

1.1.2.1 Emergence of Extra- Multidrug Resistant (XDR) TB

In 2006, Center for Disease Control (CDC), the World Health Organization (WHO) and the International Union Against Tuberculosis and Lung Disease (IUATLD) reported the results of a survey regarding drug-resistant TB conducted by 25 reference laboratories comprising the Global Supranational TB Reference Laboratory Network (2000 to 2004), the National TB Surveillance System in the United States (1993 to 2004), the national reference

laboratory of South Korea (2004), and the national MDR TB patient registry in Latvia (2000 to 2002) (CDC, 2006a). The findings indicated that 20 % of *M. tuberculosis* isolates were MDR, and 2% also were resistant to multiple second-line drugs. This highly resistant form of TB was identified in every region of the world, including the United States, where 4% of MDR TB isolates also were resistant to multiple second-line drugs.

In a report published in 2006, this highly resistant form of TB was named Extra- Multidrug Resistant or extensively drug-resistant TB (XDR TB) (CDC, 2006b). XDR TB is a subset of MDR TB that is resistant both to isoniazid and rifampin and to any fluoroquinolones drug and at least one of three second-line injectable drugs (amikacin, kanamycin(**10**), or capreomycin) (CDC, 2006b).

As of April 2007, South Africa (where HIV prevalence was estimated at 10.8% in 2005) had reported 352 cases of XDR TB, with the actual prevalence likely being much higher (Shisana. *et al.,* 2005*).*

1.1.3 Tuberculosis Pathogenesis

Mycobacterium tuberculosis is an obligate aerobic pathogen, which has a predilection for the oxygen-rich lung tissue. In general, this pathogen enters the body via the respiratory route and spreads from lung through lymphatic and blood to other parts of the body (Fun *et al.,* 2006). TB progresses through five stages. In stage one, droplet nuclei are inhaled. If these reach the alveoli infection begins. Stage two begins 7-21 days after initial infection. *Mycobacterium tuberculosis (M.TB.)* multiplies virtually unrestricted within inactivated macrophages until the macrophages burst. In the third stage T-cells recognize *M.TB.*

antigens. This results in T-cell activation and the liberation of cytokines including gamma interferon (IFN).

The liberation of IFN results in the activation of macrophages. These activated macrophages are now capable of destroying *M.TB*. It is at this stage that the individual becomes tuberculin positive. It is also at this stage that tubercle formation begins. *M.TB.* can persist within these tubercles for extended periods. Stage four involves replication and growth of tubercles that are surrounded by inactivated or poorly activated macrophages. The growing tubercle may invade a bronchus causing spread to other parts of the lung (pulmonary TB). Invasion of a blood vessel results in extrapulmonary or milliary tuberculosis. In stage five, the organisms begin to rapidly multiply extracellularly and rapidly spread to other parts of the lung or body.

1.2 Literature Review

1.2.1 Genus *Mycobacterium*

The genus *Mycobacterium* includes *M. tuberculosis, M. bovis, M. ulcerans, M. leprae, M. paratuberculosis, M. lepraemurium, M. phlei, M. smegmatis, M. fortuitum, M. marinum,* and *M. kansasii* (Bauer *et al*., 1968). *Mycobacterium tuberculosis* is the etiologic agent of tuberculosis in humans. Humans are the only reservoir for the bacterium. *Mycobacterium bovis* is the etiologic agent of TB in cows and rarely in humans. Both cows and humans can serve as reservoirs. Humans can also be infected by the consumption of unpasteurized milk. This route of transmission can lead to the development of extrapulmonary TB, exemplified in history by bone infections that lead to hunched backs. *Mycobacterium avium* causes a TB-

like disease especially prevalent in AIDS patients. *Mycobacterium leprae* is the causative agent of leprosy (Bauer *et al*., 1968).

1.2.2 *Mycobacterium Tuberculosis*

Mycobacterium tuberculosis (M.TB) is a fairly large non-motile rod-shaped, 2-4 μm in length and 0.2-0.5 μm in width. It is a facultative intracellular parasite, usually of macrophages, and has a slow generation time of 15-20 hours. Two media are used to grow *M.TB*. These are Middle brook's medium (an agar based medium) and Lowenstein-Jensen medium (an egg based medium). *M.TB*. colonies are small and buff colored when grown on either medium. Both types of media contain inhibitors to keep contaminants from outgrowing *M.TB*. It takes 3-6 weeks to get visual colonies on either type of media. Chains of cells in smears made from *in vitro*-grown colonies often form distinctive serpentine cords. Cords are most abundant in virulent strains of *M.TB*.

If a Gram stain is performed on *M.TB*. it stains very weakly Gram-positive or not at all. *Mycobacterium* species are classified as acid-fast due to their impermeability by certain dyes and stains. Once stained, acid-fast bacteria retain dyes when heated and treated with acidified organic compounds. One acid-fast staining method is the Ziehl-Neelsen stain. Acid-fast bacilli appear pink in a contrasting background.

Over 60 % of the mycobacterial cell wall is lipid. The high concentration of lipids has been associated with impermeability to stains and dyes and resistance to many antibiotics, killing by acidic and alkaline compounds, osmotic lysis via complement deposition, lethal oxidations and with survival in macrophages. The property is utilized in isolation of mycobacterium from other microorganisms in biological specimens. Resistance to

antimicrobials in *Mycobacteria* typically results from point mutations in the bacterial chromosome, resulting in changes to the antibiotic target that renders it no longer susceptible to the drug in question.

1.2.3 Treatment of Tuberculosis

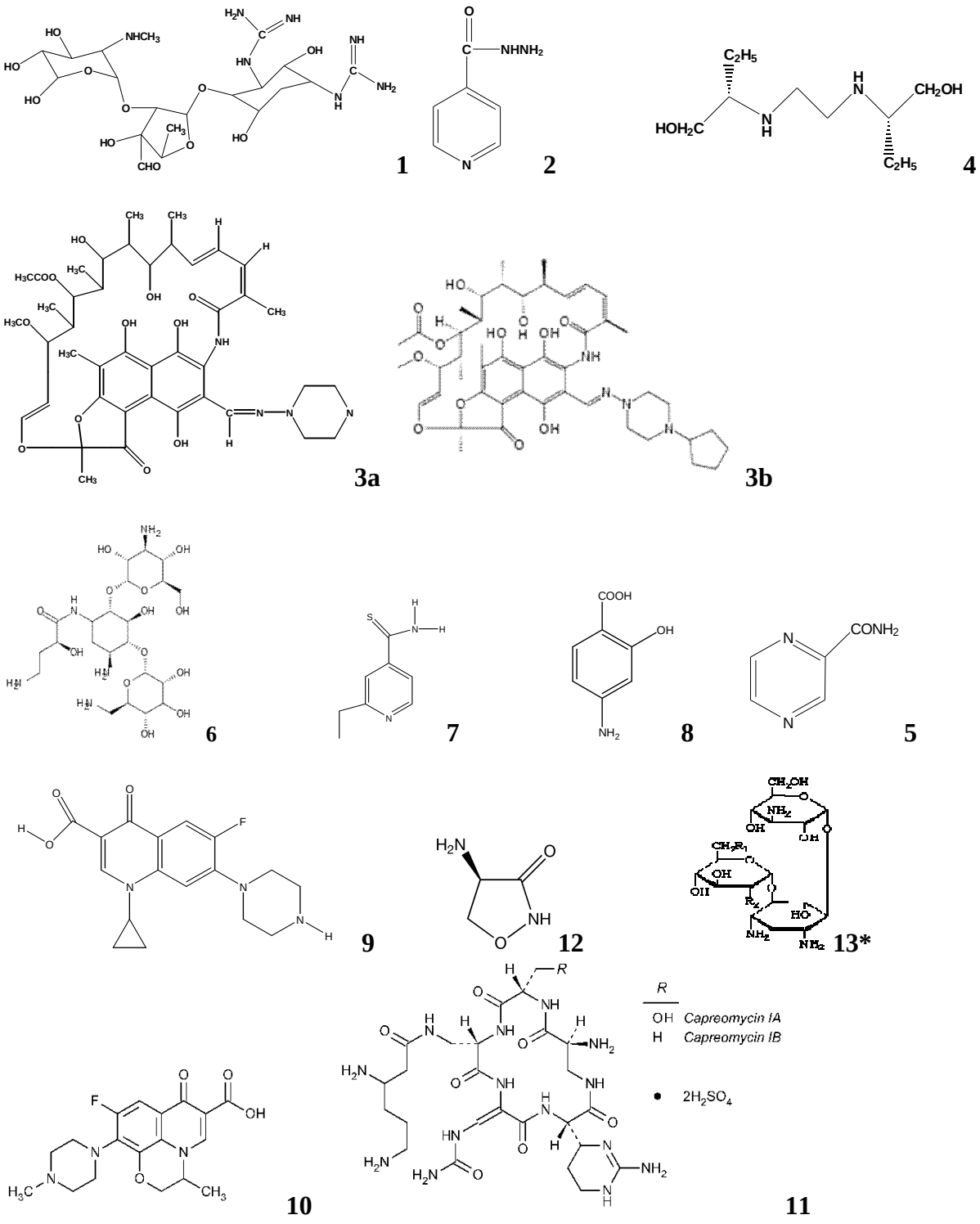
1.2.3.1 Current Conventional Drugs

Most of the current drugs used in TB treatment were discovered over 30 years ago (Duncan, 2003). These are streptomycin (**1**), p-aminosalicylic acid (**8**), Isoniazid (**2**), cycloserine (12), kanamycin, Rifampicin (**3**), ethionamide (**7**), Ethambutol **(4**) and Pyrazinamide (**5**). (Duncan, 2003; Singh and Kapoor, 1998). Streptomycin (**1**) was the first drug used successfully to treat tuberculosis. It is a protein synthesis inhibitor. It is a polar aminoglycoside that must be administered parenterally. The drug could be ototoxic (auditory and/or vestibular damage) and is only used in drug combinations for treatment of life-threatening TB.

Isoniazid (**2**) inhibits enzymes in biosynthesis of very long chain fatty acids (mycolic acids) and mycobacterial cell walls. It is the single most important drug used in tuberculosis management and it is a component of most drug combination regimens. Resistance can emerge rapidly if the drug is used alone. Its toxic effects include neurotoxicity (peripheral neuritis, restlessness, muscle twitching and insomnia) and hepatotoxicity (hepatitis, jaundice). The drug is well absorbed orally and is metabolized by the liver through acetylation. Patients may be fast or slow inactivators of the drug. Fast acetylators require higher dose.

Rifampicin (**3a**) is a derivative of rifamycin acts by interfering with the DNA-dependent RNA polymerase of bacterial cells and hence ultimately stops protein synthesis. If used alone, resistance develops rapidly. Rifapentine (**3b**) is a new rifamycin derivative that is more potent than rifampicin (Pascale *et al.*, 2000). Ethambutol (**4**) inhibits cell wall biosynthesis through arabinosyl transferases although its mode of action remains to be elucidated fully. Pyrazinamide (**5**) inhibits Mycobacterial metabolic processes but its mode of action remains to be elucidated fully. It is well absorbed orally and distributes to most tissue including the CNS. Its adverse effects include visual disturbances, headache and confusion. Resistance develops rapidly if the drug is used alone.

Antimycobacterial drugs are frequently associated with unpleasant side effects. This makes patient compliance in therapy difficult. To help overcome that problem, which undoubtedly contributes to the emergence of resistant strains, many health authorities are recommending Directly Observed Therapy, DOTS for short. Treatment under 'DOTS' (directly observed therapy, short-course) consists of the daily administration of four drugs, namely, isoniazid, pyrazinamide, rifampin, and ethambutol, for two months and a follow-up, thrice-a-week treatment of isoniazid and rifampin for another four months (Kenneth, 2005).



* Specific kanamycins differ in groups at R_1 and R_2 e.g. R_1= NH_2 and R_2 = OH in kanamycin A

Figure 1: Conventional TB treatment drugs

Cases that are resistant to the first-line drugs are subjected to the second-line antimycobacterial agents (usually more toxic) (Trevor *et al.*, 1998). These include:-

(i) **Amikacin (6)** – for MDR or streptomycin resistant strains

(ii) **Ethionamide (7) -** a congener of INH

(iii) ***P*- Aminosalicylic acid (PAS) (8)** – rarely used because of toxicity and primary resistance.

(iv) **Ciprofloxacin (9) and Ofloxacin (10) -** fluoroquinolones that are effective against resistant strains (but have enhanced toxicity.

(v) **Capreomycin sulphate (11) and cycloserine(12) -** highly toxic and hence rarely used.

(vi) **Kanamycin (13)**

The vaccine for TB is called BCG (Bacillus of Chalmette and Guerin, after the two Frenchmen who developed it). BCG consists of a live attenuated strain of the avirulent *Mycobacterium bovis*. It is 60-80 % effective in children.

1.2.3.2 Current Problems and on Going Efforts in TB Drug Development

Bacterial resistance to antibiotics is the principal obstacle to their successful therapeutic use. With time the resistant strain is disseminated in the community leading to a situation in which most cases are resistant and alternative treatment must be adopted (Canetti *et al.*, 1963). Mutations in the genes coding for proteins involved in the target biochemical processes and in the activation of pro-drugs confer drug resistance. Non compliance by the patient may also result in drug resistance.

The appearance of multi-drug resistant (MDR) strains has prompted the quest for new antimycobacterial agents. These should inhibit targets different from those of currently used drugs (Guido *et al.*, 2005). In addition, new agents should inhibit targets involved in persistence or dormancy and hence shorten the lengthy TB therapy. TB progresses faster in HIV positive patients and the two diseases may not be treatable at the same time because the required two sets of drugs can interfere with each other (France, 2006). There is need therefore to develop TB treatment drugs that do not interfere with the only available HIV and Aids management drugs.

TB drug development is a slow lengthy process mainly due to the nature of the pathogen. Its slow growth rate, the necessary duration of the infection models, and the requirement for specialized physical containment facilities all hamper progress. Various organizations and groups are currently involved in efforts towards TB drug development. These include the UNICEF-Word Bank-WHO special Programme for Research and Training in Tropical Diseases (TDR), Stop TB partnership Working Group on New TB Drugs (WGNDs) and The Global Alliance for TB Drug Development (GATB) among others.

Launched in 2000, GATB aims to overcome the natural barriers to TB drug development among academic institutions, government research laboratories, non-governmental organizations, the pharmaceutical industry and contract research houses.

In their strategic plan for 2006 - 2015, WGND identified one of the areas of strategic importance as 'basic discovery biology to identify and validate new targets and identify candidate compounds using effective screens and creative medicinal chemistry" to stimulate TB drug development by providing a framework in which the various elements of the process may be brought together. The WGND's vision is to have new TB regimens that will

achieve cure in 1-2 months or less, be effective against MDR-TB, compatible with antiretroviral drugs used for treating HIV/AIDS and effective against latent TB infection. In addition, new regimens must be affordable and easily managed in the field.

The recent emergence of extensively drug-resistant tuberculosis (XDR TB) has further complicated the TB treatment problem (MedicineNet.com, 2008). XDR TB is resistant to almost all drugs used to treat TB, including the two best first-line drugs: isoniazid and rifampin. XDR TB is also resistant to the best second-line medication: fluoroquinolones and at least one of the injectable drugs; amikacin (**6**), kanamycin (**13**), or capreomycin (**11**)). (MedicineNet.com, 2008). WHO and CDC have formulated action plans to combat XDR TB both nationally in the USA and internationally. Among the recommendations is to accelerate research in new TB drug development. (WHO, 2007;

CDC, 2009)

1.2.3.3 Tb Drug Susceptibility Testing

Reliable TB drug susceptibility testing (DST) is of fundamental importance in TB treatment and control programs because it enables clinicians to design effective multi-drug treatment regimens (Mitchison, 2005; Heifets and Desmond, 2005). During the 1960s, there was much discussion about the methods used in drug susceptibility tests because of discrepant results between laboratories. The World Health Organization (WHO) called two meetings with international participants to discuss the techniques and their uses. These meetings reported accounts of three different methods for performing DSTs; the absolute concentration method, the resistance ratio method and the proportion method (Canetti *et al*, 1963, 1969).

Even today it is well-documented that TB drug-susceptibility testing (DST) is one of the most difficult procedures to standardize in the mycobacteriology laboratory. Discrepant results occur for many reasons, even in the most competent laboratories. (Kent and Kubicka 1985). Mycobacterial drug susceptibility tests may give varied results due to differences in culture media, inoculum size, organism viability, and the critical drug concentrations (Montalbine and Collins, 1971). Clumping of inoculum suspension has also been reported as a major factor especially in the proportion method (Mitchison, 2005).

In the *Direct method* a set of drug containing and drug-free media are inoculated directly with concentrated specimen while in the *Indirect method* a pure culture is inoculated in drug containing and drug-free slopes either in egg-based Lowenstein-Jensen or agar based 7H11 medium. The *Absolute concentration method* involves inoculation of media containing graded concentrations of the drug(s) to be tested with standardized inoculums initially grown on drug free media. Resistance is expressed in terms of the lowest concentration of a given drug that inhibits growth (MIC) to less than one per cent twenty or fewer colonies.

Resistance ratio method compares the drug susceptibility of a given strain of tubercle bacilli with that of a standard one (usually $H_{37}R_v$). Resistance is expressed as the ratio of the MIC of the test strain to that of the standard strain in the same set. *Proportion method* enables estimation of mutants resistant to a given drug. Several 10-fold dilutions of inoculums are planted on both controls (drug-free) and drug containing media. The proportion of bacilli resistant to a given drug is the number of colonies on drug-containing media divided by those on drug free media. The proportion method is currently the method of choice and its principle is being applied in several rapid testing methods which include BACTEC 460, MGIT 960, MB/BacT, and ESPII. Among other DSTs reported are: - E-test (AB BIODISK),

Micro well Alamar blue, Mycolic acid index susceptibility testing, Microscopic observation of broth cultures, Micro colony detection, Phage amplified B (Pha B) assay, and Luciferase reporter phage assay.

Choice of method to use depends on factors such as the availability and cost of materials and equipment, time required until results are acquired, reproducibility of results, and biosafety concerns. Currently it is recommended that DST with the primary (first-line) drugs isoniazid (INH) (**2**), rifampin (RIF) (**3**), ethambutol (EMB) (**4**) and pyrazidamide (PZA) (**5**) be performed on the initial isolate from all patients. All rifampin-resistant or organisms resistant to any two first-line drugs should then be tested for susceptibility to secondary drugs including fluoroquinolones (**9, 10**), amikacin, kanamycin and capreomycin (**11**). (CLSI, 2003; WHO, 1997). WHO experts have coordinated the establishment of National Reference Laboratories for routine drug resistance or susceptibility testing and Superanatural Laboratories for maintenance of standards and quality control. The specificity and sensitivity of the test used should compare favorably with conventional methods (Laszlo *et al.*, 1983; Siddiqi *et al*., 1985).

1.2.3.4 Discordant Results

Current methods for susceptibility testing of *M. tuberculosis* complex (MTBC) are based on proportion methods, which rely on a bacteriologic definition of drug resistance that was developed in recognition of the difficulties in defining clinical resistance for Mycobacteria. These methods provide qualitative results of "susceptible" or "resistant," defining resistance as growth of greater than 1% of an inoculum of bacterial cells in the presence of a "critical" concentration of the drug. The critical concentrations of drugs were established on an empiric basis and adopted by international convention.

The critical concentration represents the lowest concentration of drug that inhibit 95% of "wild strains" of *Mycobacterium tuberculosis* (MTB), and not inhibiting strains of MTB isolated from patients unresponsive to therapy and considered resistant. (CLSI, 2003). Although Clinical and Laboratory Standards Institute (CLSI) has published some guidelines for standardizing the test procedure and selecting the panel of drugs to be tested, there are technical challenges inherent in the testing methodology. These limitations in the TB DST methods can result in differences in inter- and intra-lab results. (Laszlo *et al.*, 1997).

Laszlo and others (1997) listed some of the factors contributing to the reproducibility challenges as :- (1) Bacterial population (isolate vs. subculture) Re-testing may result in a false susceptible result if the isolate is repeatedly subcultured, as a result of differential growth kinetics in the bacterial population. The slower-growing resistant population may be lost with time.

(2) Different inoculation methods (size, clumps). (3) Different methods or media. (4) Methodology not standardized. (5) Cross-contamination. (6) Transcription or labeling errors. (7) Problem strains: some isolates are more difficult to grow. (8) Intrinsic drug related

problem; that the accuracy of susceptibility testing results (performed under the best circumstances) varies with the drug tested: it is most accurate for rifampicin and isoniazid and less for streptomycin and ethambutol.

1.2.4 *In vitro* Screening of Compounds in TB Drug Development

There are two basic approaches to develop a new drug for TB: (a) synthesis of analogues, modifications or derivatives of existing compounds for shortening and improving TB treatment and (b) searching for novel structures that the TB organism has never been presented with before (Dharmarajan *et al.*, 2006). A number of approaches may be taken to identify lead compounds that inhibit the activity of a particular target. The most comprehensive approach is high throughput screening especially when there are no other clues to guide discovery. An alternative, rational approach may be followed when there is detailed information available on the interaction between the target and a ligand such as its substrate, often in the form of the three-dimensional structure. This can be used to build a map of the active site and in silico docking experiments conducted to identify a small number of compounds for testing.

1.2.4.1 Solid Media *In Vitro* Screen

Canetti *et al.,* (1963 and 1969) have described the major steps of the method as:-

i. Preparation of the drug dilutions; Usually, a 1% stock solution of the drug is prepared in an appropriate solvent.
ii. Preparation of the culture medium. Lowenstein-Jensen medium is commonly used

iii. Incorporation of drug solution in the media to attain the required uniform drug concentration in the culture media.

iv. Coagulation of the media in a slanting position in flowing steam at 82-85ºC for 40-60 minutes.

v. Preparation of the inoculum suspension of *M. tuberculosis*. The adjustment is made by comparing the turbidity of the suspension with that of a standard barium sulfate solution equivalent to 1 mg/ml of wet bacterial mass. The bacterial suspension obtained is diluted 1:50 in Dubos medium. This gives a suspension containing 0.02 mg of bacteria per ml (about 200 000- 1 000 000 organisms per ml. A loopful (diameter: 3mm) of the suspension contains

2000 – 10 000 organisms and is usually used as the inoculum. (NB; 85-100 loops = 1ml).

vi. Incubation of the cultures at 37ºC until readable colonies are obtained after about four weeks.

vii. Reading of the cultures. Less than 20 isolated colonies is considered effective inhibition.

1.2.4.2 Liquid Media *In Vitro* Screen

The principles used in antimycobacterial susceptibility testing (AST) in liquid media are commonly adopted; the liquid media used include Middlebrook, 7H9 and 7H10. The method of proportion (MOP) compares colony counts on drug containing and drug free media (CLSI, 2003). Daily counting of colonies or colony forming units has been shown to

correlate well with daily monitoring of growth indicators or growth units generated by automated systems like the BACTEC MGIT 960 (Leonid *et a*l., 1986).

1.2.4.3 Target Organism in Primary Drug Screening

The choice of target organism in TB drug screening depends on factors such as available laboratory facilities, safety concerns and time availability. Testing against drug-resistant and MDR strains of *M. tuberculosis* is not critical in primary screening, because these strains are not resistant to multiple antibiotics by virtue of a single mechanism, but as a result of specific step-wise mutations to individual drugs. (Guido *et al.*, 2005).

The commonly used strain, *M. tuberculosis* $H_{37}Rv$, has a drug susceptibility profile which is fairly representative of the majority of drug susceptible clinical isolates. Because it is a virulent strain, *M. tuberculosis* $H_{37}Rv$ should only be handled in a biosafety level 3 laboratory (BL-3). Personnel working within the BL-3 lab must wear protective gear, most importantly a respirator, which will minimize the risk of infection by aerosolized *M. tuberculosis*.

In absence of level 3 laboratory one should work with avirulent surrogate organisms such as the rapidly growing saprophytic *Mycobacteria*. One such species is *M. smegmatis* (ATCC 607). Such organisms, however, only possess a limited degree of similarity to *M. tuberculosis* with regard to drug susceptibility. Another alternative to working with virulent strains of *M. tuberculosis* is to use the slow growing, avirulent strain known as *M. tuberculosis* $H_{37}R_a$ (ATCC 25177) or the commonly used vaccine strain, *M. bovis* BCG (ATCC 35743). These organisms are more closely related to *M. tuberculosis* $H_{37}R_v$ than the rapid-growing Mycobacteria with respect to both drug susceptibility profile and genetic

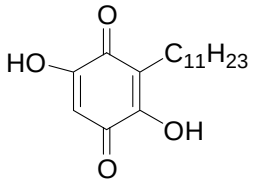
composition. Working with these organisms requires only the use of a class 2 biosafety cabinet and sound microbiological techniques.

1.2.5 Isolating Antimycobacterials from Plants.

Isolation of phytocompounds has been appreciated as a fundamental process in the global war against microbial diseases (Addy, 2005; Guido *et al.*, 2005). Isolation of antimicrobials from plants involves collecting of plant material, drying, grinding, extraction, separation and purification. The isolates are identified and characterized through spectroscopic, chromatographic and X-ray diffraction methods.

Advantages of compound isolation include enabling of product concentration, determination of mechanism of action, pharmacokinetics studies, enhancement of activity through structural modification of active molecules, guided synthesis of compounds and hence in conservation of medicinal plants. Disadvantages of compound isolation include loss of original activity, loss of synergistic effects, possible increase in toxicity and increased cost of drug due to increased investment in labor, time and equipment. The Myrsinaceae family is a good example of plants that have received extensive chemical study since the early 20^{th} century. It has been chemotaxonomically characterized by presence of alkylated hydroxybenzoquinone derivatives and oleane/ursane skeleton based triterpenoids. (Ahmed *et al*, 1977).

Paranjpe and Gokhale (1932) isolated embelin **(107)** as a major constituent of *E. ribes*. The same compound was later isolated from petroleum and ether extracts of *M. africana* berries by Krishna and Varma (1936). Manguro (1994) has detailed the history of isolation and structure determination of embelin (**107**).



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Pharmacological effects of 2, 5-dihydroxy-1, 4 benzoquinones have been reported by Midiwo et *al* (1990; 1995). Embelin (**107**) and other benzoquinones were also found to be active against Rhizobia CI AT 652 gram positive cocci (Manguro,1994). The same researcher reported good antibacterial activity by the dimethylether derivative of embelin and *E. variestis* (Muls) anti- feedant activity of embelin.

Dale and Greenway (1961) reported five Kenyan medicinal species of the *Myrsinaceae* family. These were *Embelia schimperi* Vatke, *Embelia keniensis* R.E. Fries, *Myrsine Africana* L., *Maesa lanciolata* Forsk and *Rapanea melanphloes* Mez. These plants were used in indigenous medical systems for treating some endemic diseases such as those caused by worms and bacteria (Kokwaro, 1976; Dale and Greenway, 1961). Huang (1980), reported *in vitro* inhibitory effect of *E. ribes* seeds extract on *Mycobacterium tuberculosis* and attributed the activity to embelin (**107**), barganin and guacentia. Structural modification of natural embelin yielded derivatives with antibacterial and antifungal activities of up to 10µg/ml. These include 6- iodoembelin which was more effective as an antibacterial than embelin and the diacetate of haloembelin which was more potent as a fungicide (Tukannen, *et al*, 1983). Embelin derivatives of aromatic amines were also reported to show good antifungal activity (Rao *et al.*, 1984).

In the first meeting of the West African Network of Natural Products Scientists (WANNPRES) held in Accra Ghana in August 15-20, 2004, success in treatment of HIV/Aids and accompanying opportunistic infections including TB was reported. Cocoa

plant root and stem bark extracts were reported to have been used specifically for TB treatment in HIV positive patients (Addy, 2005).

In another study involving Mexican plants used traditionally in treatment of respiratory diseases, activity against MDR and several other strains of *Mycobacterium tuberculosis* was reported. Plants evaluated include *Artemisia ludoviciana, Chamaedora tepejilote, Lantana hispida, Juniperus communis,* and *Malva parviflora* (Adelina *et al.*, 2003). Anti-Mycobacterial activity of carbazole alkaloids and fatty acid lactones isolated from *Micromelium hirsutum* , acetylchavicole acetate (ACA) from *Alpinia galanga* and ergosterol-5,8-endoperoxide from *Ajuga remota* has also been reported (Guido *et al.*, 2005).

The role of polyketides (complex natural lipids synthesized by plants, fungi and bacteria) in mycobacterial cell structure and virulence has been reported (NIAID, 2003). The anti-mycobacterial activity of thiolactomycine (a small natural product molecule) has been studied in structure-based designs at GlaxoSmithKline (NIAID, 2003). By 2003 NIAID TB research scientists had begun a collaborative project involving the screening of ethnobotanically selected natural products for anti-tubercular activity at the National Institute for Pharmaceutical and Research Development (NIPRD) in Abuja.

Midiwo *et al.* (1990; 1992; 1993; 2002) have described five Kenyan Myrsinaceae species that are rich in 'ubiquitous' benzoquinones. These are *Maesa lanceolata, Myrsine africana, Rapanea melanophloeos, Embelia schimperi and Embelia keniensis* The researchers reported isolation of embelin (**107**), maesanin (**109**), emodin (**111**). Embelin (**107**) was earlier reported from work by Ogawa and Natori (1968) and Rao and Venkateswarlu (1962).

Manguro (1994) again reported the golden- yellow plant secondary metabolite (**107**) from *M. Africana* parts and reported 10.4% yield from the plant's fruits.

Xiao-hua and McLaughlin (1989) isolated emodin (**111**) and nepodin (**118**) and other compounds from ethanol extracts of the roots of *Myrsine Africana.* They found nepodin (**118**) to have lower cytotoxic effect on brine Shrimp than emodin (**111**). In 1994, Manguro reported isolation of maesoquinone (**110)** from ethyl acetate extracts of the fruits, leaves, stem and root barks of *Maesa lanceolata* The researcher also synthesized several 3-alkyl homologues (**108**) in order to determine the "role of chain length in insect antifeedant, antibacterial, nematicidal, antifungal and phytotoxicity effects." Short chain length was attributed to low BA.

Naphthoquinones like plumbagin (**117**) have been reported by Harbone (1967) and Zenk *et al*. (1969). Activity against *Mycobacterium tuberculosis* by anthracene derivatives has been reported by Ranganatha and Shadri (1955) and Masakazu (1952). The former attributed low BA activity to increased molecular size and increased number of hydroxyl groups (e.g. emodine,(**111**).

Maesanin (**109**) was isolated from the fruits and bark of *Maesa Lanceolata* and reported as a novel compound by Midiwo *et al*. (1990). Manguro (1994) reported activity of the compound against nematodes, E. coli and Rhizobia. Ogawa and Natori had reported Maesoquinone (**110**) in 1968. Manguro (1994) reported the benzoquinone derivative from ethyl acetate extracts of fruits,leaves, stem and root barks of *M. Lanceolata.* The highest concentration was in the fruit extract. The compound, at a concentration of 50µg/ml, was inactive against the microganisms used in Manguro's study; Rhizobia CI AT 899 Gram-ve cocci, Rhizobia CI AT 652 Gram+ve cocci, Rhizobia CR 477 Gram+ve cocci, Rhizobia

Viking I Gram-ve cocci, Pseudomonas SPP Gram+ve rod , Rhizobia B/7 Gram-ve rod, E. coli YM 101 Gram-ve., Rhizobia B36, *Bacillus subtilis*, *Salmonella typhi*, *Streptococcus feacacus*, *Xannthomonas comprestis* and *Micrococus luteus*. The compound however caused 62.42% mortality on nematodes.

The anthraquinone used in this study is 1,3,8-Trihydroxy-6-anthraquinone (**125)**. It had been isolated from *R. abyssinicus* by Midiwo and Rukunga (1985). Manguro (1994) isolated the same along with chryosopanol (**126**), 2-hydroxychrysophanol (**127**) and physcion (**128**) from *M. Africana* dichromethane and ethyl acetate extracts. Antraquinones have been reported to elicit varied BA; bacteriastatic, fungicidal and tumoricidal (Powell, 1984; Kasuke and Nagayo, 1951), purgative effect on Rhesus Monkey (Loewe and Hbacker,1941) and on rats (Beccari,1939). Ranganatha and Shadri (1955) tested a number of anthraquinones derivatives against *Mycobacterium tuberculosis* and found some to be active. They observed that activity was lower for molecules with higher number of hydroxyl groups and bigger sizes like purpurin (**129**) and emodin (**130**).

Masakazu (1952) has reported activity of some anthraquinones against *in vitro* tubercle bacilli. He also reported activity of anthraquinones glycosides against pathogenic fungi. The MIC obtained for emodin (**130**) in the current study would correspond to 1:20,000 (assuming media density to be approximately equal to water density). This is a low activity relative to the activity reported by Masakazu. This observation is in agreement with the observations of Ranganatha and Shadri (1955). The naphthoquinone used in this study is plumbagin (**131).** It was reported from studies of Plumbaginaceae spp (Harbone, 1967) and Droseraceae (Zenk *et al*., 1969). The fungicidal, antibacterial, insecticidal, phytotoxic,

cytotoxic and anticarcinogenic activities of naphthoquinones have been reported.(Evans, 2002)

The naphthalene derivative used in this study (**132)** was isolated from *M. africana* by Mclaughlin and Xiao-Hua li' (1989) and latter by Manguro (1994). Named nepodin (**132**) it had been reported from ethanol extracts of the roots of *M. africana* and found to be non cytotoxic to brine shrimp by Mclaughlin and Xiao-Hua li' (1989). In the same study emodin (**130**) and 2- hydroxychrysophanol (**127**) were reported as lethally cytotoxic to brine shrimp. Manguro (1994) isolated nepodin (**132**) at 0.0001% yield from the same dichloromethane extract that contained anthraquinones. Compound number **134** was isolated as a minor component of chloroform fractions of the aerial surface exudates of *Polygonum senegalense* (meism) by Midiwo *et al* (1992). The researchers reported the antifeedant activity of the compound towards the ravenous insect *Schistocerca gregaria*. (Midiwo *et al.,* 1992). Compound number **136** is one of the flavonoids constituting the aerial surface exudates of *Polygonum senegalense.* (Midiwo *et al*, 2002). Some Polygonaceae plants have been widely used as ethno-anthelmintics (Kokwaro, 1976). The leaf of *Polygonum senegalense* is up to 17% surface exudates and about thirteen non polar flavonoid derivatives have been isolated from it. (Midiwo *et al.*, 2002)**.** Diterpenoids used in the study were isolated along with other compounds from *Psiadia puntualata* (**146, 147**) by Midiwo *et al.* (2002). Composite leaf decoction from the plant has been used in treatment of colds, fevers, abdominal pains and removal of ectoparasites from cattle (Kokwaro, 1976).

1.2.6 Structure Activity Relationship Studies (SARs)

1.2.6.1 History

The science of SARs dates back to the 19th century and has improved with development of methods of structural determination in Chemistry. As early as the 1860s, scientists were sometimes able to relate similarities in structure to a particular biological activity (BA). For instance, in 1869, Crum- Brown and Fraser observed that tertiary amines with varying pharmacological properties tended to show similar pharmacological properties when quarternised (Crum-Brown and Fraser, 1868-9). However, attempts to establish general principles that could guide rational drug development or to relate a particular functional group to a particular biological response have been largely unsuccessful. For example, varied biological activities have been reported for compounds with same functional groups. Same general BA has also been frequently reported for compounds that are unrelated chemically (Burger, 1958). The frequent failure to find a simple relationship between chemical structure, physical properties and biological action has been attributed to the complex nature of biological systems (Daniels 1943).

1.2.6.2 Background Information on Drug Action

The physicochemical properties of a compound are measurable characteristics by which the compound may interact with other systems. Biological response to a drug is a consequence of the interaction of the drug with the living system, causing some change in the biological processes present before the drug was administered.

1.2.6.3 Theories of Drug Action

Drugs are either agonists (stimulants) or antagonist (block stimulation) on specific biological receptors. Clark (1926) and Gaddum (1937) proposed that biological action (BA) is proportional to the number of receptors occupied – the **occupancy theory of drug action**. Ariens and Simonis (1964) and Stephenson (1956) modified this idea by proposing that drug-receptor interaction takes place in two independent steps:-

(1) Combination of drug and receptor (**affinity**)

(2) Production of effect (**efficacy**)

In this case biological action is related to the number of drug receptor complexes. Agonists and antagonists have same affinity but only agonist has efficacy. Drugs with lower receptor affinity may possess equal efficacy. Such drugs are required in higher concentrations for the same BA than those with higher receptor affinity. Structural changes of a drug molecule can also lead to reductions in efficacy with retention of affinity. Such derivatives show reduced BA but are good antagonists of active derivatives of the same series. For instance, modification of acetylcholine structures with bulky non polar groups to either end of the molecule may lead to the complete antagonistic activity found in the parasympatholytic compounds like atropine.(Ariens

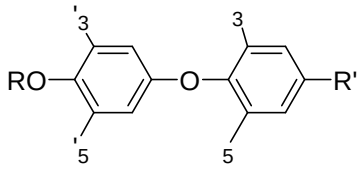
and Simonis, 1964). Croxatto (1956) and Paton (1961) contrasted the occupancy theory by proposing the **rate theory of drug action**; excitation by a stimulant drug is proportional to the rate of drug receptor combination rather than to the number of receptors occupied.

Belleau (1964) explained the mode of action of acetylcholine at muscarinic receptor through the **macromolecular perturbations theory**. The theory proposes that interaction of small molecules (substrate or drug) with a macromolecule (protein of a drug receptor) may lead either to specific conformational perturbations (SCP) to non specific conformational perturbations (NSCP). SCP results in specific response of an agonist (potency). If a NSCP occurs, there is no stimulation and antagonist action may result. A drug possessing features capable of both SCP and NSCP produces an equilibrium mixture of the two complexes and a partial stimulation action occurs.

1.2.6.4 Specificity of Drug Receptors

The high degree of structural specificity necessary for strong interaction between a receptor protein and a drug is demonstrated by studies of Sterling (1964). Table 1 below gives his findings on the structural requirements for strong binding of thyroxin analogues to albumin.

Table 1: Relationship Between Structure of Thyroxin analogs and binding by Thyroxin Binding Albumin (adopted from Sterling 1964).



SUBSTITUENTS

Compd. No.	R	3',5'	3,5	R'	Association Constant
14	**H**	**I**, **I**	**I**, **I**	**$CH_2CH(NH_2)COOH$**	**500,000**
15	CH_3	**I**, **I**	**I**, **I**	$CH_2CH(NH_2)COOH$	20,000
16	H	I,H	**I**, **I**	$CH_2CH(NH_2)COOH$	24,600
17	H	**I**	**I**, **I**	CH_2CH_2COOH	160,000
18	H	**I**	**I**, **I**	CH_2COOH	100,000
19	H	**I**	**I**, **I**	COOH	72,000
20	H	**I**, **I**	**I**, **I**	$CH_2CH_2NH_2$	32,000
21	H	Cl, Cl	Cl, Cl	$CH_2CH(NH_2)COOH$	23,000
22	H	$(NO_2)_2$	$(NO_2)_2$	$CH_2CH(NH_2)COOH$	6,600
23	H	H, H	**I**, **I**	$CH_2CH(NH_2)COOH$	6,400
24	H	H, H	I,H	$CH_2CH(NH_2)COOH$	5,060
25	H	H, H	H, H	$CH_2CH(NH_2)COOH$	660

As seen the specific structural requirements for maximum binding of thyroxin analogs to a thyroxin binding fraction of serum albumin are:-

A diphenyl ether nucleus; four iodine atoms; a free phenolic hydroxyl group ; an alanine side chain or anionic group separated by three carbon atoms from the aromatic nucleus.

The resulting association constant of 500,000 was reduced significantly if any of this changed. Compounds with single aromatic rings showed reduced association constants (≈ 10).

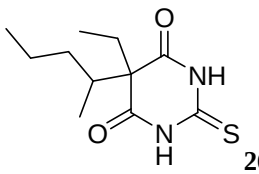
1.2.6.5 Penetration of Drugs Through Biological Membranes

The extent of absorption or penetration of a drug through biological membranes influences its biological activity and is dependent on factors like dissolution rate, solubility in polar and non polar solvents and partitioning between polar and non polar phases. All biologically active substances must have or acquire, through chemical modification, some minimum solubility in the polar extra cellular fluids (Daniels, 1943). Factors that determine the dissolution rate of a drug, and its transfer from one phase to another, affect its absorption (Wagner, 1961).

Penetration of biological membranes may be active or passive. Polar molecules of a given size may pass through aqueous pores in membranes. Molecules that resemble normal metabolites may utilize existing transport systems. Lipid soluble compounds diffuse through the lipoidal membrane Diffusion through pores for ions is reduced by hydration (Overton, 1901); (Schanker,1960). This makes diffusion through lipoidal membrane more significant and common for most drugs.

Lipid solubility of weak acids and bases is influenced by pK_a of the acid or/base and the pH of the surrounding media; - the lower the degree of ionization of a compound at a given pH, the higher its lipid solubility. That lipid solubility is the physical property governing the passage of uncharged molecules across membranes barriers is supported

by the observation that three barbiturates with similar pK_a values were absorbed at rates proportional to their lipid/water partition coefficients (K = Chloroform/Water) of the unionized forms:- Thiopental (**26**) (pKa = 7.6; K = > 100) was absorbed very rapidly, the less lipid-soluble secobarbital (**27**) (pKa = 7.9; K = 23.3) less rapidly and barbital (**28**) with its poor lipid solubility (pKa = 7.8; K = 0.7) was absorbed very slowly (Schanker *et al.,* 1957; Hobgen,1957).



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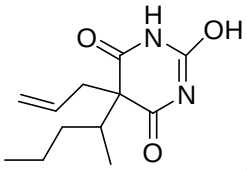

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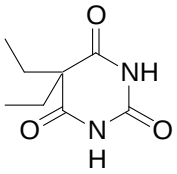

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1.2.6.6 Influence of Specific Physicochemical Properties on Biological Activity

1.2.6.6.1 Solubility and Partition Coefficients

In homologous series of undissociated or slightly dissociated compounds like alcohols, gradations in intensity of biological action have been observed; normally the lower members of homologous series show a low order of activity. This increases with increase in the length of carbon chain, passes through a maximum then decreases rapidly with further increase in length of carbon chain (Daniel, 1943; Coulthard *et al.,* 1930; Dodds, 1939). The initial increase parallels increased lipid solubility and hence availability of compound to the cell. The observed decrease has been attributed to decreased solubility in the aqueous extra cellular fluid in which cells are immersed (Daniel, 1943). The size of the molecules that shows maximum activity in a given series depends on the organism's sensitivity (Albert, 1968).

Antibacterial activity (*Staph. aureus*) of normal aliphatic alcohols increases from methyl through octyl then decreases in higher members. Branched alcohols are more water soluble and have lower lipid/water partition coefficients. They have lower biological activity e.g. n-hexyl alcohol is more than twice as active as 2° hexyl alcohol and 5 times as active as the 3° hexyl alcohol. (Winterstein, 1926) found that partition coefficients in series of esters of *p*-hydroxybenzoic acid were parallel to a BA as shown in table 2 below. The correlation between biological activity (fermentation and bacterialcidal) are both significantly correlated to partition coefficients of the esters as seen from the SPSS analytical output (table 3).

In his study of narcotics, Meyer (1891) observed that efficiency of narcotic agents is dependent on their partition coefficients in a mixture of water and lipid substance (Falk 1944). This has been found to be true for many compounds (Winterstein and Die Narcose 1926 ; Meyer. *et al.,* 1920). Table 2 below shows the change in values of two biological activities with partition coefficient values of some esters of p-Hydroxybenzoic Acid.

Table 2: Some Biological Activities And Partition Coefficients of Esters of p-Hydroxybenzoic Acid; Adopted from Winterstein (1926).

Ester	Compd. No.	Inhibition of fermentation	*Staphylococus. aureus* bacterialcidal	Partition coefficient
Methyl	**29**	3.7	2.6	1.2
Ethyl	**30**	5.3	7.1	3.4
n- Propyl	**31**	25.0	15.0	13.0
i- Propyl	**32**	15.0	13.0	7.3
n- Butyl	**33**	40.0	37.0	17.0
Amyl	**34**	53.0	……	150.0
Allyl	**35**	15.0	12.0	7.6
Benzyl	**36**	69.0	83.0	119.0
Phenol	**37**	1.0	1.0	……

The inhibition of fermentation and the *Staphylococus aureus* bacterialcidal activity of the esters are significantly and strongly correlated to their corresponding partition coefficient values as shown by the SPSS nonparametric Correlation output in table 3 below.

Table 3: SPSS Nonparametric Correlations of some biological activities with the partition coefficients of some esters of p-hydroxybenzoic acid.

			Inhib. of fermen.	*Staph. aureus*	Partition
Spearman's rho	Inhib. of fermen.	Correlation	1.000	.994**	.970**
		Sig. (2-tailed)	.	.000	.000
		N	9	8	8
	Staph. aureus Bacterialcidal	Correlation	.994**	1.000	.964**
		Sig. (2-tailed)	0.000	0.000	0.000
		N	8	8	7
	Partition coefficient	Correlation	.970**	.964**	1.000
		Sig. (2-tailed)	0.000	0.000	0.000
		N	8	7	8
**. Correlation is significant at the 0.01 level (2-tailed).					

1.2.6.6.2 Mole Refraction

To explain why compounds with high partition coefficients show hypnotic properties Wulf and Featherstone (1957) correlated biological activity to molecular size. They argued that energy of intermolecular attraction is approximately proportional to the polarizability (**mole refraction**) of the molecules of the anesthetic agent and that a correlation exists between the constants "a" and "b" in the van de Waal's equation and the presence or absence of anesthetic potency; that a critical "size" (van der Waals "b") related to molecular volume was necessary for the anesthetic molecule.

Molecular volume ("b" values) for some anesthetic agents are:- N_2O, 4.4; Xe, 5.1; Ethylene, 5.7; cyclopropene, 7.5; chloroform, 10.2; ethyl ether, 13.4. Substances with values less than 4.4 were not potent e.g. water, 3.05; Oxygen, 3.18 and nitrogen, 3.91.

Such physical properties as solubility in oil, distribution between oil and water, the vapour pressure ("thermodynamic activity") of the pure liquid or the partial pressure of hydrate crystals were found to correlate with the anesthetic activity and to the Van der Waals attraction of molecules of anesthetic agents for other molecules.

1.2.6.6.3 Thermodynamic Activity

Compounds with different functional groups often display similar biological activity e.g. narcotic action. This is indicative that mainly physical, rather than chemical properties are responsible for biological activity. That equilibrium exists between the extra cellular and the biophase is indicated by the numerous relationships between biological activity and physical properties in homologous series; - in many such series the potency increases as the series is ascended. Fuhner (1904) found this decrease in equitoxic concentration to be a geometric progression (1, 1/3, $1/3^{2}$, $1/3^{3}$ …) with increase in number of carbon atoms.

Certain but not all physical properties also change according to a geometric progression in ascending a homologous series. These include vapour pressure, water solubility, capillary activity and distribution between immiscible phases. Since logarithms represents a geometric progression, a plot of the logarithms of the value of these various properties against the number of carbon atoms gives straight lines as illustrated in Table 4 and Fig. 2 below.

Table 4: Logarithmic values of some properties along a series of normal primary alcohols

No. of C atoms	Log of Value				
	I	II	III	IV	V
1	5.0	6.1	6.7	7.0	
2	4.5	5.8	6.3	6.8	
3	3.8	5.4	5.8	6.4	
4		5.0	5.4	5.8	6.1
5		4.5		5.3	5.5
6		4.0		4.8	5.0
7		3.4		4.2	4.2
8		3.0		3.7	3.8

I- partition coefficient between water and cotton seed oil

II- vapour pressure at 25˚C

III- concentration reducing surface tension of water

IV- toxic concentration for *C. typhosus*

V- water solubility

Figure 2 below illustrates the graphical trends of the properties in table 4 above along the series of compounds. The attribute these physical properties have in common is that they involve distribution between heterogeneous phases;

i. Solubility – solid or liquid and saturated solution

ii. Surface activity – surface and solution.

iii. Vapour pressure – liquid and vapour

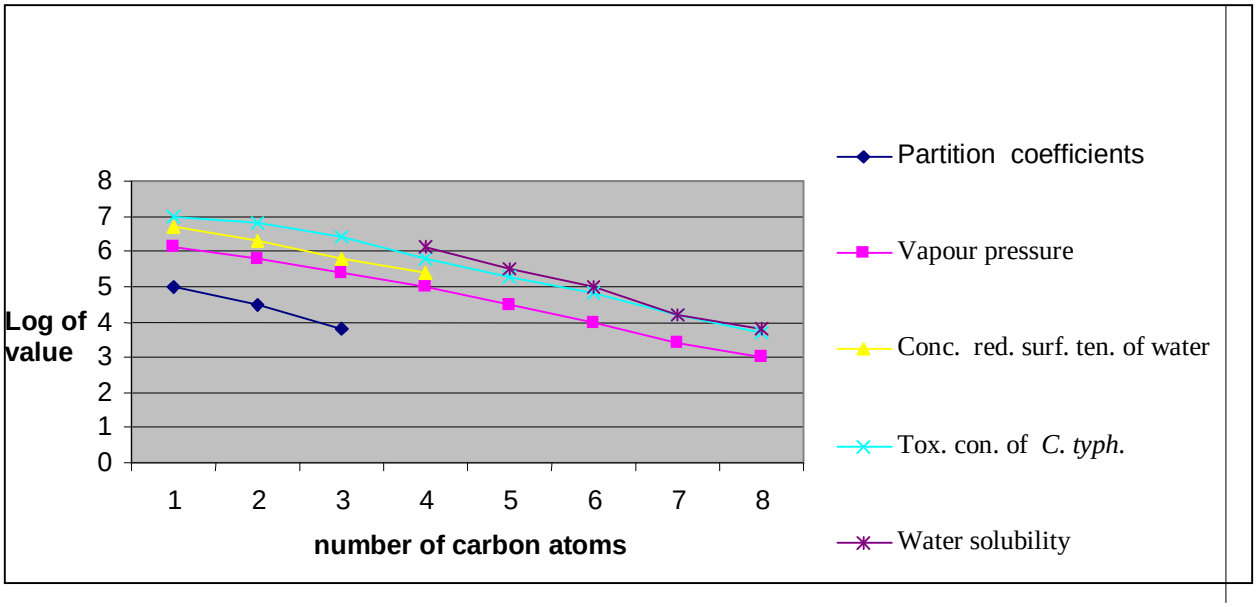


Figure 2: Change in some properties of normal primary alcohols with number of carbon atoms

According to Ferguson (1939) BA of this type (log) must hence involve equilibrium between extra cellular fluids and biophase and results from the relation,

Log K = ($F°_2 - F°_1$) / RT

where k is the distribution coefficient and F_1^0 and F_2^0 partial molal free energies of the substance in standard states in phases 1 and 2, respectively. Each additional CH_2 group hence gives rise to a constant increment in the difference between the partial molal free energies . Ferguson advanced the concept that it is unnecessary to define the nature of the biophase or receptor, nor is it necessary to measure the concentration at this site. If equilibrium conditions exist between the drug in the biophase and that in the extra cellular fluids, although the concentrations in each phase are different, the tendency to escape from each phase is the same. In such a system, the partial molal free energy for

the substance must be equal in each phase ($F°_1 = F°_2$) since this serves as quantitative measure of the escaping tendency from that phase.

The **degree of saturation** of each phase is a reasonable approximation of the tendency to escape from that phase, and this may be called the **thermodynamic activity**. In case of a gas it is approximately equal to $\mathbf{P_t/P_s}$ where $\mathbf{P_t}$ is that partial pressure of a gas necessary to give a B.A and $\mathbf{P_s}$ is the saturated vapour pressure. In case of a solution, the thermodynamic activity is approximately equal to $\mathbf{S_t/S_o}$ where $\mathbf{S_t}$ is the concentration necessary to give a BA and $\mathbf{S_o}$ is the solubility of the substance. Since thermodynamic activity is the same in both the biophase and the extra cellular phase, measurements made in the latter, which is accessible, may be directly equated with the former which is inaccessible.

Ferguson (1939) has given further evidence to show that B.A of various types closely parallels the thermodynamic activity of compounds. In members of homologous alcohols, he observed this with respect to:-

i. Inhibition of development of sea urchin eggs.
ii. Bactericidal action against *B. typhosis*
iii. Tadpole narcosis
iv. Hemolysis of OX blood

It also holds for the action of several types of compounds with respect to narcosis, bactericidal and insecticidal action: - bactericidal concentration of an alcohol varies from 10.8 to 0.0034 moles per liter in going from methyl to octyl alcohol while the thermodynamic activity varies only from 0.33 to 0.88 (Ferguson, 1939); for a variety of depressant gases and vapors, the isonarcotic concentrations varied from 100 to 0.5 per

cent by volume but the thermodynamic activity (ratio of partial vapor pressure to saturation pressure) varied from only 0.01 to 0.07 as shown in the table below (Table 5). Table 5 also illustrates that anesthetic action of compounds in man parallels thermodynamic activity.

Table 5: Isoanaesthetic concentration of gases and vapours in man at 37°C

Substance	Vapour Pressure*(mm) p_s	Anesthetic[§] Concentration (V%)	Partial Pressure at anesthetic conc. (760x c/100)=P_t	Approximate Thermodynamic Activity P_t/p_s
Nitrous oxide	59,300	100	760	0.01
Ethylene	49,500	80	610	0.01
Acetylene	51,700	65	495	0.01
Ethyl chloride	1,780	5	38	0.02
Ethyl ether	830	5	38	0.05
Vinyl ether	760	4	30	0.04
Ethyl bromide	725	1.9	14	0.02
1,2-Dichloroethylene	450	0.95	7	0.02
Chloroform	324	0.5	4	0.01

*from data by Ferguson (1939) and the handbook of chemistry and physics, chemical rubber company.
[§]from data in Goodman and Gilman (1965).

The data shows that although the anesthetic concentration changes by 200-fold the thermodynamic activity changes only slightly. Compounds whose BA parallels

thermodynamic activities are said to be structurally non specific and exhibit the following characteristics:-

i. BA is related directly to the thermodynamic activity.
ii. Same BA is observed if thermodynamic activity is same even when compounds differ widely in chemical properties.
iii. In general substances which are present in the same proportional saturation have the same thermodynamic activity and the same degree of biological activity.
iv. Saturated solutions of different substances should have the same biological effect.

Structurally specific compounds are effective in lower concentrations than the non specific ones. Equilibrium is involved in the action of both specific and non specific compounds;

i. External phase – biophase
ii. Drug – receptor
iii. Drug – enzyme

The bonds in such interactions include covalent, ionic, ion-dipole, dipole – dipole, hydrogen, Van der Waals and hydrophobic. In case of the structurally specific agents the bonds are likely to be stronger and the equilibrium shifted to the side favoring maximum BA. Whether a drug is structurally specific or non specific is of fundamental importance in developing new therapeutic agents. This can be decided by determining the thermodynamic activity necessary to produce the useful BA ("Ferguson Value", P_t /P_s, S_t / S_o) being studied using several different chemical types and comparing the values for the new compounds. If the new agents have comparable values, they most probably are

structurally non specific and minute variations in structure usually would not be expected to produce marked changes in BA.

1.2.6.6.4 Steric and Stereochemical Features

The ability of a molecule to produce potent analgesia has been related to the relative spatial positioning of a flat aromatic nucleus, a connecting aliphatic or alicyclic chain, and a nitrogen atom which exists largely in the ionized form at physiological pH. (Beckett and Casy 1954). Staggered conformations are favored more than skew or eclipsed ones. The favored conformation with respect to an atom like nitrogen in the chain may change when a methyl or another heavier group is substituted to the carbon bearing the nitrogen atom e.g. the favored conformation of (3 – phenyl ethylamines with respect to nitrogen is dependent on atoms bonded to the carbon bearing the nitrogen**.** The overall result is a reduction in the average distance between the aromatic group and the basic nitrogen atom in a methyl substituted (3 –amylethylamines).

This steric factor influences the strength of the binding interaction with biologic receptor. The altered stereochemistry of 3- amylethylamines has been used to partially account for the slow rate of metabolic deamination. In addition to adding polar character to a segment of a chain ester, amide groups introduce a planar configuration that is resonance stabilized and mainly in the *cis* form. Such configuration full extended chains like in succicnyl choline:-

The molecule is readily hydrolyzed by plasma cholinesterase. Intra molecular interactions may favour a given conformation. For instance, the terminal positive charges on the polymethylene bis – quartenary ganglionic blocking agents

hexamethonuim and the new muscular blocking agents decamethonuim make it most likely that the ends of this molecule are maximumly separated in solution. In some cases, dipole – dipole interaction appear in structures in solution hence methadone may exist partially in a cyclic form due to dipolar attraction between the basic nitrogen and carboxyl group (Beckett, 1954).

In such a conformation it closely resembles the confromationally more rigid potent and analgesics, morphine, meperidine and their analogs and it may be in this form that interacts with the analgesic receptors.Intra molecular bonds may stabilize a particular conformation of a drug in non aprotic solvents. This is not significant in biological systems where drugs are suspended in aqueous phases.

1.2.6.6.5 Conformational Flexibility

It has been suggested that conformational flexibility of open chain molecules permits multiple biological effects to be produced by single molecule interacting in different and unique conformations with different receptors. Hence a acetylcholine interacts with muscarinic receptor of postganglionic parasympathetic nerves and with acetylcholinesterase in the full extended conformation and with nicotinic receptor at the ganglia and neuromuscular in a more folded structure (Martin – Smith *et al.*, 1967; Kier, 1968). Molecular orbital calculations have indicated that histamine may exist in two extended conformations (A & B) of nearly equal and minimal energy (Kier, 1968) rather than the predicted coiled form (C) involving inter molecular hydrogen – bonds (Niemann and Hayes, 1942). It proposed that histamine acts on smooth muscle in

conformation A and induces gastric acid release through conformation B. This hypothesis has however not been tested.

1.2.6.6.6 Optical Isomerism

(-) Amino acids are either tasteless or bitter while(+) amino acids are sweet ; (-) ascorbic acid has good antiscorbutic properties while positive ascorbic acid has none; (-)epinephrine is 12 to 15 times more active as vasoconstrictor than (+) epinephrine. Cushny (1926) accounted for this difference by assuming that the optical enantiomers reacted with an optically active receptor site to produce diastereoisomers with different physical and chemical properties. Easton and Steadman (1938) proposed that only one enantiomer is capable of superimposing and hence causing BA on a receptor site.

Easson and Steadman (1938) explained the differences in BA of enantiomers of epinephrine as due to differences in ease of attachment to the receptor surface. This is consistent with the observation by Blaschko (1950) that desoxyepinephine which lacks the alcoholic hydroxyl group has about the same pressor effect as (+) epinephrine.

1.2.6.6.7 Isosterism

The concept was introduced by Langmuir (1919). While seeking a correlation to explain similarities in physical properties of non isomeric molecules he defined Isosteres as compounds or groups of atoms having same number and arrangement of electrons. Isoelectric isosteres (with same number of electrons and total charge) were predicted to possess similar physical properties e.g. N_2 and N_3 and NCO. Grimm (1925) advanced the concept by introducing the "Hydride Displacement Law":- By adding a hydrogen atom to another atom (or group),one obtained a combination (pseudo atom) with some physical properties similar to those of the atom (or group) which has one more electron than that from which the "pseudo atom" was derived.

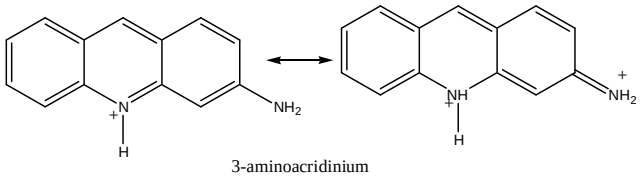
Friedman (1950) has defined bio-isosteric compounds as those that possess similar steric, electronic and BA .The general principles of isosterism are frequently applied in the structural alteration of compounds known to possess BA – for more selective drugs and understanding of drug. Free and Wilson (1964) proposed a purely empirical mathematical approach for correlating and predicting activity based on substitutents on a fundamental structural unit; the series must be on a clear portion of the usual parabolic curve of a homologous series. This method has been used in studies of substituent effects on the ability of the parent phenylethylamine structure to inhibit uptake of norepinephrine by the isolated rate heart (Ban and Fujita, 1969).

1.2.6.6.8 Ionization

BA of molecules that require to be undissociated for maximal effect is pH dependent; the antibacterial activity of benzoic acid, salicylic acid, mandelic acid etc is greater in acid media and increases close to 100 times in going from neutral to acidic solution (pH 3) due to high concentration of undissociated molecules at low pH. (Rahn and Conn, 1944). The antibacterial action of the phenols is greatest at a PH below 4.5 and the activity again increases at a pH above 10 (Kuroda, 1926). This increase at high pH has been attributed to partial oxidation of the phenol to a more quinone. Solubility and partition coefficients of acids and bases may be altered greatly by changes in pH; Cocaine hydrochloride is freely soluble in water (1.04) while the free base is poorly soluble (1:600). The free base partitions well in organic solvents while the salt is poorly partitioned. Some compounds show increased BA when their degree of ionization is increased. Some compounds are thought to act outside the cell.

Albert and co- workers (1945) studied ionization of a number of acridine compounds at pH 7.3 and observed that effective antibacterial action required 75% ionization at 20˚C or 60% ionization at 37˚C. Acridine cations were shown to be responsible for B.A. The undissociated molecules, anions or zwitterions were inactive. Table 6 below shows some of the acridines studied. The base strength of the heterocyclic nitrogen is affected by amino group substitution:-

i. Base strength increases through resonance stabilization of the ion by amino group in the 3, 6 or 9 position leading to higher B.A.



3-aminoacridinium ions

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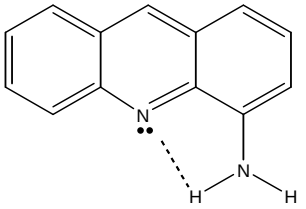
ii. Substitution of NH_2 group in the 4 position leads to base weakening intermolecular hydrogen bonding :-



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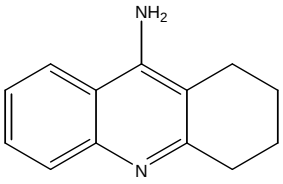
iii. Substitution in the 1&2 positions permits no resonance stabilization and base strength remains low.

Apart from NH_2 substitution BA of aminoacridines is also affected by factors affecting total flat surface area of the molecule and is lost for areas below 38 square Å;

i. MIC for 9-aminoacridine (**39**) with one ring reduced (9-aminotetrahydroacridine) is 1/5,000



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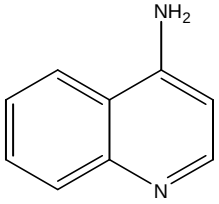
ii. MIC for 4-aminoquinoline (**49**) is < 1/5,000



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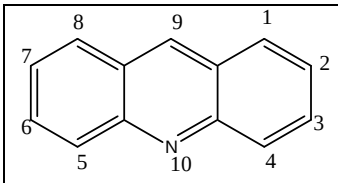
It was hence postulated that only a given flat area enables strong enough van der Waals forces with the receptor for effective BA.

Table 6: Ionization and Bacteriostatic Effects of Aminoacridines*



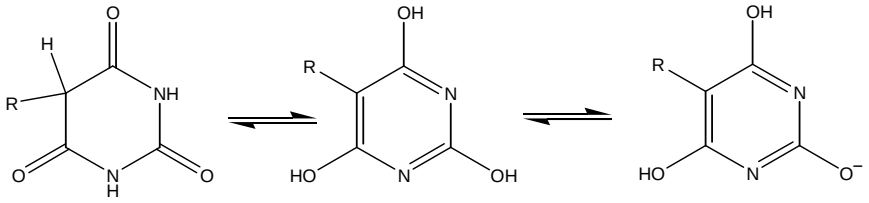
Compod. No.	Acridine	Min. bacteriostatic Conc., *Strept. Pyog*.	Per Cent Ionized (pH 7.3; 37˚
38	3-NH_2	1/80,000	73
39	9-NH_2	1/160,000	99
40	3,6-diNH_2	1/160,000	99
41	3,7-diNH_2	1/160,000	76
42	3,9-diNH_2	1/160,000	100
43	4,9-diNH_2	1/80,000	98
44	4-NH_2	1/5,000	<1
45	2-NH_2	1/10,000	2
46	1-NH_2	1/10,000	2
47	4,5-diNH_2	<1/5,000	<1
48	2,7-diNH_2	1/20,000	4

*Adopted from Albert (1968)

A large number of substances showing marked antibacterial activity are only similar in possessing cations of high molecular weight (150 or more) and being ionized as pH 7. This includes aliphatic amines, quaternary ammonium compounds, diamines, amidines,diamidines, guanidines, biguamidines, pyridinium compounds among others.

Highly ionized anionic compounds normally show no significant BA. probably due to the predominantly anionic nature of living cells. For instance, most bacteria have an isoelectric point of about 4 and are anionic at pH of 7 or more.

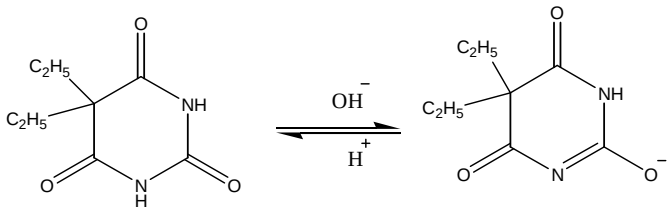
Minor changes in structure can produce significant changes in the degree of ionization of weak acids and bases and this may be responsible for presence or absence of BA.; 5,5 disubstituted barbituric acid derivatives are CNS depressants while barbituric acid (**50**) (pK_a= 4.0) and its 5-monosubstituted derivatives (e.g.5-ethylbarbituric acid (**51**); (pK_a= 4.4) are inactive .Barbituric acid and its 5-monosubstituted derivatives are stronger acids because of delocalization of the extra electron pair in a completely aromatic structure which can stabilize the barbiturate ion:-



Barbiturate ion

R = H for Barbituric acid (**50**) and -C_2H_5
for 5-Ethylbarbituric Acid (**51**)

At physiological pH of 7.4 the compounds are 99.9% ionized and do not effectively penetrate the lipoidal barrier of the CNS. In contrast, the 5, 5 disubstituted barbituric acid (**52**) derivatives cannot assume fully aromatic character:-

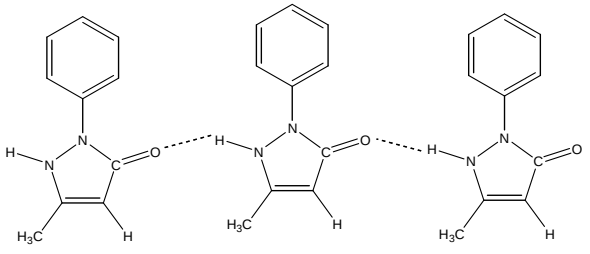


5,5-Diethylbarbituric acid (Barbital) 5,5-Diethylbarbiturate ion **52**

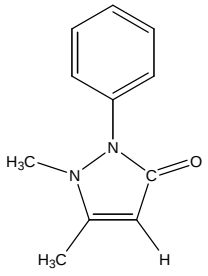
They are therefore much weaker acids (pK_a 7.0 to 8.5) and are less than 50% ionized at physiological pH and easily penetrate the lipoidal CNS tissue.

1.2.6.6.9 Hydrogen Bonding

In a number of cases, hydrogen bonding may be correlated with biological activity; 1-Phenyl-3-methyl-5-pyrazolone(**53**) shows no analgesic properties while 1-phenyl-2, 3-dimethyl-5-pyrazolone(**54**) is a well known analgesic agent. The former has a melting point of 127^0 C has low water solubility and is only slightly soluble in ether. The latter has a lower melting point of (112^0 C), is soluble in water (1:1) and moderately soluble in ether (1:43). These differences have been explained as arising from formation of a linear polymer through intermolecular hydrogen bonding in the first molecule.:-



Intramolecular hydrogen bonded1-Phenyl-3-methyl-5-pyrazolone **53**



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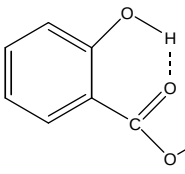

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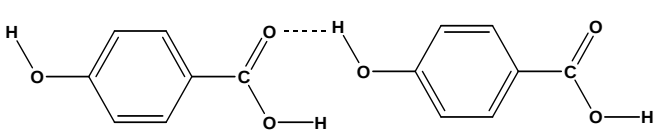
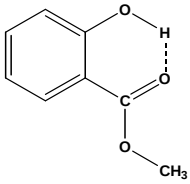


p-Hydroxybenzoic Acid (dimer) **56**



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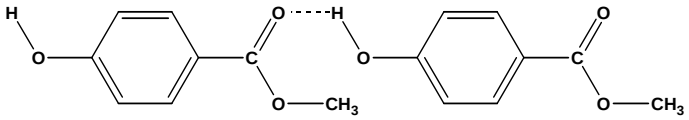


Methyl p-Hydroxybenzoate (dimer) **58**

o-Hydroxybenzoic acid (salicylic acid) (**55**) is antibacterial while p- hydroxybenzoic acid (**56**) is inactive. The reverse is true for the esters (methylsalicylate and methyl -p-hydroxybenzoic acid). The differences may be accounted for through hydrogen bond formation. Salicylic acid is the only one of the three hydroxybenzoic acid isomers (m-, o-, p-) that can form intramolecular hydrogen bonds. The m-and p- isomers can only form intermolecular Hydrogen bonds. Salicylic acid (**55**) with an intramolecular hydrogen bond has the phenolic hydrogen masked, but the carboxylic acid group is free and can function as antibacterial agent similar to benzoic acid. Hydroxybenzoic acid (**56**), on the other hand, must form intermolecular hydrogen bonds leading to a high degree of association and thereby lowering its antibacterial activity.

Other differences between the two isomers include acidity (-o > -p), water solubility –o < -p), partition coefficient (benzene/water) (-o> -p). For the esters opposite effect in bactericidal effect is observed. Methyl salicylate (**57**) has extremely weak antibacterial activity, but Methyl p-Hydroxybenzoate shows good action. Methyl salicylate, through intramolecular hydrogen bond formation has the phenolic hydroxyl group masked. Although intermolecular hydrogen bonding is possible in esters of p-Hydroxybenzoic acid (**58**) evidence from partition coefficients and bacterial activity data suggest that the molecules are not highly associated and the phenolic hydroxyl group is unmasked.

1.2.6.6.10 Chelation

Many drugs form chelate complexes with metals. Apart from affecting the major action of a drug, chelate formation may be responsible for significant side effects; e.g. the anemia produced by administration of the hypotensive agent hydralazine (Apresoline) (**59**) has been attributed to its ability to complex with iron (Prerry and Schroedes 1954). Dimercaprol (**60**) and the antitubercular drug isonicotonic acid hydrazine **(INH) (2)** tend to induce histamine like actions probably due to complexing with a copper catalysed enzyme responsible for the destruction of the histamine (Bruns and Stiiltgen 1951).

INH (**2**) may function as a chelating agent in inhibiting the growth of *Mycobacterium tuberculosis*. The drug is active chelating agent and nonchelating derivatives like 1-methyl-1- isonicotinoyl hydrazine are inactive (Cymerman – Craig *et a.,l* 1955). SARS of chelates as antibacterial agents have been described by Albert and Co-workers (1945; 1947; 1951). 8-Hydroxyquinoline (oxine) (**61**) has antifungal and antibacterial properties associated with removal of trace metals essential for metabolism of the organisms. (Albert, 1945).

A study of the 7 isometric monohydroxy-quinolones showed that only the 8-Hydroxyl isomer was active in inhibiting the growth of microorganisms and that the same isomer was the only one to form metal chelates. A study of derivatives of 8-Hydroxyquinolones and related analogs led to the following generalizations on SARS. All the active compounds (antibacterial) form metal chelates but not all chelating structures are biologically active as illustrated in figure 3 below .

Both the 8-methyl ester and the 1- methyl derivatives of 8- hydroxyquinoline which are unable to form chelates show no antibacterial effect. Substitution of a mercapto for the hydroxyl group in oxine gave an active chelating agent which is also active as an antibacterial. The substitution of a methyl group in the 2- position of oxine gives an active chelating agent in vitro but the compound is relatively inactive as an antibacterial. The decreased activity was attributed to lack of penetration of the cell, or interaction with the cell receptor due to steric hindrance. The introduction of a highly ionizable group in oxine (e.g. 8 – hydroxyquinoline – 5-sulfonic acid) does not alter the chelating property in vitro but the antibacterial activity is lost, presumably due to the inability of the ions to penetrate the cell wall.

A high partition coefficient appeared essential for antibacterial activity. It was established that oxine and its analogues acts as antibacterial and antifungal agents by complexing with iron or copper. In absence of these metals oxine was non toxic to microorganisms. In a study to determine whether oxine (**61**) acted within or on the cell surface, Albert and Co-workers (1954) observed that activity of mono- aza and alkylated mono-aza oxines paralleled the oil/water partition coefficients, suggesting that the compounds penetrate the bacterial cell for action.

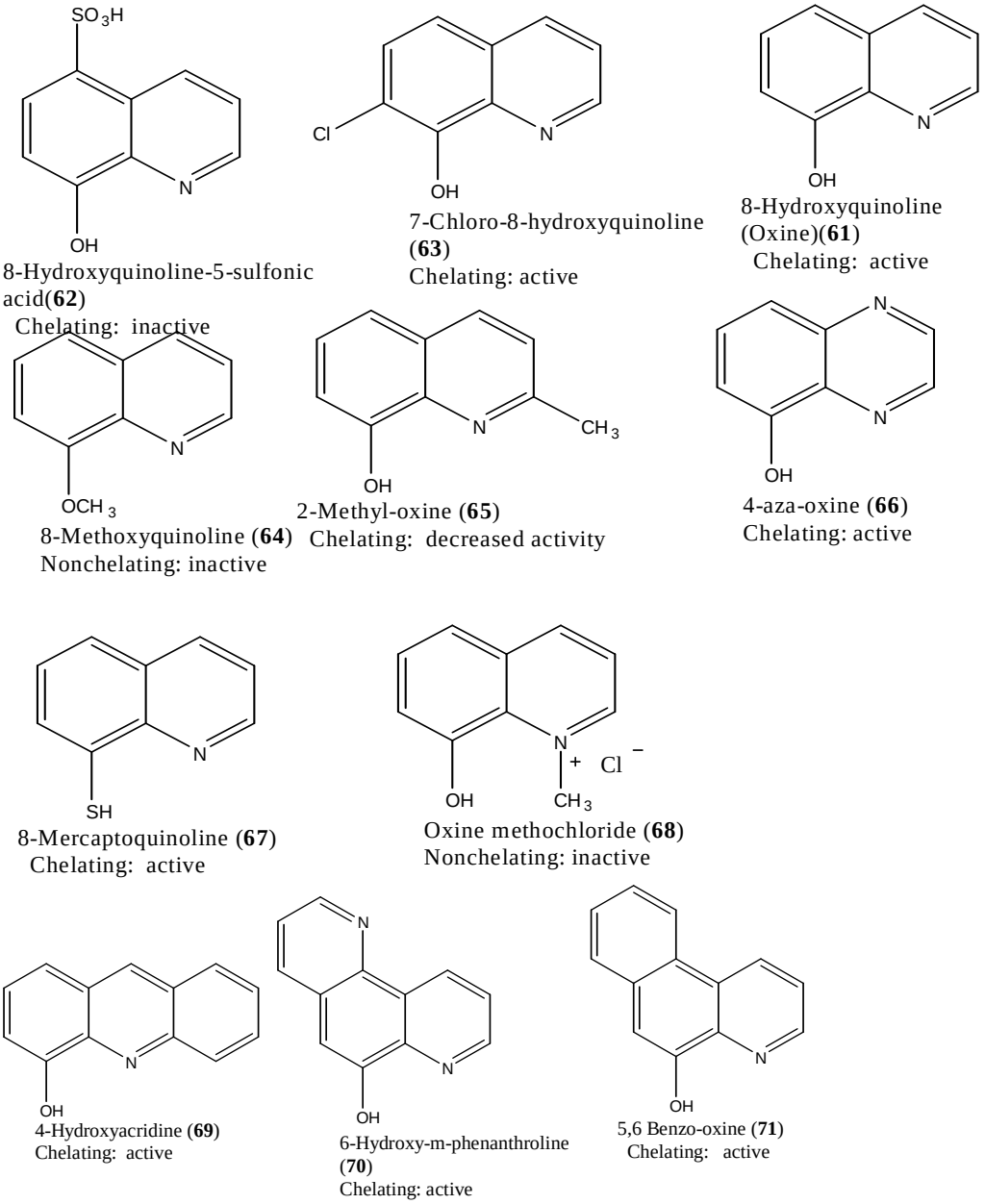


Figure.3: Information on chelate formation and biological activity of some 8-hydrxyquinolone derivatives

Beckett (1958) have however suggested that the site of action may be on the cell surface. The presence of an excess of either iron or oxine leads to loss of the antibacterial action (Rubbo *et al.,* 1950); the growth of *Staph. aureus* in untreated meat broth is completely inhibited by oxine at 10^{-5}M but this concentration has no effect on

the organism when suspended in distilled water. In the meat broth oxine combines with trace amounts of iron and was active. When concentration of oxine was increased to $1.25x10^{-3}$M, the inhibition of growth disappeared. This was attributed to shifting of the equilibrium from the unsaturated and toxic (1:1- and 2:1- complexes) to the saturated non toxic 3:1-oxine-ferric complex as shown in Fig. 4 below :-

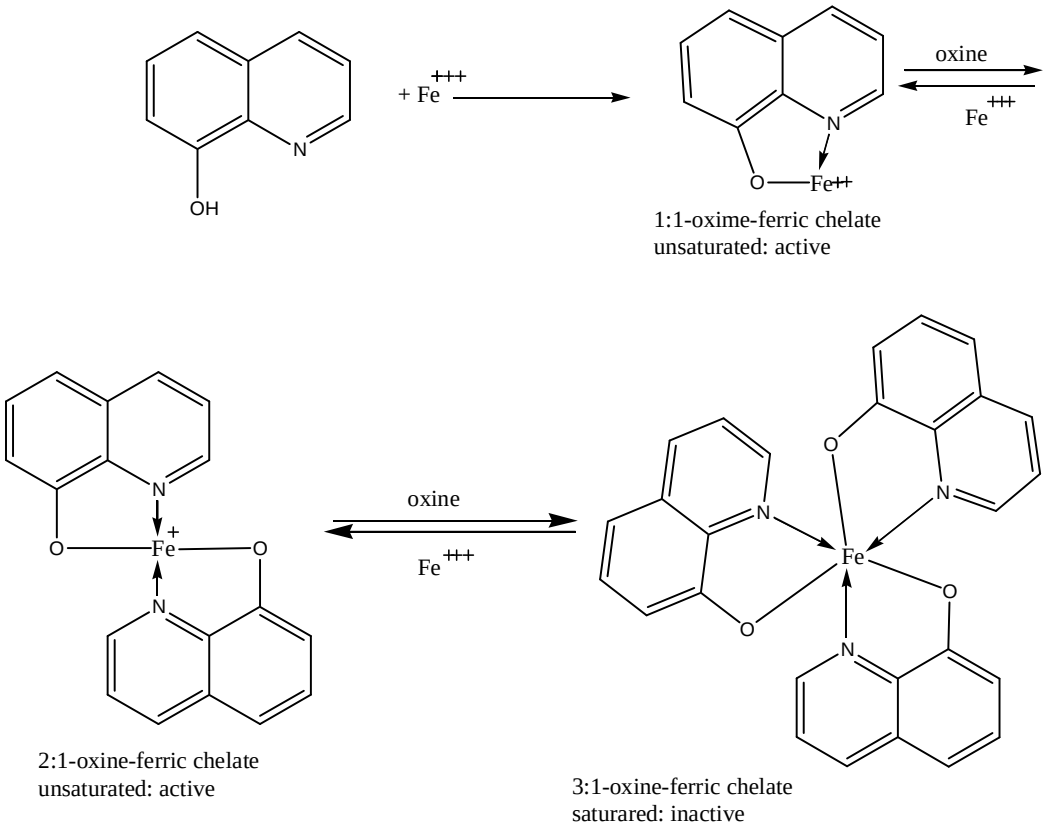


Figure 4: oxine-iron chelates

Inhibition of growth occurred again when the concentration of iron was increased to $1.25x10^{-3}$M since the equilibrium is shifted from the saturated 3:1 complex to the unsaturated 1:1 and 2:1 complex which are toxic. The antibacterial properties of oxine-iron complexes are antagonized by metals that form more stable complexes. For

instance, the addition of low concentrations of cobalt sulphate ($^{1}/_{25,000}$M) completely inactivates the antibacterial action of (0.1µM) oxine-iron solutions (Rubbo *et al.,* 1950).

1.2.6.6.11 Oxidation – Reduction Potentials

Paye and Robinson (1943) studied the relationship between bacteriostatic activity and the normal redox potentials of substituted quinones. They found no simple relation between the reduction potentials (E'_o) of 20 substituted quinones and their observed bacteriostatic activity. Breyer *et al.,* (1944) also found no detailed correlation between reduction potentials of a number of acridines and their antibacterial activity. Tozer *et al* (1969) correlated the anthelmintic activities of a series of substituted phenothiazines with possession of a redox potential.

1.2.6.6.12 Surface Activity

Surface activity is the lowering of the surface tension or surface energy of water by amphipathic molecules that are less polar than water. The surface – active ions of intermediate to high molecular weight (150 to 300) show the same electrical and osmotic properties in dilute solutions as equivalent concentrations of inorganic electrolytes. The ions are therefore monomeric in dilute solutions. As the concentration of the surfactant is increased a critical point is reached at which the molecules associate (become polymeric) to form micelles. This happens at the critical micelle concentration (CMC) which differs for each surfactant. According to Albert (1968), drugs act in their monomolecular form, but at the CMC and above the micelle competes with the microorganism for the monomers, thereby reducing the effective antibacterial concentration. He also suggested

that mixed micelle formation may be responsible for the phenomenon of therapeutic interference.

The antihelminitic activity of hexylresorcinol is reported to be increased by low concentration of soap. If the soap concentration is kept below the CMC, a 1:1 association of the phenol and soap occurs which facilitates the penetration of phenol through the surface of the worm. If the CMC is exceeded, the micelle competes favorably with the worms for the phenol and there is decreased activity (Valko and Dubois, 1944).

1.2.6.7 The Hansch Approach and QSARS

A quantitative measure of the importance of partition behaviour on drug action has been introduced. Hansch (1964) has determined the effects of substituent groups on distribution between water and the non polar solvent 1-octanol. The distribution coefficients for the parent compound e.g. phenoxyacetic acid ($C_6H_5OCH_2$ COOH) and a derivative e.g. 3-trifluoromethyl phenoxyacetic acid (3-CF_3-$C_6H_5OCH_2$ COOH) are measured and a value, π, for the substituent trifluoromethyl group is determined by the difference between the logarithm of the distribution coefficient:-

$$\pi_{CF3} = \log P_{CF3} - \log P_H$$

Where P_{CF3} is the partition coefficient of the 3–trifluoromethyl derivative, and P_H that of the unsubstituted parent compound. The π values taken in conjunctions with the Hammett Sigma (σ) values (measure of the electronic contributions of substituents relative to hydrogen) have been used effectively in correlating chemical structure, physical properties and biological activities. (Jaffe, 1953).

Hansch has assumed that a rate limiting condition for many biological responses involves the movement of the drug through a large number of cellular compartments made up essentially aqueous or organic phases. The molecule possessing solubility and structural characteristics such that the sum of the free energy changes is minimal for many partitionings made between phases, including adsorption-desorption steps at solid surfaces, will have ideal lipohydrophylic character and will most easily reach its site of action. The π value is a measure of the substituents contribution to solubility behaviour in such a series of partitions. Table 7 below lists some typical substituent constants arranged in order of decreasing contribution to the lipophilic character when substituted in the three positions of phenoxyacetic acid.

Values of π and sigma (σ) are approximately constant and additive in a variety of different aromatic systems, as long as no strong group interactions occur. Therefore the substituent constant for a polysubstituted aromatic compound are approximately equal to the sum of π and σ values for individual substituents. The additive character of these constants has been demonstrated by good correlations obtained by the action of polysubstituted phenols on gram-positive and gram-negative organisms, the action of thyroxin analogs on rodents and the carcinogenic activity of derivatives of dimethylaminoazobenzene and aromatic hydrocarbons and benzacridines (Hansch and Fujita, 1964). A different set of π values has been obtained for substituents attached to an aromatic nucleus (Iwasa *et al.,* 1965). In a homologous series, if functional groups are removed by two or more methylene (CH2) groups, interaction is small and values may usually be determined additively. Both the methyl and methylene(-CH_2-) groups have an

additive π value of about +0.5; thus π values for homologous series substituted in the three positions of the phenoxyacetic acid are: H=0, CH_3 = 0.51, C_2H_5= 0.97, n- C_3H_7 = 1.43, n – C_4H_9 = 1.

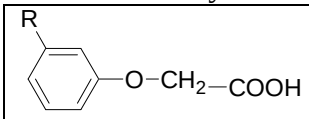
Relative to hydrogen = 0, a positive value for π means that the group enhances solubility in non polar solvents, a negative value that solubility in a polar solvent is enhanced. A positive value for σ denotes an electron - attracting effect; a negative value denotes electron-donation by the group. Thus the methyl group is typical of the alkyl groups in enhancing non polar solubility (π = +0.51) and is electron donating (σ = -0.17). By contrast the acetamido group (CH_3 – CONH -) as a substituent strongly enhances water solubility (π = -0.79) and is a weak electron acceptor (σ = +0.10). Replacement of a hydrogen, a methyl or a halogen by the trifluoromethyl group increases lipophilicity and BA: - CF_3>CH_3; SCF_3>SCH_3; OCF_3>OCH_3; SO_2CF_3>SO_2CH_3

In relating the application of the pi (π) and sigma (σ) substituent constants to biological activity (1/C) Hansch (1963) derived the equation:-

$$\mathbf{Log\ (^1/_C) = -k\pi^2 + k'\pi + \rho\sigma + K''}$$

Where C is the concentration of drug necessary to produce the biological response, K, K' and K" are constants for the system being studied, ρ is the reaction constant, π the substituent constant for solubility contribution and σ the substituent constant for electronic contributions. In this form, contributions by steric factors are assumed to be constants as substituents are varied.

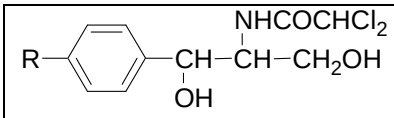
Table 7: Constants for Solubility (Π) and Electronic (σ) Effects of 3-Substituents In Phenoxyacetic Acid . (Data From Hansch 1963, 1964)



Compd. No.	R	π	σ
72	n-C_4H_9	+1.90	- 0.15
73	SCF_3	+1.58	+ 0.51
74	SF_5	+1.50	+0.68
75	n-C_3H_7	+1.43	- 0.15
76	OCF_3	+1.21	+0.35
77	I	+1.15	+0.28
78	CF_3	+1.07	+0.55
79	C_2H_5	+0.97	-0.15
80	Br	+0.94	+0.23
81	SO_2CF_3	+0.93	+0.93
82	Cl	+0.76	+0.23
83	SCH_3	+0.62	-0.05
84	CH_3	+0.51	-0.17
85	OCH_3	+0.12	-0.27
86	NO_2	+0.11	+0.78
87	H	0	0
88	COOH	-0.15	+0.27
89	$COCH_3$	-0.28	+0.52
90	CN	-0.30	+0.63
91	OH	-0.49	-0.36
92	$NHCOCH_3$	-0.79	-0.02
93	SO_2CH_3	-1.26	+0.73

Hansch (1953) did show (table 8) application of this to compare antibacterial activities of Chloromycetin and a series of its analogues. When substituent constants π and σ and relative observed antibacterial activities are substituted in the equation **Log A = -Kπ^2 + K'π + ρσ + K''**, the system and the reaction constants which best fit the experimental data are:- **Log A = -0.54π^2 + 0.48 π+ 2.14 σ + 0.22**

Table 8: Analogs Of Chloromycetin Tested Against *Staphylococcus aureus*



Compd. No.	R	σ	Π	Calculated Log A	Observed Log A
94	NO_2	0.71	0.06	1.77	2.00
95	CN	0.68	-0.31	1.47	1.40
96	SO_2CH_3	0.65	-0.47	1.27	1.04
97	$COOCH_3$	0.32	-0.04	0.89	1.00
98	Cl	0.37	0.70	1.08	1.00
99	$N\equiv N\text{-}C_6H_5$	0.58	1.72	0.69	-0.78
100	OCH_3	0.12	-0.04	0.46	0.74
101	$NHCOC_6H_5$	0.22	0.72	0.76	0.40
102	$NHCOCH_3$	0.10	-0.79	-0.28	-0.30
103	OH	0	-0.62	-0.29	< - 0.40
104	COOH	0.36	-0.16	0.90	< - 0.40

σ = Hammett sigma constant for 3- substituents A = activity relative to Chloromycetin = 100

A comparison of observed antibacterial activities and those calculated from the derived equation showed excellent correlation (r= 0,789; $P < 0.05$).

From these data, it is concluded that a strong electron – attracting group enhances activity ($\sigma_{N02} = +0.71$) as does a moderately lipophilic group ($\sigma_{N02} = 0.06$). The hypnotic activities of a variety of drugs, including barbiturates, tertiary alcohols, carbamates, amides and N, N-diacylureas have been correlated with their distribution behaviour using the model nonpolar – polar system, **octanol /water** (Hansch *et al* 1968). The most active depressant drugs, of all classes, have partition coefficients of about 100/1 (log P = 2) in the **octanol / water** system. All effective hypnotics contain a very polar non ionic portion of the molecule as illustrated by their large negative π values.

i. 5,5 – unsubstituted barbituric acid $\pi = -1.35$

ii. Hydroxyl (OH) $\pi = -1.96$

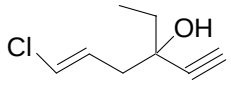
iii. Carbamate (-OCO-NH_2) $\pi = -1.16$

iv. Carboxamide ($CONH_2$) $\pi = -1.71$

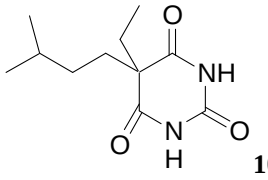
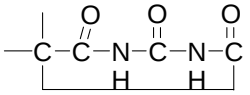
v. N, N – diacylurea (-CONHCONH – CO-) $\pi = -1.68$

In addition they possess hydrocarbon or halogenated hydrocarbon residues which are sufficiently lipophilic to provide the intact molecule with non ionic surface – active character and a distribution coefficient (log P) in the range of 1 to 3. Examples of additive nature of the Hansch substitute constant π in estimating the partition coefficient (Log P) and the closeness of the value to the ideal coefficient for hypnotics (log P = 2) for amobarbital (**105**) and ethchlovynol (**106**) are illustrated below:-

Ethchlovynol (placidy) (**106**)

 **106**	Substituent/constituent group				Σπ= Log P
	C-OH	C≡CH	CH_3CH_2	ClHC=CH	
π	-1.16	0.84	1.00	1.32	2.00

Amobarbital (Amytal) (**105**)

 **105**	Substituent/constituent group			Σπ= Log P
		CH_3CH_2	(CH_3)	
π	-1.35	1.00	2.30	1.95

In addition to the correlations based upon electronic and solubility constants for substituents, parameters for steric contributions of substitutents have been applied (Kutter and Hansch 1969). Steric constants (Es) derived from substituent effects on the rates of hydrolysis of aliphatic esters or ortho- substituted benzoic acid esters, or calculated values based upon Van Der Waals radii, have been used to correlate structure activity relationships in substituted phenoxyethyl / cyclopropylamine **(R – $C_6H_4OCH_2CH_2N$-$CH(CH_2)_2NCH(CH_3)_2$)** mono amide oxidase inhibitors. The reduced activity produced by *meta* substitution was best correlated with steric inhibition of fit to the enzyme surface.

1.2.7 Statement of the Problem

The rate of TB infection has increased greatly especially among HIV positive people. Current TB treatment takes too long and considerable efforts are required to ensure compliancy. More cases of MDR and XDR strains of *Mycobacterium tuberculosis* are being reported and drug-drug interference between current Aids management drugs and TB treatment drugs complicates health care for HIV positive TB patients.

1.2.8 Justification

New anti-*mycobacterials* that are more potent, faster and non interactive with Aids drugs are needed. Screening of ethnobotanically-selected natural products has proved to be a good method in drug discovery especially when combinatorial and high throughput techniques are not possible. Kenyan natural products Chemists have isolated, purified and characterized many phytocompounds guided by existing ethnobotanical knowledge and chances of getting promising leads for *M. tuberculosis* from the currently existing libraries are high. Generation of plausible QSARs from the existing libraries would aid in guided synthesis and structure modification aimed at BA optimization.

1.2.9 Null Hypothesis

There is no chance of discovering antimycobacterial leads through low-throughput screening of a relatively few ethnobotanically selected phytocompounds.

1.2.10 Objectives

1.2.10.1 Main Objective

The aim of this study was to establish the *in vitro* anti TB activities of some ethnobotanically selected phytocompounds and the relationship between structural differences in a series of compounds to observed biological activity differences.

1.2.10.2 Specific Objectives

The specific objectives of this study were:-

i. To select phytocompounds with an aim of covering a wide chemical space while ensuring inclusion of compounds that fit into one or more congeric series.

ii. To experimentally determine MIC values for test compounds and some conventional TB treatment drugs against the $H_{37}R_v$ and the MDR strains of *Mycobacterium tuberculosis.*

iii. To statistically compare the experimental results with available literature values.

iv. To correlate structure with activity using either SARs or QSARs for congeric series compound members.

v. To predict activity of unscreened compounds using QSARs generated for congeric series members.

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Reagents, Organisms, Apparatus, Equipment and Facilities

Mycobacterium tuberculosis, $H_{37}R_V$ and MDR strains were obtained from the Central Reference Laboratory (CRL) for TB in Nairobi. The isolates used had been identified and characterized through routine drug sensitivity testing (DST) in the reference laboratory. To remain viable throughout the study period, the organisms were subcultured on a regular basis on LJ solid media slopes. The LJ media was prepared following the procedure outlined by CLSI (2003). During inoculation, colonies were carefully picked from the slopes using sterile plastic loops. They were placed in pre-prepared, beads containing, sterile water in vortex bottles. Vortex shakers were used to break up the growth into a homogeneous suspension. Opacity of the suspension was adjusted to required standard through visual comparison with a pre-prepared acidic $BaSO_4$ suspension of a defined MF standard. The diluent for this adjustment was either sterile water or MGIT broth media supplement. Sterile bottles or falcon tubes were used in the dilution procedures. Sterilization of reagent containers and water was achieved by use of autoclaves. Inoculation was done either by use of 3mm plastic loops, calibrated micropipettes and pipette tips or disposable Pasteur pipettes depending on purpose of inoculation. All inoculated solid media screw bottles were always placed in bottle holding racks before incubation at 37˚ C in the walk-in incubator of the CRL. Liquid media MGIT tubes were incubated either in the walk-in incubator or in the BACTEC MGIT 960 system. All the procedures involving handling MTB were performed in biosafety cabinets or hoods. All the necessary safety precautions were adhered to; wiping

of working areas with phenol before and after procedures, wearing safety masks and gloves, illuminating working areas with UV light after procedures etc. Non mycobacterial micro was always ruled out or confirmed through Acid Fast Smear Microscopy (AFM) procedures. (CLSI, 2003)

2.1.2 Test Compounds and Conventional Drugs

Test compounds were obtained from the Natural Products Laboratory of Prof. Jacob. O. Midiwo at the Department of Chemistry University of Nairobi. The continuous collection of phytocompounds and related synthetic derivatives since the 1980s has enabled the laboratory accumulate a rich library of compounds with a great potential for further research. The four TB treatment Conventional drugs used in the study (Isoniazid (2), Streptomycin (1), Rifampicin (3), and Ethambuto (4) were obtained at the CRL. The structures of the test compounds used in this study are shown in Fig. 5 to Fig.12 below.

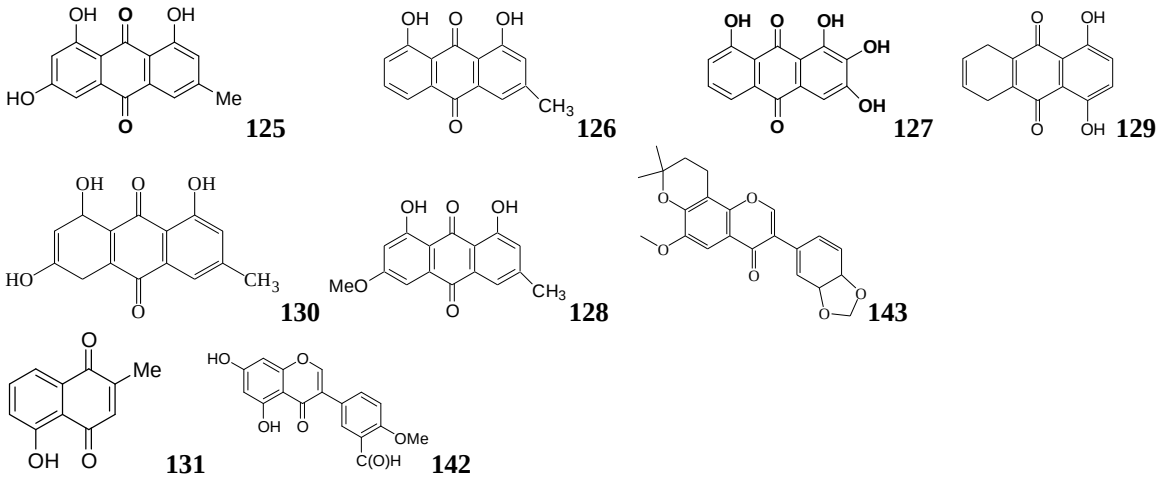
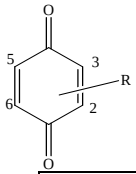


Figure 6: structures of anthraquinones (**125-130**), Naphthoquinone (**131**) and isoflavones (**142** and **143**)



Compound Number	R_2	R_3	R_5	R_6
107*	OH	$(CH_2)_{10}CH_3$	OH	H
111	OH	$(CH_2)_{12}CH_3$	OH	H
109*	OH	$(CH_2)_9CHCH(CH_2)_3CH_3$	OMe	H
110*	OH	$(CH_2)_9CHCH(CH_2)_3CH_3$	OH	Me
112	OH	$(CH_2)_{10}CH_3$	OMe	H
113	OH	$(CH_2)_{12}CH_3$	OMe	H
114	-C(O)Me	$(CH_2)_{13}CHCH(CH_2)_3CH_3$	-OC(O)Me	Me
115	-C(O)Me	$(CH_2)_{10}CH_3$	-OC(O)Me	H
116	OMe	$(CH2)_{10}CH_3$	OMe	H
117	OH	CH_2CH_3	OH	CH_2CH_3
108*	OH	CH_2CH_3	OH	H
118	OH	$(CH_2)_2CH_3$	OH	H
119	OH	$(CH_2)_4CH_3$	OH	H
120	OH	$(CH_2)_6CH_3$	OH	H
121	OH	$(CH_2)_8CH_3$	OH	H
122	OH	$(CH_2)_{14}CH_3$	OH	H

*Compound number 107, 109, 108 and 110 were screened in both LJ solid media and MGIT liquid media. The rest were only screened in the MGIT liquid media.

Figure 5: Structures of benzoquinones

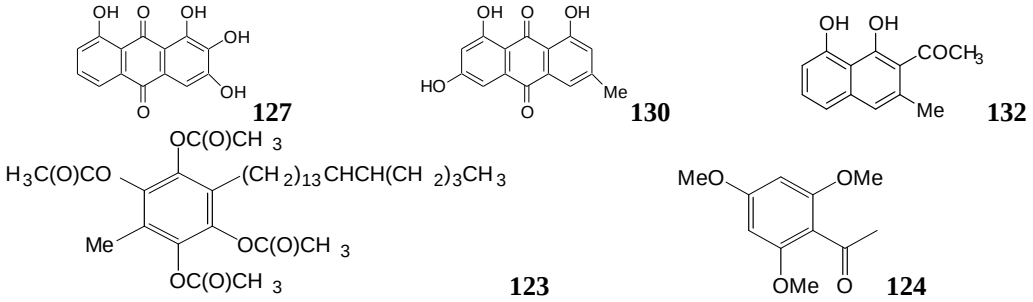


Figure 7: Structures of Naphthalene derivatives (127, 130 and 132) and Benzoquinone derivatives (123 and 124)

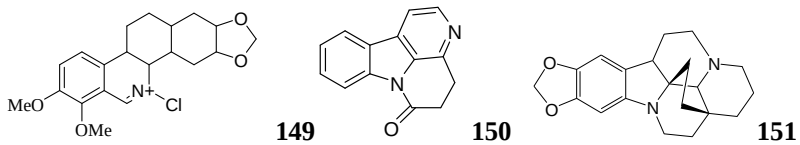
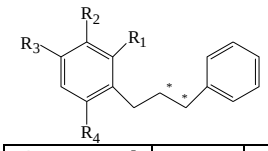
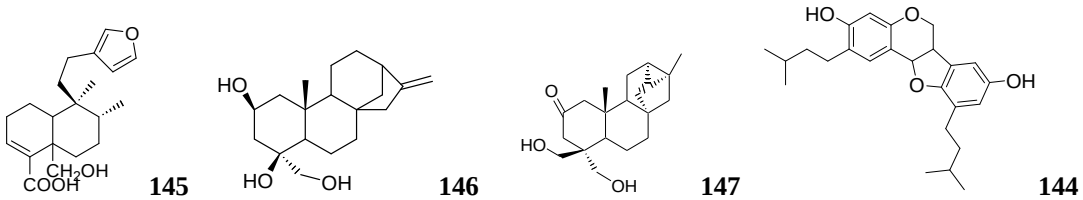


Figure 9: Structures of alkaloids



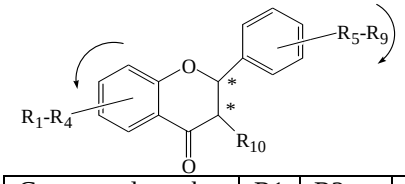
Compound	R1	R2	R3	R4	*-*
133	-OMe	-H	-OMe	-OH	C=C
134	-OMe	-H	-OH	-OH	C=C
135	-OH	-OMe	-OMe	-OH	C=C
136	-OH	-OMe	-OMe	-OH	C─C

Figure 8: Structures of chalcones (133, 134, 135) and dihydrochalcone (136)



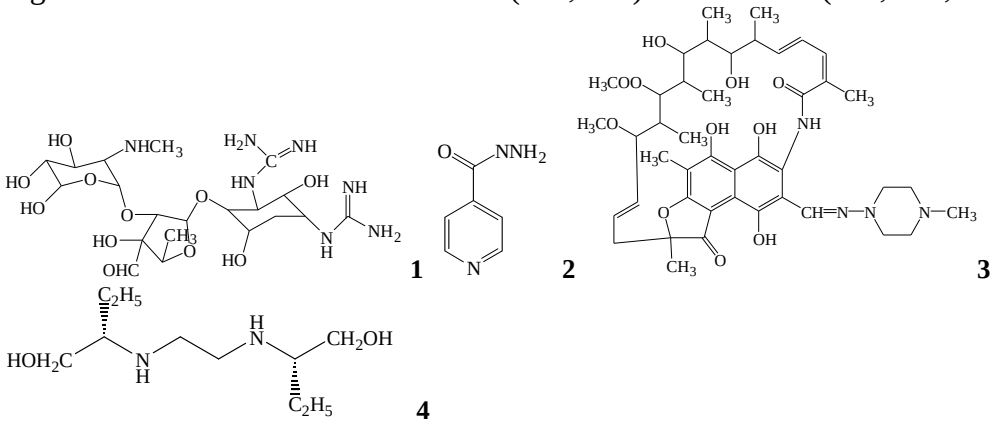
145 **146** **147** **144**

Figure 10: Structures of diterpenoids (**145**, **146**, **147**) and Pterocarpan (**144**)



Compound number	R1	R2	R3	R4	R5	R6	R7	R8	R9	R10	*─*
137	-H	-OH	-H	-OH	-H	-H	-H	-H	-H	-H	C─C
138	-H	-OH	-OMe	-OH	-H	-H	-OMe	-H	-H	-OMe	C─C
152	H	OH	OMe	OH	H	H	OMe	H	H	OMe	C=C
139	-H	-OH	-H	-OH	-H	-OMe	-OMe	-OMe	-OMe	-H	C═C
140	-H	-OMe	-H	-OH	-H	-H	-OH	-H	-H	-OH	C═C
141	-H	-OH	-H	-OH	-H	-H	OMe	-H	-H	-OH	C═C

Figure 11: Structures of the flavanones (137, 138) and flavones (139, 140, 141 and 152)



1 **2** **3** **4**

Figure 12: Structures of conventional drugs

2.2 METHODOLOGY

2.2.1 Compound Selection and Purity

For the solid media preliminary screening, compound selection was done at random from the existing library series. This was done so as to cover a wide chemical space and because all the library members had not been rejected as promising leads in earlier studies (WHO/TDR, 2003) and had been isolated from plant extracts with known antimicrobial activity. The selection also aimed at obtaining at least one congeric series within the library of *alkaloids, lignans, and derivatives of cinnamic acid, flavanones, benzoquinones, chalcones, dihydrochalcones, flavones, anthraquinones, naphthalene derivatives, isoflavones, isoflavenes, isoflavanones, rotenoids, and ptercarpans*. Twenty seven compounds (**107**,**109**.**110**,**108**,**125**,**132**,**131**,**133**,**134**,**135**,**136**,**137**,**138**,**152**,**139**,**140**,**141**, **142**,**143**,**144**,**145**,**146**,**147**,**148**,**149**,**150** and **151**) were chosen for initial screening on solid media. Fourteen benzoquinones (**114**,**121**,**112**,**107**,**116**,**119**,**111**,**115**,**117**,**120**,**113**,**118**,**122** and **108**) and a benzoquinone derivative (**123**) were later selected for screening in liquid broth media. All the compounds purity was confirmed through TLC. The four conventional drugs (**1**, **2, 3** and **4**) were selected because they are commonly used in the CRL and have well established methods for TB drug susceptibility testing.

2.2.2 Preparation of Stock Solutions or Suspension

The choice of solvent for stock solutions preparation was guided by:-

i) Compatibility with the mainly aqueous LJ and MGIT MTB growth media which favoured a water miscible or polar solvent.

ii) Low antimicrobial activity which discouraged use of organic solvents as would be guided by compound extraction procedures. Water was hence the solvent of choice but DMSO and Ethanol/methanol mixtures were used when solubilization in water proved difficulty.

2.2.2.1 Stock Solutions for Conventional Drugs

Stock solutions for screening conventional drugs were prepared as follows:-

(i) Isoniazid (**2)** (critical concentration= 0.2μg/ml)

A 1mg/ml stock solution was made by dissolving 0.020g in 1ml water and then diluting to 20ml. The stock solution was sterilized through micro filtration. A 20 μg/ml solution (a) was then made by diluting 0.2ml of the stock solution to 10ml with water. Another dilution of 5ml of solution (a) to 10ml was done to obtain a 10 μg/ml solution (b). Solution (c) of 1 μg/ml was made by diluting 1ml of solution (b) to 10ml with water.

(ii) Rifampicin (**3**) (Critical concentration = 40 μg/ml)

Stock solution (a) of 2mg/ml was made by dissolving 20mg of the drug in 2ml DMSO before diluting with water to 10ml. The stock solution was sterilized through microfiltration. From this, solutions (b) and (c) of concentrations 1mg/ml and 1mg/ml respectively were prepared using water as the diluent.

(iii) Ethambutol (**4**) (Critical concentration = 2.0 μg/ml)

Stock solution was made by dissolving 20mg of the drug in 2ml water and then diluting the solution to 20ml using water. The stock solution was sterilized through micro filtration. Solutions (a), (b) and (c) of concentrations 0.2mg/ml, 01mg/ml and 0.01mg/ml respectively were then made through appropriate dilution with water.

(iv) Streptomycin (**1**) (Critical concentration = 4.0 μg/ml)

Drug solutions were made in a similar way to that of Ethambutol.

2.2.2.2 Stock Solutions for Test Compounds

Stock solutions of the test compounds were made by dissolving 10mg of each test compound in about 2ml of DMSO and then topping up with water to 10ml. The stock solutions were sterilized through micro filtration and subsequent dilutions done with sterile water. Suitable volumes of the 1mg/ml stock solutions were diluted with sterile water to prepare a 0.5mg/ml and 0.1mg/ml solutions for each compound.

2.2.3 *In Vitro* Bioassays

2.2.3.1 Solid Media Screen

The absolute concentration method is a WHO recommended solid media method of drug susceptibility testing that is appropriate for MIC determination. The method has been described by (Canetti *et al.*, 1963, 1969) and was adopted in this project for determination of test compounds' and conventional drugs' MIC values in Lowenstein-Jensen solid media. The major steps in the absolute concentration method are:

i. Preparation of the drug dilutions; usually, a 1% stock solution of the drug is prepared in an appropriate solvent.
ii. Preparation of the culture medium; procedure for preparation of Lowenstein-Jensen media is outlined in laboratory manuals (CLSI 2003).
iii. Incorporation of drug solution in the media to attain the required uniform drug concentration in the culture media

iv. Coagulation of the media in a slanting position in flowing steam at 82-85ºC for 40-60 minutes

v. Preparation of the inoculum suspension of *M. tuberculosis*. The adjustment is made by comparing the turbidity of the suspension with that of a standard barium sulfate solution equivalent to 1 mg/ml of wet bacterial mass. The bacterial suspension obtained is diluted 1:50 in Dubos medium. This gives a suspension containing 0.02 mg of bacteria per ml (about 200 000 to 1 000 000 organisms per ml. A 3mm diameter loopful of the suspension contains 2000 to 1000 organisms and is usually used as the inoculum. (NB; 85-100 loops = 1ml).

vi. Incubation of the cultures at 37ºC until readable colonies are obtained after about four weeks.

vii. Reading of the cultures. Less than 20 isolated colonies is considered effective inhibition; the inhibition that reduces expected growth by 99% or more.

2.2.3.1.1 Inoculum Preparation

Mycobacterium tuberculosis H_{37}RV strain was subcultured on LJ solid media slopes on a weekly basis to ensure viability. In the first three months of subculturing, different inoculum concentrations were used in order to determine the concentration that gave growth of easily countable colonies. The exercise also aimed at determining the most appropriate time to obtain viable organisms for subculturing or drug sensitivity testing. Most viable inoculums were obtained between the second and the third week of incubation. Easily read colonies were obtained from inoculums of concentrations corresponding to 0.5MF or lower. When required for media inoculation, two to three weeks old colonies were carefully picked from

the LJ slopes, added to the sterile water in beads containing Votexing bottles and the growth shaken into a homogenous mixture on the vortex machine. The homogeneous MTB suspension was allowed to stand for about 20 minutes before being adjusted to the required concentration. Concentrations of most inoculums used in the study were always adjusted to 0.5MF.

2.2.4.1.2 LJ Media Preparation and Inoculation

Drug-containing and drug free control LJ media slopes were prepared not more than two days before inoculation. Stock solutions of the test compounds, conventional drugs and solvents were always prepared not more than one week before media preparation. Drugs or solvents were added to freshly prepared egg based media to attain the required concentration in each incubation bottle. The medium (with drug or without drug in controls) was then coagulated in a slanting position in flowing steam at 85ºC for 60 minutes. A sterile loop was used to scrape as many colonies as possible from a viable subculture growth of not more than 14 days old. The colonies were suspended in a sterile McCartney bottle containing 1.0 ml water and 3 - 4 glass beads (3.0 mm diameter).This mixture was homogenized on a Vortex mixer for 2 - 3 minutes to break up the larger clumps. After standing undisturbed for 20 minutes, the supernatant was carefully transferred to another sterile bottle and left to sit for a further 15 minutes. Its opacity was adjusted through addition of water and visual comparison to that of a 0.5 McFarland standard turbidity standard. . A loopful (3mm diameter) of the suspension was used as the inoculum on drug containing and some drug free media slopes. 1:100 control slopes were inoculated with an inoculum suspension prepared through x100 dilution of the 0.5McF inoculum. The growth on the 1:100 drug free

control slopes were used as the comparison standard for defining the MIC of a compound; The MIC was defined as the minimum concentration of a compound that inhibited MTB growth by 99% hence causing growth similar or less than that of the 1:100 control slopes. The cultures were incubated at 37ºC and examined on a weekly basis for four months.

2.2.3.1.2.1 LJ Media with DMSO Solubilised Drugs and Corresponding Solvent Controls.

A 1mg/ml stock solution was prepared for each of the test compounds through ten times dilution of a 10 mg/ml DMSO solution using sterile de-ionized water. Drug-containing media and DMSO solvent controls were prepared in triplicate set for each compound as shown in tables 9 and 10 (appendix I).

2.2.3.1.2.2 Media Containing Water Solubilised/Suspended Test Compounds

About 10 mg of test compound was suspended in 1-2 ml water. To achieve solubilization, six drops of DMSO were added to each drug suspension in water except in the completely water soluble compound number **108**. The volume of DMSO was arrived at on trial solubility testing that aimed at achieving compound solubilization in minimum DMSO v/v concentration. The compounds suspended well or dissolved completely in the resulting solution. This increased the volume of each drug by about 0.2 ml. Further dilution was through addition of sterile water to final concentration of 1mg/ml. The drug solutions were then incorporated into the LJ media components before incipation as shown in table 11 in appendix I. By the time the drug solutions or suspensions were added to the LJ media components, DMSO had been diluted by a factor of more than 500 and the media was

basically aqueous. Plain LJ media was prepared in sterile conditions and mixed in the incubation bottles with drug solutions following recommended procedures CLSI (2003).

2.2.3.1.2.3 Media Containing Ethanol/Methanol Solubilised Drugs and Corresponding Solvent Controls.

Solubilization was done following the WHO protocol on Rifampicin by using methanol and ethanol. Compound number **147,143,150,145,146** and**107** were found to be relatively highly soluble in methanol/ethanol mixture. 10 mg of each dissolved well in a 1:1, 2 ml methanol/ethanol mixture and were diluted with water to form a 1mg/ml stock solution (i). Two other dilutions, (ii) and (iii) were made by diluting solution (i) to 0.1mg/ml and 0.01mg/ml respectively through addition of sterile water. The solutions were incorporated in media in triplicate sets before incipation as shown in table 12 (appendix I).

For compounds **149,140,139 and 148,** 10 mg were not completely soluble in 2 ml 1:1 methanol/ethanol mixture. A further 2ml of the Methanol/ethanol mixture was added before diluting the resulting solution with sterile water to 10ml of 1mg/ml stock solution (i). Two dilutions were made with addition of water to form solutions (ii) and (iii) of concentrations 0.05 and 0.005 mg/ml respectively. The solutions were dispensed in media in triplicate sets as shown in table 13 (appendix I). In preparing ethanol/methanol solvent controls, 5ml ethanol was added to 5 ml methanol to make stock solution (a). This was used to make stock solutions (b) and (c) through serial consecutive x10 dilutions with water. The stock solutions were added to media before incipation to triplicate sets as shown in tables 14(a) and 14(b) (appendix I).

2.2.3.1.2.4 Conventional Drugs- Containing LJ Media

Isoniazid, Ethambutol and streptomycin were dissolved in water to make 1mg/ml stock solution (a). Rifampicin solution was made in methanol/ethanol mixture. Dilutions were made such that concentration range of each drug in media centrally covered its literature critical concentration value as shown in the tables below:-

Isoniazid (**2**) (critical concentration = 0.2 µg/ml in LJ media); dilution of stock solution (a) was done to obtain solutions (b), (c) and (d) of concentrations 0.1 mg/ml, 0.01 mg/ml and 0.001 mg/ml respectively. Triplicate sets of drug in media were made as shown in tables 15 to 18 in appendix I.

2.2.3.1.2.5 Blank Controls

Blank controls were set up for the purpose of monitoring any changes in the media that could not be attributed to drug/compound, solvent or inoculum. These consisted of six drug/compound, solvent and inoculum free LJ slopes.

2.2.3.1. 2.6 Positive Controls

These were set up for the purpose of monitoring uninhibited MTB growth on LJ media. They consisted of sets of drug and solvent free LJ media slopes that were inoculated with MTB inoculums of at different concentrations as described under LJ Media preparation and inoculation in section **2.2.3.1.2** above.

2.2.3.2 Liquid MGIT Media Screening

The principles used in antimycobacterials susceptibility testing (AST) in liquid media were adopted; the method of proportion (MOP) compares colony counts on drug containing and drug free media (CLSI. 2003). Daily counting of colonies or colony forming units has been shown to correlate well with daily monitoring of growth indicators or growth units generated by automated systems like the BACTEC MGIT 960 (Leonid B. H. *et al.*, 1986). The liquid media used in these experiments was the modified BACTEC MGIT Middlebrook 7H9 Broth supplemented with BACTEC MGIT growth Supplement in a 7 ml Mycobacteria Growth Indicator Tube (MGIT). The 16 x 100mm round bottomed MGIT tubes contain a fluorescent compound (Tris 4, 7-diphenyl-1, 10-phenanthroline ruthenium chloride pentahydrate) in a silicone rubber base on the bottom (BD 2007). The fluorescent compound is sensitive to the presence of oxygen dissolved in the broth. Initially the large amount of dissolved oxygen quenches emissions from the compound and little fluorescence is detected by the MGIT detection system. Latter, actively respiring microorganisms consume the oxygen and allow the fluorescence to be detected and the tube is reported as instrument positive.

Tubes entered into the BACTEC MIGIT 960 system are continuously incubated at 37˚C and monitored every 60 minutes for increasing fluorescence. It requires approximately 10^5 to 10^6 CFU/ml for the fluorescence to be high enough when the system reports positive growth (BD, 2007). Tubes that remain negative for a minimum of 42 days and a maximum of 56 days and which show no visible signs of growth are removed from the instrument as negatives and sterilized before discarding. The principles of the radiometric proportion method were used to define MIC in this study. They state that the concentration of the drug

which produces a daily GI increase and final GI reading lower than that in the 1:100 control can be considered the concentration inhibiting more than 99% of the bacterial population.

2.2.3.2.1 Preparation of Stock Solutions and Their Incorporation into MGIT Tubes

(a) Test Compounds

Stock solutions of all the test compounds were prepared by weighing 10 to 20mg of the compound, dissolving in 2ml DMSO and then diluting with water to the required concentration. Test compound-containing tubes were prepared as shown in table 23 (appendix II).

(b) Conventional Drugs

The incorporation of various components into MGIT tubes before incubation is shown in tables 19 to 22 (appendix II).

2.2.3.2.2 Inoculum Preparation and Inoculation of the MGIT Media

Pure H37Rv and MDR M. tuberculosis isolates had earlier been identified and characterized through regular routine work at the CRL. Viable organisms maintained during the study period through regular subculturing of H37Rv and MDR MTB strains on solid LJ media. On each day of inoculation, a 0.5 McFarland turbidity standard of each strain was prepared as described in the BACTEC^{TM} MGIT 960 user's manual AST instructions (BD 2007). Inoculation of tubes containing test compounds and conventional drugs was done using 0.5mF suspensions instead of the 1:5 dilution suspensions normally recommended and used for conventional drugs susceptibility testing with the MGIT 960 system (BD 2007). By

using the undiluted 0.5 mF inoculum the chances of reporting MICs that are lower than the true values were highly reduced. In drug susceptibility testing with the MGIT 960 system, the chances of reporting false susceptibility results are reduced by using drug critical concentrations that are lower than those used in the conventional **method of proportions** (MOP) (BD 2007). Since no critical concentrations have been developed for the test compounds used in this study, false susceptibility results were avoided through use of a more concentrated inoculum that was determined by trial and error using the conventional drugs. Inoculation was done immediately after other components had been added to the MGIT tubes. H37Rv and MDR inoculums were used for each drug concentration or positive control tubes. Various sets of tubes were prepared as shown in appendix II.

2.2.3.2.3 Controls for the Liquid MGIT Media Screen

The controls used in the MGIT media experiments were for monitoring drug/compound unrelated changes in the MGIT media during incubation period (Blank controls), non compound/ non drug inhibitory effects (solvent inhibitory effects controls), inoculum viability and inoculum dilution effects (Positive controls) and MTB strain susceptibility (Conventional drugs controls).

a) Blank Controls

A triplicate set of six inoculum free and drug/compound free MGIT tubes were treated as described in table 26 appendix II before incubation along with other experimental set ups.

b) Controls for Solvent Inhibitory Effects

DMSO was used in stock solutions preparation in the liquid media screen. MGIT tubes containing DMSO at different concentrations were inoculated with undiluted 0.5mF inoculums as described in table 25 appendix II.

c) Positive Controls-MTB-Inoculated Drug Free MGIT Tubes

The positive controls for uninhibited growth were set up to establish MTB viability and for the purposes of setting a criteria for MIC definition. Undiluted and diluted inoculums were used in different experimental set ups as described in table 24 (appendix II) and table 37 (appendix III).

2.2.3.2.4 Preliminary Liquid Media Screen

In the preliminary liquid media screening the BACTEC MIGIT 960 system was not available for immediate incubation because of the volume of routine DST work in the laboratory. MGIT tubes were hence incubated at 37º C in the walk-in incubation room. Mycobacterial growth was monitored visually on a weekly basis and recorded for a period of six weeks. The MGIT system was latter available for growth determination.

2.2.3.2.5 MGIT Growth Units Monitoring Liquid Media Screen

The exercise aimed at screening the test compounds and conventional drugs at concentrations closer to their MICs as guided by the preliminary liquid media screening results. The incubation was done in the MGIT 960 system and tubes were monitored daily by printing the instrument's ongoing tubes status report and reading growth units at a specific time. During the period of rapid growth, instrument report was printed two or three times in a day. MGIT tubes were prepared as shown in tables 27 to 36 in appendix III.

2.2.4 Quantitative Descriptors of Physicochemical Properties

CS ChemDraw Ultra software was used to generate values of physicochemical properties like LogP, Molecular Refraction (M.R.) and Critical Volumes (C.V.) for the test compounds and conventional drugs as calculated using Crippen's fragmentation method (Crippen, 1987). Chemical names for the test compounds were also generated using the software. This information is in the results section of this report.

2.2.5 Generation of SARs and QSARs

Methods commonly used in generation of SARs and QSARs in organic pharmaceutical chemistry were adopted in this study (Singh and Kapoor 1998; Bloom and Lauback, (1962); Burger 1970; Crippen and Ghose 1987; Daniels and Jorgensen 1971; Manguru 1994). The methods require that a congeric series of closely related compounds be identified. SARs generation involves relating change in activity within a congeric series to corresponding variation in compound structure. QSAR generation involves generating statistically valid equations or relationships between the activities of compounds in a congeric series and values of identified physicochemical parameters. Plausible SARs and QSARs can guide in selection of untested compounds for similar studies or in prediction of structural changes that could increase or decrease activity of a compound.

CHAPTER THREE

3.0 RESULTS, DATA ANALYSIS, SARS AND QSARS

3.1 Results for the Solid LJ Media Screen.

3.1.1 Controls

3.1.1.1 Positive Controls- MTB-Inoculated Drug Free LJ Slopes

Good MTB growth was observed in the positive control slopes within two weeks after incubation at 37˚C in the walk in incubator. The growth of the H37Rv MTB strains was in form of cream colonies that decolorized the green LJ media. The growth on the slopes inoculated with the more dilute inoculum was always distinctly less dense than that on the slopes inoculated with undiluted inoculum. This made it possible to detect significant inhibition in drug/compound containing media by comparison with growth on the control slopes inoculated with diluted inoculum. The growth from a x100 diluted 0.5mF inoculum (usually 10 to 50 cfu) was taken to represented 1% of growth arising from undiluted inoculum. A drug/compound containing slope with a growth similar to this, after inoculation with undiluted inoculum, was hence considered to have a 99% inhibition of the normal growth. The drug/compound concentration that achieved this inhibition was taken to be the MIC.

3.1.1.2 Blank Controls- Drug and Inoculum Free LJ Slopes

Drug and inoculum free LJ slopes were set up and incubated like other set ups for the purposes of monitoring unexpected contamination or any inoculum or drug unrelated changes in the LJ media. Well cocked blank controls remained unchanged for the entire

incubation periods. When loosely cocked, the media dried up and started cracking within two weeks.

3.1.1.3 Controls for Solvent Inhibitory Effects

The solvents used in the preparation of the stock solutions for the test compounds are DMSO, a 1:1 Ethanol/methanol mixture and water. Controls for solvent inhibitory effects had been set up as shown in table 10 (0.5-10.0% v/v DMSO) and table 14 (0.01-8.0% v/v Ethanol/methanol mixture) in appendix I. DMSO was found to cause 99% inhibition of H37Rv growth at LJ media concentration of 3.0% v/v and was hence assigned a MIC value of 3.0%v/v. The 1:1 Ethanol/methanol was found to inhibit H_{37}Rv at MIC value of 2.75%v/v. The two inhibitory concentrations are above the highest solvent concentrations attained in the LJ media when the two solvents were used for compound solubilization; 1%v/v for DMSO and 1.6%v/v for ethanol/methanol mixture. The solvents did not therefore contribute significantly to any observed growth inhibition on MTB in the drugs/compounds containing LJ media slopes.

3.1.2 MICs for Test Compounds and Conventional Drugs

The minimum concentration (µg/ml) of each compound or drug in the LJ media that inhibited growth to more than 99% after a four weeks incubation period was defined as its minimum inhibitory concentration (MIC) (Leonid *et al.,* 1986). This was determined by comparing the growth on drug free and drug containing LJ slopes at the end of four weeks of incubation at 37˚C. The drug free control slopes used for comparison had been inoculated with a 1:100 dilution of the 0.5mF inoculum used on drug containing slopes. The highest

drug concentration that inhibits growth of the 0.5mF inoculum to produce growth similar to that produced by the 0.5/100 mF inoculum is assumed to inhibit 99% of bacterial growth (Canetti 1969; Leonid *et al.,* 1986) and is taken as the MIC of the compound. The colony count on the 0.5/100 mF inoculated slopes ranged between 10 and 50 cfu. Where no distinct drug concentration could be used to assign MIC value, the average of the highest concentration that does not inhibit and the lowest concentration that inhibits is taken as the MIC. The MIC values are designated MIC aq , MIC dm and MIC eth/meth for solutions of compounds whose stock solutions/suspensions were prepared in water, DMSO or 1:1 ethanol/methanol solvent mixture respectively. Five triplicate experiments were performed for each compound (data in appendix IV). The mean MIC value for each compound (n= 15) was determined using MS Excel (see Tables 38 to 41 in appendix V) and the results are Summarized in Tables 43 and 43 below.

Table 43. Summary of MIC values of test compounds from the LJ solid media screen.

Compd. No.	MIC aq (µg/ml)	MIC dm (µg/ml)	MIC eth/meth (µg/ml)
107	49.0 ±2.8	35.3±1.9	14.7±1.9
109	50.0±2.8	40.7±3.1	.
110	40.9±3.1	41.3±4.0	.
108	62.3±3.3	70.0±2.8	.
125	50.3±3.0	51.7±3.1	.
132	84.7±3.7	40.3±2.9	.
131	40.3±3.2	42.3±3.3	.
133	37.9±3.3	58.4±3.7	.
134	53.7±3.4	49.7±4.2	.
135	48.3±2.3	41.0±4.2	.
136	49.7±3.7	34.3±2.9	.
137	63.3±3.7	73.0±4.4	.
138	59.3±3.3	53.0±3.0	.
152	51.0±3.1	59.0±3.3	.
139	48.0±3.3	15.0±3.5	7.4±1.1
140	59.7±3.2	37.7±3.6	15.3±3.5
141	26.0±3.2	32.7±4.3	.
142	36.3±3.0	37.0±2.9	.
143	37.9±2.9	74.7±3.5	40.3±2.4
144	81.7±4.4	74.3±4.8	.
145	36.7±3.1	37.4±1.4	31.0±3.9
146	66.8±3.2	37.6±1.6	39.7±3.0
147	23.3±2.5	42.9±3.3	42.3±3.9
148	.	.	15.8±2.9
149	50.3±3.2	31.3±4.1	16.0±2.7
150	49.3±3.2	42.0±3.6.0	30.0±3.2
151	38.7±1.8	36.2±5.5	.

Table 44. Summary of MIC values of conventional drugs in LJ media

drug	RIF(3)	STR(1)	EMB(4)	INH(2)
Mean MIC µg/ml	45.7±3.1	4.95±0.9	2.65±0.4	0.6±0.2

3.2 Results for Preliminary Screening in the MGIT Liquid Media

The results in this section are for MGIT tubes that were initially incubated in the normal walk-in incubator and latter on entered in the BACTEC MGIT 960 instrument for monitoring of growth as mentioned in the methodology section. Although the results were not used in determination of compound MIC values they were a useful guide in the choice of concentrations to be used in further screening.

a) Visual Inspection of MGIT Tubes

The visual inspection of MGIT tubes was done for about four weeks. Some growth appeared as creamy or whitish clumps, others as cloudiness in the solution, others as transparent colonies on the tube walls and others as tiny or fine white specks or suspension. Whatever the morphology there was a continuous increase and clearness of growth with time. Sometimes it was difficult to tell with certainty the presence of Mycobacterial colonies in the MGIT tubes

b) Preliminary MGIT Instrument Readings

Readings were obtained within a few hours of placing the tubes in the MGIT system. The results were of a qualitative nature indicated by positive (+) reading for substantial growth or no reading for insignificant or no growth in the MGIT tubes as shown in tables 46 and 47 in appendix VI. Lack of growth was an indicator of inhibitory effect by the compound in the MGIT tube.

While most of the visual observations agreed with the MGIT readings, some discrepancies were noted. For instance there appeared to be no growth in tubes containing JM 12 at all concentrations by visual inspection. MGIT system however recorded growth in all the JM 12 containing tubes.

3.3 Growth Units Monitoring Results for the MGIT Liquid Media Screen.

3.3.1 H37Rv Inoculated Positive Controls.

The inoculum concentrations used in are shown in Table 54 below.

Table 54: Inoculum concentrations in MGIT positive controls

Tube set	Pa	Pb	Pc	Pd
H37 Rv Inoculum concentration	0.5mF	0.5/100 mF	0.5/200 mF	0.5/500 mF

Fig. 18 below shows Log of growth units plotted against time in days for various drug – free tubes.

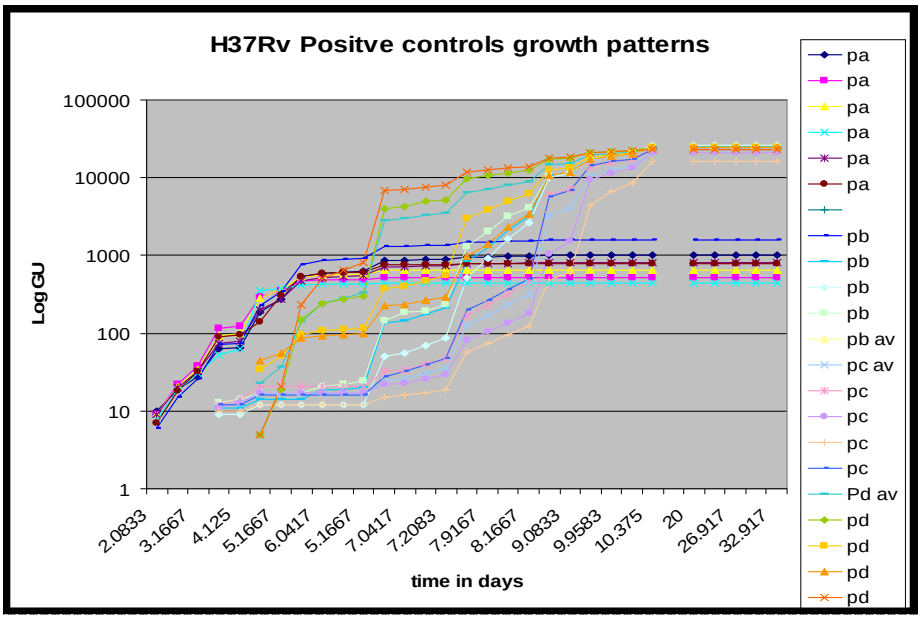


Figure 18: *M. tuberculosis* H_{37}Rv growth patterns in drug free tubes

Growth in tubes with undiluted inoculum (set pa) started early and maximum Log growth units (GU) was attained early. Growth for the diluted inoculum was registered after three days but was more rapid than that of the undiluted one. The diluted inoculum attained higher maximum growth units than the undiluted one. Growth patterns for tubes with similar inoculation were very similar and it was found appropriate to take the

average of each set for a clearer picture of the trends in growth. This is shown in Fig. 19 below.

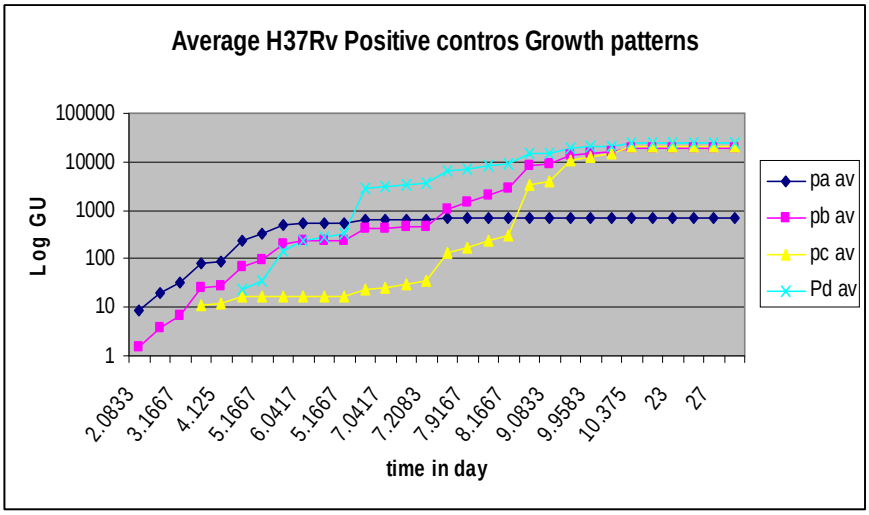


Figure 19: Average growth of *M. tuberculosis* $H_{37}Rv$ in drug free tubes

From the growth patterns observed with the various positive control dilutions, inhibition of growth by compounds was associated with:-

i) GU values that are equal or lower than those of the 1:100 diluted inoculum between the 2nd and the 5th day of incubation.

ii) GU values that did not rise above those of the undiluted inoculum before the 9th day of incubation.

3.3.2 MDR Inoculated Positive Controls.

Tubes were labeled like those used for the $H_{37}Rv$ strain except but in a different colour. The processes of drug addition, inoculation and incubation were done same time as for the $H_{37}Rv$ tubes. Fig. 40 below shows the plot of logarithm of growth units against time in days for tubes inoculated with various concentrations of MDR with no drugs added.

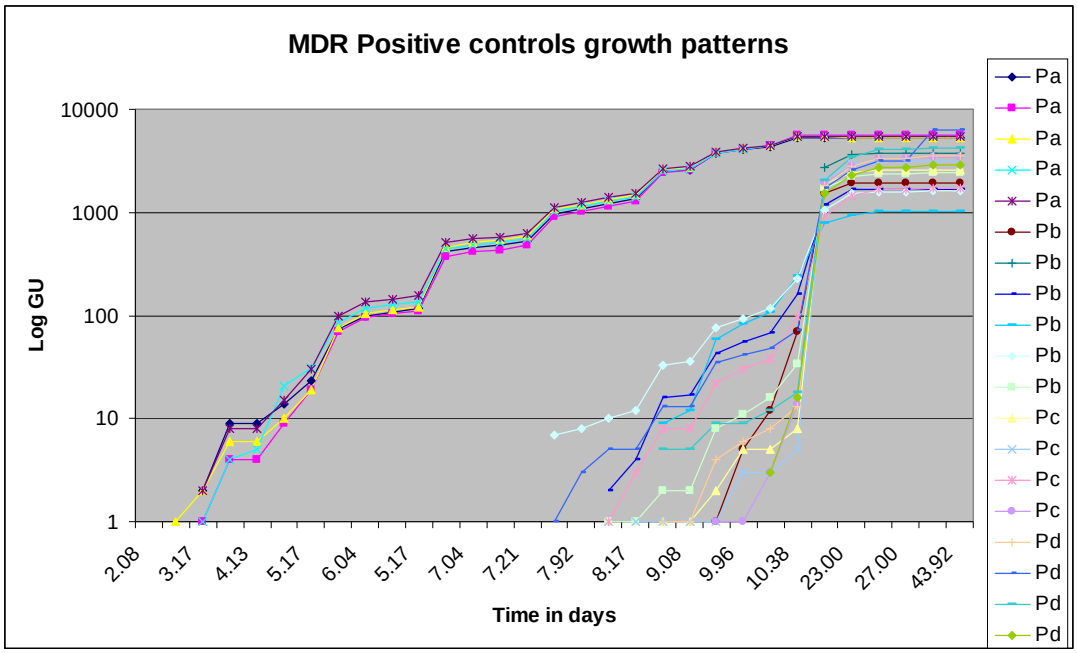


Figure 40: MDR growth in drug free MGIT tubes

The average growth units were calculated for each set of inoculum concentration and logarithms of the averages plotted against time in days as shown in Fig. 41 below:

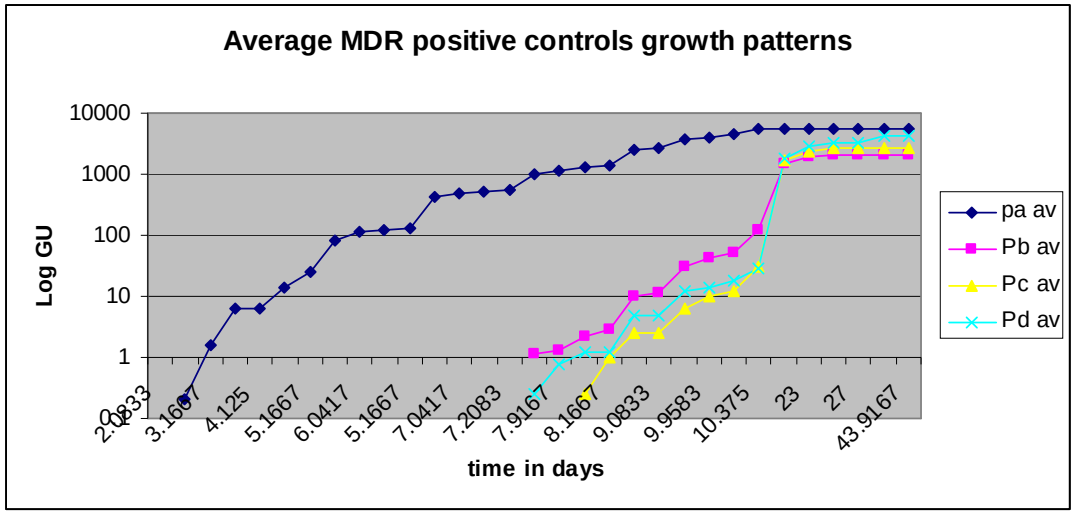


Figure 41: Average MDR growth in drug free MGIT tubes

Growth inhibition was associated with:-

i. GU registered latter than those of the undiluted inoculum

ii. GU values equal to or less than those of the 1:100 diluted inoculum between the 7th and the 20th day of incubation

3.3.3 MTB Strain Susceptibility Controls

MGIT tubes were treated as illustrated in tables 19-22 in appendix I. The resulting MTB growth patterns in tubes containing conventional drugs at different concentrations are shown in Fig. 36 to Fig. 39 for H37Rv strain and figures 58 to 61 for MDR strain in appendix V. The patterns show an inhibitory effect that increase with drug concentration in media. The MIC values deduced from the growth patterns are given in Table 60a below. The results are in good agreement with recorded MTB inhibitory effects of the four conventional drugs (BD 2007). Since the drugs used in these experiments have some established and recorded anti- TB activity then uninhibited growth in test compounds containing MGIT tubes was attributed to compound inactivity at the concentration used

Table 60a: MGIT MIC values of conventional drugs.

Drug	LJ MIC (µg/ml)	MGIT MIC (µg/ml)
Rifampicin.	45.7	7.5
Streptomycin.	4.95	3.25
Ethambuto	2.64	2.5
Isoniazid	0.59	0.8

3.3.4 Controls for Solvent Inhibitory Effects

The growth patterns in these tubes are shown in Fig. 50 for MDR and fig. 35 for H37Rv (Appendix VII**).** The MIC of DMSO as derived from these set ups was 1.35% v/v for

H37Rv strain and 1.5% v/v for MDR strain. Since the concentrations of DMSO in the compounds/drugs containing tubes were always far much below 1.35% v/v, then any inhibitory effects in those tubes could only be attributed to the compound/drug. DMSO growth patterns also indicated a bactericidal activity which was not a common feature with other set ups.

3.3.5 Blank Controls

Six triplicate sets of drug and inoculum free MGIT tubes were incubated along with other tubes for the purpose of monitoring any drug or inoculum unrelated changes (Table 26, appendix II). These tubes remained unchanged throughout the incubation period and no growth units were recorded by the instrument from the tubes. This implies that changes in MGIT tube contents or growth units arising from such tubes could only be attributed to other additives into the liquid media.

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3.3.6 MGIT MICs Determination from MGIT Growth Units Patterns.

Bacterial growth in MGIT tubes was monitored through recording of growth units (GU) readings from the BACTEC MGIT 960 instrument on a daily basis for a period of four weeks. Logarithms of GU-time curves were plotted for controls and compound containing tubes as illustrated by Fig. 1 below. For this particular example, MGIT tubes contained compound number JM 27(120) at concentrations abbreviated p, q and r (P=35 µg/ml, q = 65 µg/ml, r = 105 µg/ml). Pa and pb were drug free control tubes that differ in concentration of inoculum used (Pa = 0.5mF inoculum, P_b = 1/100 0.5 mF inoculum)

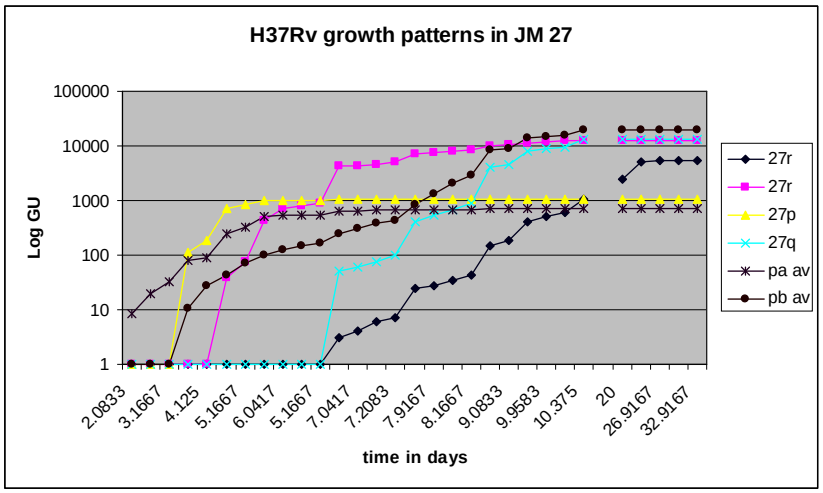


Figure 1: Growth patterns of H37Rv MTB in various concentrations of compound number 120 (JM 27)

A compound's MIC was defined as its concentration in µg/ml that produced a growth units plot that is similar to that of the 100 folds diluted inoculum control (Leonid *et al* 1986). For this example, 35 < MIC < 65 µg/ml, and a value of 50 ± 15 µg/ml was assigned. The growth patterns for tubes containing other compounds and those containing conventional drugs are shown in appendix VII. Tables 61 and 62 below show the summary of MIC values of H37Rv and MDR TB strains of each compound used in the liquid media MGIT screen as derived from the growth patterns.

Table 61: MGIT H37Rv MIC values

Relative position	Compound number	MGIT MIC µg/ml
1	IZ (2)	0.8 ±0.2
2	EM (4)	2.5±0.5
3	ST (1)	3.3±1.2
4	114	5.0±1.0
5	121	7.5±2.5
6	Rf(3)	7.5±2.5
7	123	10.0±5.0
8	112	15.0±5.0
9	107	15.0±5.0
10	116	15.0±5.0
11	119	32.0±2.5
12	111	35.0±5.0
13	115	42.5±7.5
14	117	50.0±15.0
15	120	50.0±15.0
16	113	60.0±20.0
17	118	85.0±15.0
18	122	107.0±2.0
19	108	109.0±4.0

Table 62: MGIT MDR MIC values

Relative position	Compound number	MGIT MIC in µg/ml
1	ST (1)	2.5±0.5
2	IZ (2)	2.7±0.9
3	EM (4)	15.0±2.5
4	116	20.0±5.0
5	114	27.5±7.5
6	Rf (3)	30.0±10
7	112	32.9±2.9
8	123	40.0±5.0
9	115	50.0±15.0
10	107	50.0±15.0
11	117	55.0±10.0
12	121	60.0±2.1
13	113	60.0±10.0
14	111	65.0±5.0
15	122	105.0±5.0
16	120	106.0±15.0
17	119	110.0±5.0
18	118	115.0±15.0
19	108	120.0±5.0

3.4 General Results Analysis

3.4.1 Variation of Compound Activity with Stock Solution Solvent in the LJ Media Screen.

Three solvents were used in stock solution preparation in the LJ solid media screen. Although the resulting solvent concentration in LJ media was far much below inhibitory levels, compound activity was still found to be affected by the solvent used in stock solution/suspension preparation as seen in Fig. 63 below. The figure shows relative trends of MIC values for compounds whose stock solutions were prepared in three different solvents.

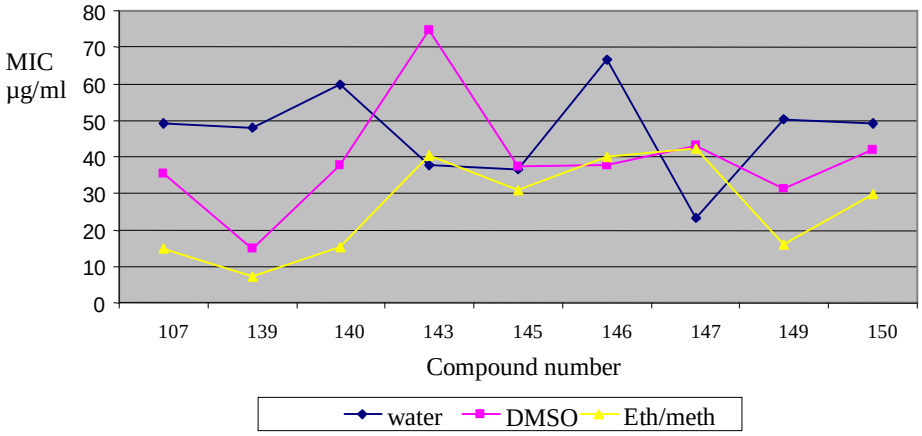


Figure 63: Trends in the LJ- H37Rv MIC values for compounds solubilised in different solvents

.

The DMSO and Ethanol/Methanol curves are similar in many sections and are both different from that of water. This observation is supported by SPSS regression analysis of the MIC values; weak and insignificant negative correlation between the data from water and the other two media ($r = -0.233$ at $P < 0.546$ level (2-tailed) for water/DMSO and $r = -0.350$ at $P < 0.356$ (two- tailed) for water/Eth/met.) and positive, stronger and statistically significant ($r = 0.767$ at $P < 0.05$ level (2-tailed) correlation between MIC values obtained in DMSO and those in ethanol/methanol mixture.

3.4.2 Variation of Compound Activity with Media.

Table 5 below shows the H37Rv MIC values of the six compounds that were screened in both the LJ and MGIT media. The Pearson product moment correlation coefficient for the two series of MICs is high ($r = 8.1$ and $r^2 = 0.65$) indicating that trends in MIC values are similar in the two media.

Table 63: LJ and MGIT MIC values of some compounds.

Compound	LJ MIC (µg/ml)	MGIT MIC (µg/ml)
Rifampicin.	45.7	7.5
Streptomycin.	4.95	3.25
Ethambuto	2.64	2.5
Isoniazid	0.59	0.8
107	35.3	15
108	70	109

The value for the F statistic (F = 0.40) for differences in the MIC values in the two media is not significant (P < 0.1), indicating that MIC values are not significantly dependent on the type of media.

3.4.3 Activities of Conventional Drugs in Relation to Literature Values.

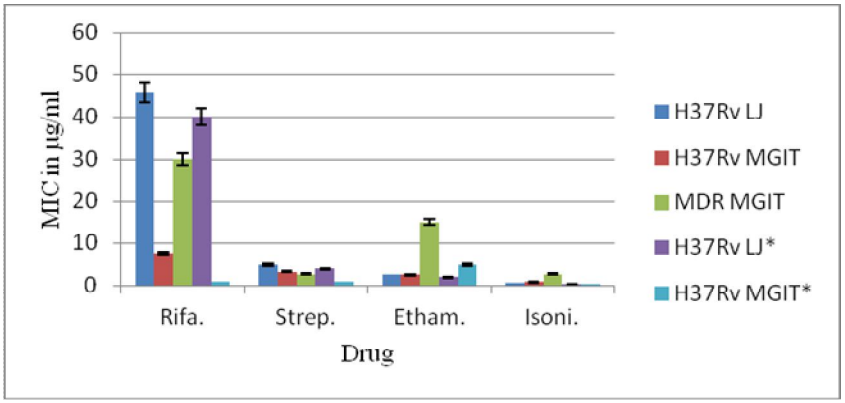
Fig. 75 below shows the various MIC values generated in the study for the four TB treatment drugs along with their respective conventional critical concentrations (Laszlo *et al.*, 1997; BD, 2007) in the solid and liquid media. In the figure, series 1,2,3 and 4 represent Rifa. Strept., Ethamb. and Isoniazid respectively.The first three sets (LJ H37Rv, MGIT H37Rv and MGIT MDR) are the experimental data from this study while the last two (Cr.H37LJ and Cr.H37MGIT) are the litrature critical concentation values for the drugs. The correlation between the experimental data and the literature values was analyzed using SPSS non parametric methods. Significant correlations were obtained between,

i. LJ $H_{37}Rv$ and MGIT $H_{37}Rv$ (r = 1 ; P < 0.01, two tailed)

ii. LJ $H_{37}Rv$ and LJ critical concentration (r = 1 ; P < 0.01 two tailed,)

iii. MGIT $H_{37}Rv$ and LJ critical concentration (r = 1 ; P < 0.01 two tailed,)

The relatively good correlation between experimental values and literature values (except with MGIT critical concentrations) adds credibility to procedures adopted in determining test compounds' MIC values. MDR MIC values do not correlate significantly with any of the other MIC values.



*Critical concentration values

Figure. 75: Relative MIC and critical concentration values of conventional drugs

The weak correlation of MDR values with other values is in good agreement with expectation; resistant strains normally have higher MIC values for a given drug than susceptible strains. Hatsumi *et al* (2006) have reported MTB strains with a broad range of susceptibility to Rifampicin (0.39 µg/ml to more than 50 µg/ml). The MIC values generated in this study for the four conventional drugs were in good agreement with most literature values as seen in table 6 below. This gave credibility and validity of the results of the study.

Table 80: Some literature activity values of the four conventional drugs used in the study

Drug	MIC(susceptible strain) (µg/ml)	MIC (low level resistance strain) (µg/ml)	MIC (high level resistance strain) (µg/ml)
Rifampicin(3)	$< 0.39^{a}$, $\leq 1^{b,e}$	12.5^{a}, $(2\text{-}32)^{b}$	$\geq 50^{a}$, $\geq 64^{b,c}$, $\geq 512^{b}$, (40,80,120, 160, 200, > 200)^{d}
Streptomycin(1)	1.0^{e}	$(1.0 - 4.0)^{e}$	$> 4.0^{e}$
Isoniazid(2)	0.1^{e}	$(0.1\text{-}0.4)^{e}$	$> 0.4^{e}$
Ethambuto(4)	5.0^{e} ; 1.9^{f}	7.5^{f}	60^{f}

a- Hatsumi *et al* (2006) ; b – Hideaki *et al* (1996); c – Prahaj *et al*(2004); d- Taha *et al* (2009); e – BD (2007) MGIT critical concentrations; f - Leonid *et al* (1986)

3.4.4 Relative Activities of Non Congeric Series Compounds

The non series class of compounds are those that do not have a common structural template and hence for which no attempt was made in generation of SARs or QSARs. The class was composed of one anthraquinones (**125**), a naphthoquinone (**131**), nepodin (**132**), two isoflavones (**142**) and (**143**), a Flavanone derivative (**124**) a pterocarpan (**144**), three diterpenoids; (**145**), (**146**) and (**147**) and four alkaloids; (**148**), (**149**), (**150**) and (**151**).Their structures are shown in figures 6 to 10 . The MS Excel analysed results for the compounds are shown in Tables 41(a), 41(b) and 41(c) in appendix V and a summarized form in Fig. 16 below.

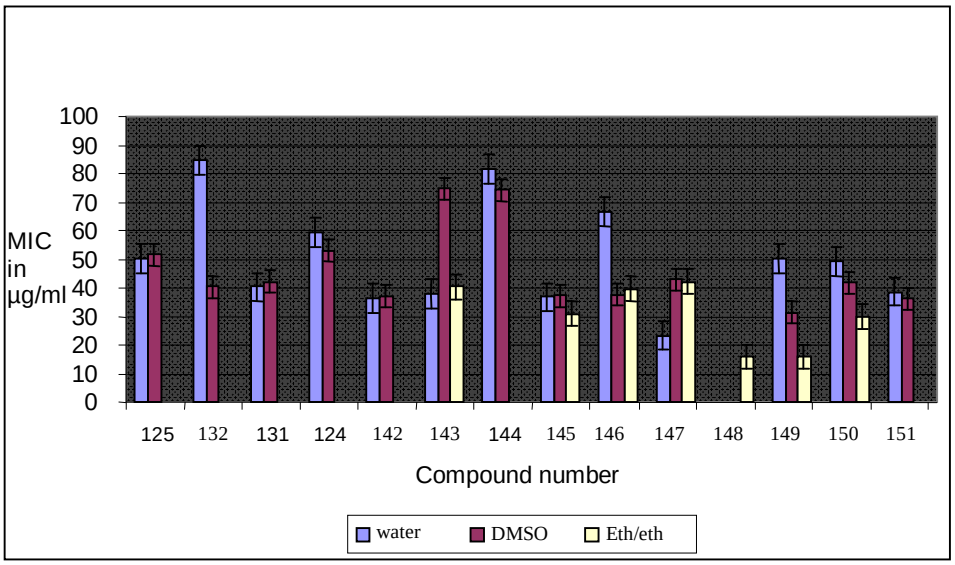


Figure 16: Relative activities of non congeric series compounds

The strongest activity was observed in the ethanol/methanol media for the alkaloids (**148** and **149**). The lowest activity is observed in the water media for the nepodin (**132**) and pterocarpan (**144**). There is no clear pattern in compound activity but the alkaloids appear more promising.

3.4.5 Variation of MICs with MTB Strains

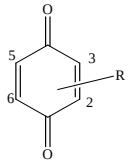
Compound potency was highly dependent on the strain used. However more test compounds were generally more potent on the H37Rv strain than on the MDR strain. This is in agreement with other studies on various Mycobacterium species and MTB strains.(Leonid *et al.*,1985; 1986; 1988 ; Charles 1986).

3.5 Relative Activities of Congeric Series Members and SARs Deduction

Classes of compounds with members that formed well defined congeric series are the benzoquinones, Chalcones and the Flavanones.

3.5.1 SARs of the Benzoquinone Series

The general template for the benzoquinone series members is shown below.



3.5.1.1 LJ Solid Media Screen.

Short alkyl side chains at position 3 reduce antimycobacterial activity. Longer chains increase activity up to a given maximum after which chain lengthening reduces activity. Methyl group at position 6 increases activity. 5-hydroxy compounds are more active than 5-methoxy compounds.

3.5.1.2 MGIT Liquid Media Results

a) Effect of the 3-alkyl chain length in 2,5-dihydroxy substituted Benzoquinones with no substituent at position 6. These were JM1 (107), JM2 (111), JM24 (108), JM25 (118), JM26 (119), JM27 (120), JM28 (121) and JM29 (122). Activity against both

H37Rv and MDR strains increased with increase in chain length to a maximum at C9 and then decreased with further lengthening of the alkyl group.

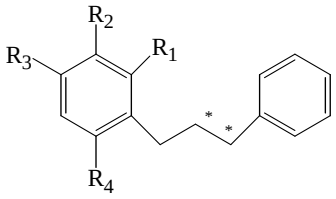
b) Short alkyl substituents at position 6 increases activity of 2,5-dihydroxy substituted benzoquinones with another alkyl group at position 3. This is observed in activities of JM23 (117) and JM24(108).

c) Effect of substitution of an OH with an OMe group at position 5. (JM1(107) to JM6(112) and JM2(111) and JM9(113)). This decreases compound activity against both MTB stains (from 35μg/ml to 65 μg/ml for H37Rv and 65 μg/ml to 70 μg/ml. However, substitution of both OH groups at position 5 and 2 with OMe groups increased activity as observed from activity of JM1(107)and JM14(116) for both MTB strains.

d) Methyl esterification of the 2, 5 hydroxyl groups reduces activity as seen from activity of JM1 (107)and JM13(115). 2,5 methyl esters with long unsaturated 3-alkyl groups and a methyl substituent at position 6 are more active than position 6-unsubstituted 2,5- methyl esters with shorter saturated 3-alkyl substituents (JM12 (114) and JM13 (115)).

3.5.2 SARs of Chalcones (133, 134, and 135) and Dihydrochalcone (136) in MGIT Screen

The general template of the four member series is shown below and also in Fig. 7 in the experimental section



SARs of the series are:-

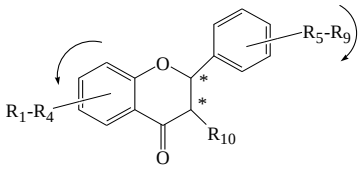
i. OH at position 3 in the first ring increases activity (134 is more active than133).

ii. A higher OH: OMe ratio for the first ring substitution increases activity. The ratio is highest for 134 and lowest for 133.

iii. A double bond between the two rings reduces activity as seen in activities of 135 and 136.

3.5.3 SARs of Flavanones Series from MGIT Screen

The series template is shown below and also in figure 10 in the experimental section.



SARs of this series were not very clear probably due to the relatively large number of substitutions in relation to only a few series members **(137, 138, 139, 140, 141)** screened.

3.6 Regression of Activity of Congeric Series Members with Physicochemical Properties in QSARs Generation

3.6.1 Benzoquinone Series

3.6.1.1 Physicochemical Parameter Values

The Table 7 below shows various CS ChemDraw generated physicochemical parameter values of the all the fifteen benzoquinones used in the study along with their MGIT MIC values.

3.6.1.1.1 QSARs for LJ Media Screen

Four benzoquinones (**107,108,109,110**) were screened in the solid LJ media. No attempt in QSAR generation was made for the solid media activity because the number of series compounds did not meet the threshold minimum of five (Cymerman-Craig, 1955).

Table 68: Some physicochemical parameters and MGIT MIC values of benzoquinones

Compound no.	Log P	MR (cm^3/mol)	Cv (cm^3/mol)	Rmm	Mpt(k)	G° (kJ/mol)	ΔHf (kJ/mol)	MGIT H37v MIC (µg/ml)	MGIT MDR MIC (µg/ml)
107	1.79	85.56	945.5	294.39	589.63	-363.37	-818.26	15	50
111	2.62	94.76	1057.5	322.44	612.17	-346.53	-859.54	35	65
112	2.15	90.31	1000.5	308.41	562.31	-323.13	-818.89	15	32.9
113	2.98	99.52	1112.5	336.47	584.85	-306.29	-860.17	60	70
114	5.62	146.15	1667.5	502.68	706.60	-530.42	-	5.0	27.5
115	2.25	103.87	1183.5	378.46	597.73	-676.79	-	42.5	50
116	2.51	95.06	1055.5	322.44	534.99	-282.89	-819.52	10	20
117	-1.2	53.11	553.50	196.2	523.26	-431.94	-685.25	50	55
108	-	44.15	441.50	168.15	488.2	-439.15	-632.5	109	120
118	-	48.75	497.5	182.06	499.47	-430.73	-653.14	85	115
119	-	57.96	609.5	210.23	522.01	-413.89	-64.42	50	110
120	0.12	67.16	721.5	238.28	544.55	-397.05	-735.70	45	106
121	0.95	76.36	833.5	266.33	567.09	-380.21	-776.98	7.5	60
122	3.46	103.97	1169.5	350.49	634.71	-329.69	-900.82	107	105
123	8.54	164.60	1915.5	588.77	725.70	-861.6	-	10	40.0

3.6.1.1.2 QSARs from MGIT Screen

Fifteen benzoquinones were screened in the MGIT liquid media as shown in Table 7. Regression analysis for QSAR modeling of MGIT liquid media activity of the benzoquinones generated the QSARs in appendix VIII. The QSARs were generated through;

i) SPSS linear regression analysis between physicochemical parameters and MIC values of the MDR strain. (QSARs 1 to 7 in appendix VIII)

ii) Curve regression analysis between

   a) logP and MIC values of the MDR strain generated QSARs 8 and 9.

b) MR and MIC values of the MDR strain generated QSARs 10 to 16.

c) Cv with activity generated QSARs 17 to 23.

d) RMM and MGIT MIC values generated QSARs 23to 31.

3.6.2 Chalcones and Dihydrochalcone Series

Four members of the Chalcone and dihydrochalcone series were screened in the solid LJ media and their solvent dependent activities are shown in Fig.14 below.

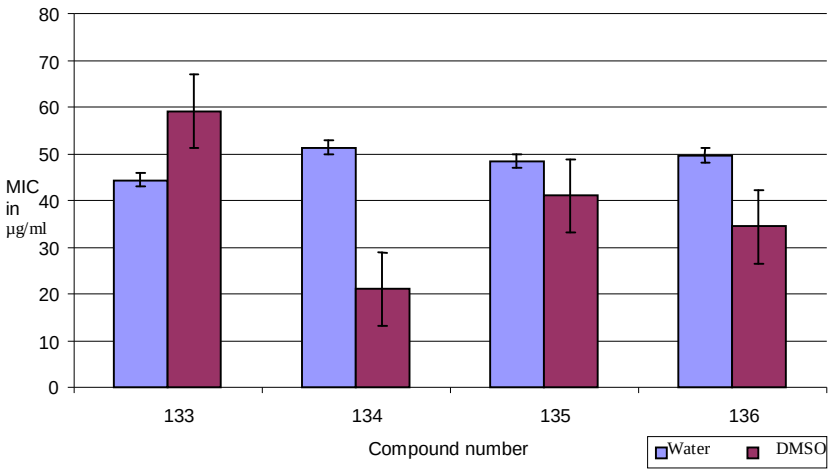


Figure 14: Variation of MIC values of chalcones and dihyroxychalcones with stock solution solvent in LJ media screen.

3.6.2.1 Physicochemical Parameters of the Chalcones Series Members.

Table 8 below gives values of some physicochemical parameters for the series along with their LJ MIC values. The SPSS correlation between physicochemical properties and LJ activity of chalcones and dihydrochalcone indicated significant and strong correlation of MIC (dm) with Mpt, G and Hf values (all have $r = 1$; $P < 0.01$). No significant correlation was found between MIC (aq) and the selected physicochemical parameters.

Table 72: Some Parameter values for the chalcones and the dihydrochalcone

Compd No.	Log P	MR (cm^3/mol)	Cv (cm^3/mol)	Rmm	Mpt(k)	G^o (kJ/mol)	ΔHf (kJ/mol)	MIC aq (µg/ml)	MIC dm (µg/ml)
133	2.94	83.73	809.5	284.31	559.76	-115.5	-381.2	37.9	58.4
134	2.68	78.3	751.5	270.28	625.46	-163.91	-394.18	53.7	49.7
135	2.55	85.54	825.5	300.31	671.48	-270.12	-558.51	48.3	41
136	2.57	83.69	845.5	302.32	676.56	-350.34	-675.73	49.7	34.3

3.6.2.2 QSARs for the Chalcones and Dihydrochalcone Series

No attempt in QSAR generation was made for this series because series members were less than five, the statistically minimum for valid QSAR generation (Cymerman-Craig, 1955).

3.6.3 Flavanone Series

The solvent dependent activities of the five members of the Flavanone series that were screened in the solid LJ media are shown in Fig. 15 below.

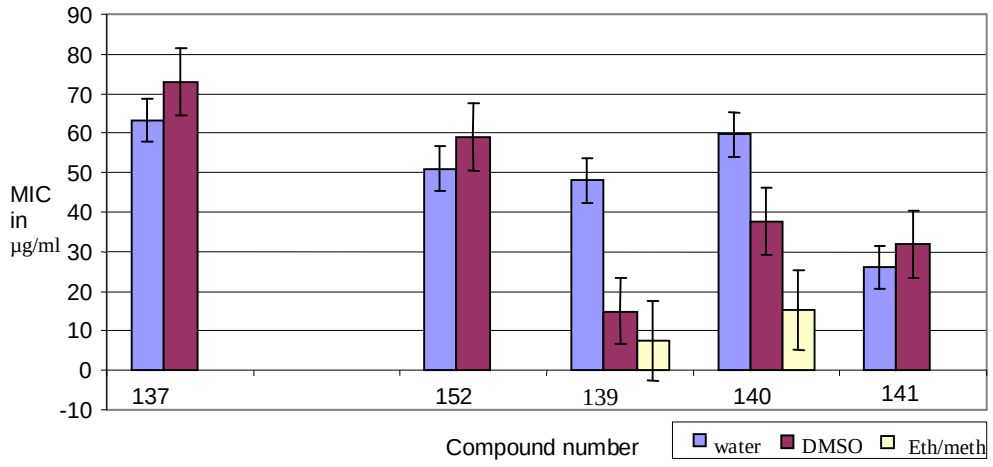


Figure. 15: MIC values of Flavanones solubilised in different solvents

3.6.3.1 Values of Some Selected Physicochemical Parameters of the Flavanones Series Members

The selected properties and their values are given in Table 9 below.

Table 74: Parameter values of the flavanones series

Compd No.	Log P	MR (cm^3/mol)	Cv (cm^3/mol)	Rmm	Mpt(k)	G° (kJ/mol)	ΔHf (kJ/mol)	MIC aq (µg/ml)	MIC dm (µg/ml)	MIC eth/meth (µg/ml)
137	2.02	69.86	668.5	256.65	656.32	-178.69	-449.02	63.3	73	.
138	1.47	91.6	889.5	346.11	777.62	-495.4	-950.88	51	59	.
139	0.99	106.64	1025.5	404.37	903.94	-681.7	-1204.02	48	15	7.4
140	1	80.13	748.5	300.26	793.2	-403.31	-710.4	59.7	37.7	15.3
141	1	80.13	748.5	300.26	793.2	-403.31	-710.4	26	32.7	.

The SPSS output for the non parametric correlational statistics is shows that MIC aq does not correlate strongly and significantly with any of the physicochemical parameters. MIC (DMSO) correlates well and significantly with only two parameters;

i. Log P ($r = 0.975$; $P < 0.005$)

ii. Mpt ($r = 0.975$; $P < 0.005$)

The two MIC values do not correlate significantly.

3.6.3.2 QSAR Generation for the Flavanone Series

Multiple linear regression SPSS analysis did not yield a good model for QSAR generation. This could be attributed to the low number of compounds in the series. It should be based on a sample that contains substantially more data points than the number of parameters in the model (MacClave 2000).

SPSS curve regression analysis between single independent variables and MICs values of the Flavanone series members generated the QSARs in section B of appendix VIII. The variables used in the QSARs generation were:-

a) LogP. for QSARs 32 to 34

b) Mpt. for QSARs 35 to 37

CHAPTER FOUR

4.0 GENERAL DISCUSSIONS, QSAR BASED PREDICTIONS, CONCLUSIONS AND RECOMMENDATIONS

4.1 Solid Media Screening

The study results indicate the strong relationship between solvent used and the observed biological activity. Activity is generally higher in Ethanol/methanol solvent mixture than in the water media. The table below (Table 81) shows the compounds with MIC values that are less or equal to 40 µg/ml (the critical concentration of rifampicin (the least potent of the conventional first line TB treatment drugs used in the study) for each of the three solvents used:-

Table 81: Compounds with MIC ≤ 40µg/ml from LJ media screening

Solvent	Compound's code and number
Water	JM114 **(131),** JM201 (**133),** JM414 **(141),** JM501 **(142),** JM521 **(143),** JM604 **(145),** JM620 **(147),** JM704 **(151)**
DMSO	JM1 **(107),** JM104 **(132),** JM208 **(136),** JM404 **(139),** JM405 **(140),** JM414 **(141),** JM501 **(142),** JM604 **(145),** JM611 **(146),** JM702 **(149),** JM74**0 (151)**
Ethanol/ Methanol	JM1 **(107),** JM404 **(139),** JM405 **(140),** JM521 **(143),** JM604 **(145),** JM611 **(146),** JM701 **(148),** JM702 **(149),** JM703 **(150)**

As seen from the table, the only compound that qualifies as a promising lead in the three solvents is compound number **145** with MIC values of 35, 37 and 30 µg/ml in water, DMSO and ethanol/methanol respectively. This indicates that the solvent plays a major role in the antibacterial activity of the compounds and a potency value obtained in one

solvent my not be reproduced in a different solvent. Data for ethanol/methanol solvent system was only generated for 10 compounds that had earlier been identified as promising using the water and DMSO media. Nine of them were again identified as promising in the ethanol/methanol media. This is relatively good reproducibility of data on promising compounds because the ethanol/methanol solvent system did not present much difficulty with regard to compound solubilization.

The relatively few promising compounds under water could be attributed to the low solubility of the test compounds in water. However, the compounds that are promising under water are probably better working Leads than those under the other solvents. This is because water presents no difficulties with regard to differentiating solvent effects from test compound activities. The six promising compounds in the water media (**110**, **131**, **141**, **142**, **145** and **147)** have relatively low Log P values except for **110**. This is observation is similar to what has been observed with majority of the conventional TB treatment drugs and hence the six compounds (**110**, **131**, **141**, **115**, **145** and **147)** are better promising Leads than the other seven in the set of seventeen promising compounds.

The solid media screen identified a relatively high number (13) of promising compounds against *Mycobacterium tuberculosis* $H_{37}Rv$. from a relatively small number of selected phytocompounds (27). This is supportive of the strength, need and importance of ethnobotanical information in searches of promising leads compounds. The number of QSARs generated from the study is also relatively high for training sets with few series compounds and whose members were selected randomly. This could be attributed to the fact that most of the phytocompounds have antibacterial activity whose strength within a given series is easily modeled through statistical regression. Inclusion of more test compounds would generate even better and more statistically plausible QSARs that can

guide compound modification or synthesis aimed at optimization of pharmacokinetic or pharmacodynamic characteristics of promising compounds.

Significant QSARs were generated for the Flavanone series as follows:-

1. MIC dm = 29.278 + 65.853 * Log (Log P) (F = 18.18; P < 0.024)
2. MIC dm = 118.603 – 89.743/LogP (F = 20.54; P < 0.020)
3. MIC dm = 45.778*LogP – 15.848 (F = 14.89; P < 0.031)
4. MIC dm = 139397.793/Mpt – 135.9897 (F=16.24; P< 0.027)
5. MIC dm = 1273.021- 184.606*Log(Mpt) (F = 17.95; P < 0.024)
6. MIC dm = 232.399 – 0.2407* Mpt (F = 18.97; P< 0.022)

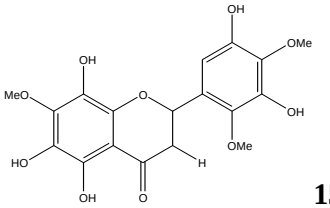
The QSARs generated related MICs of compounds solubilised in DMSO to LogP and Mpts values only. In an attempt to judge the validity and reliability of the QSARs in predicting compound activity, the parameter (LogP or Mpt) value that should correspond to an MIC value of 0.2 µg/ml was calculated. The MIC value of 0.2 µg/ml was chosen because it corresponds to that of IZH, the most potent conventional drug used in the study. The parameter values obtained are shown in Table 82 below.

Table 82: QSAR calculated LogP and Mpt for a Flavanone with MIC = 0.2 µg/ml

QSAR	LogP	Mpt	MIC(µg/ml)
1	0.361716		0.2
2	-567.318		0.2
3	0.5461		0.2
4		1023.556	0.2
5		12470416	0.2
6		965.7172	0.2

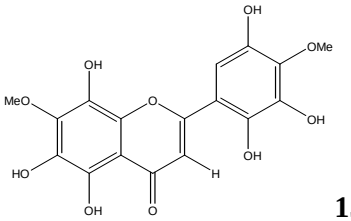
For the LogP based QSARs, the first and third are in good agreement while the second one is not. A Flavanone with a LogP value of -567.318 would be highly polar and hence not likely to have good drug-like properties (WHO/TDR., 2003). This left QSARs 1 and

3 as the only plausible models for predicting the activity of the Flavanone series members based on compound LogP values only. For the Mpt based QSARs, the ones that generated Mpt values close to those of the screened series members are QSARs number 4 and 6. These were used in predicting activity of structures that were generated from LogP based QSARs. QSAR number 1 was used to calculate the expected LogP value for compounds with an MIC value close to 0.2. The following compound structures (**152- 159**) were generated on that basis.



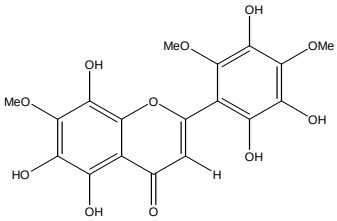
152

LogP = 0.47, MR = 93.21, Mpt = 1129.54, Cv = 938.50, MIC –qsar 1 = 7.685



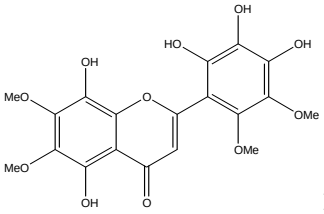
153

LogP = 0.48, MR = 89.84, Mpt = 1212.76,, Cv = 867.50, MIC-qsar1 = 8.287



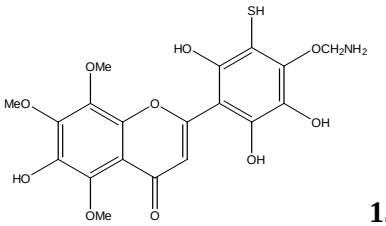
154

LogP = 0.36, MR = 96.30, Mpt = 1258.78, Cv = 941.50, MIC qsar1 = 0.592



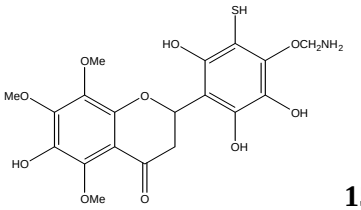
155

Logp = 0.62, MR = 101.07, Mpt = 1193.08, Cv = 999.5, MIC qsar 1 = 15.606



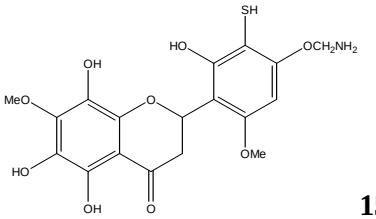
156

LogP = 0.57, MR = 110.25, Mpt = 1213.60, Cv = 1066.50, MIC qsar 1 = 13.202



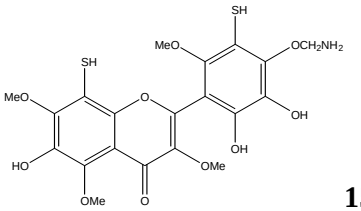
157

LoP = 0.30, MR, 108.84, Mpt = 1196.08, Cv = 1079.50, MIC qsar1 = -5.15513



158

LogP = 0.42, MR = 102.38, Mpt = 1150.06, Cv = 1005.50, MICqsar1 = 4.468



159

LogP = 0.35, Mpt = 1196.88, MR = 123.38, Cv = 1178.50, MIC qsar1 = -0.7465

The corresponding MIC value of each structure was calculated using each of the four selected QSARs and the values in table 83 below generated. While the calculated values are generally low and hence an indicator that the structures could be potent on MTB, the fact that some are less than zero indicates the limitation of various QSARs in activity prediction. The negative values could be explained by the fact that the training set used in QSARs generation did not cover a wide enough chemical space.

Table 83: Expected LJ solid media MIC values of some unscreened flavanones

Compound	**152**	**153**	**154**	**155**	**156**	**157**	**158**	**159**
Calculated qsar 1 MIC	7.68	8.287	0.592	15.606	13.202	-5.155	4.468	-0.7465
Calculated qsar 3 MIC	5.66	6.125	0.632	12.534	10.245	-2.115	3.338	0.174
Calculated qsar 4 MIC	-12.57	-21.04	-25.249	-19.15	-21.12	-19.63	-14.78	-19.52
Calculated qsar 6MIC	-39.48	-59.51	-70.589	-54.77	-59.71	-55.49	-44.42	-55.69

A wider chemical pace would be achieved by inclusion of more series members with wide variation in substitution on the basic template of the series. Fig. 75 below shows a clearer picture of the graphical patterns of the MIC values.

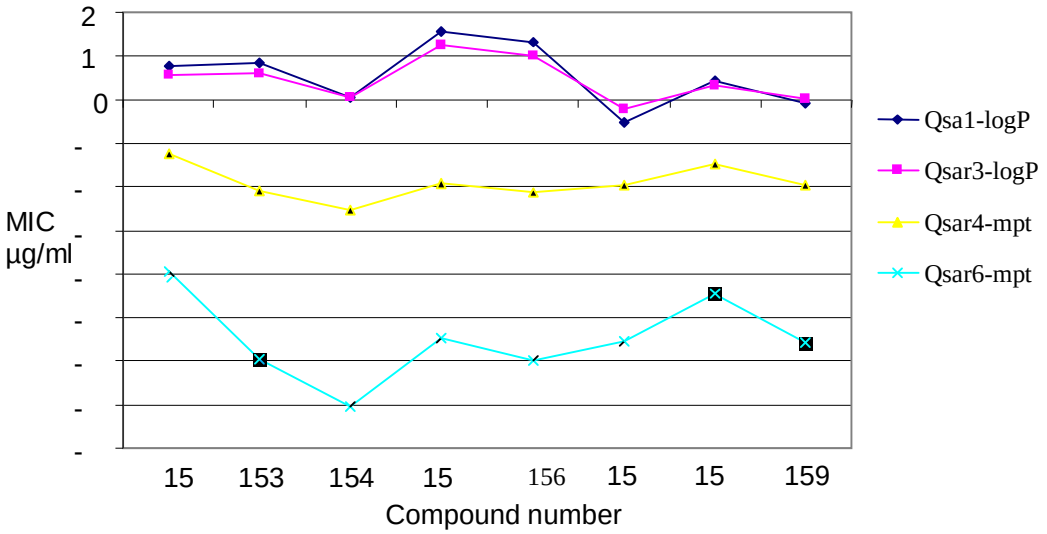


Figure. 76: Relative trends in calculated MIC values of 8 unscreened flavanones

The LogP based QSARs calculated MIC values are almost similar. They differ from those of the Mpt based QSARs by being higher for each compound. The MIC values calculated from Mpt based QSARs have similar trends but they differ widely for each compound.

The SPSS output for non parametric correlations of the various MIC values is shown in Table 84 below.

Table 84: Non parametric Correlations of predicted MIC values

			qsar1-logP	qsar3-logP	qsar4-mpt	qsar6-mpt
Spearman's rho	qsar1-logP	Correlation Coefficient	1.000	1.000**	.071	.048
		Sig. (1-tailed)	.	.	.433	.455
		N	8	8	8	8
	qsar3-logP	Correlation Coefficient	1.000**	1.000	.071	.048
		Sig. (1-tailed)	.	.	.433	.455
		N	8	8	8	8
	qsar4-mpt	Correlation Coefficient	.071	.071	1.000	.976**
		Sig. (1-tailed)	.433	.433	.	.000
		N	8	8	8	8
	qsar6-mpt	Correlation Coefficient	.048	.048	.976**	1.000
		Sig. (1-tailed)	.455	.455	.000	.
		N	8	8	8	8
**. Correlation is significant at the 0.01 level (1-tailed).						

The figures show strong and significant correlations between MIC values generated from QSARs based on same physicochemical parameter; $r = 1.000$; $P < 0.01$ for LogP based QSARs and $r = 0.976$; $P < 0.01$ for Mpt based QSARs. Correlation of MIC values generated by QSARs with different parameters is weak and insignificant.

4.2 Liquid media screening

The use of the BACTEC MGIT 960 system for MIC determination was more quantitative and more rapid than the LJ solid media method. MIC values obtained with the liquid media are generally lower than those generated with the solid media. This is in agreement with what has been observed in similar studies (Leonid *et al.*, 1986). The MGIT method also enables to distinguish bactericidal and bacteriostatic effects of test

compounds; the growth units for bacteriostatic compounds were stagnant for several days or even weeks. This was later followed by an increase in growth unit readings. This was observed with JM6 (**112**), JM23 (**117**), JM1 (**107**), JM29 (**122**), INH (**2)** and ST (**1**). for H37Rv and JM1 (**107**), JM9 (**113**), JM23 (**117**), JM28 (**121**), JM27 (**120**), JM12 (**114**), JM26 (**119**), JM13 (**115**) and EMB (**4**), ST (**1**) and INH **(2**) for the MDR strain.

The MGIT method is however more expensive than the LJ solid media method. The method generated more QSARs although only one series of compounds, benzoquinones, was analyzed. The statistically significant MGIT media QSARS for the benzoquinone series are:-

1. $MIC_{MDR} = 124.542 – 0.632MR$ ($F = 8.481$; $P < 0.012$)
2. $MIC_{MDR} = 124.545 – 0.180Rmm$ ($F = 7.838$; $P < 0.015$)
3. $MIC_{MDR} = 82.02 – 7.401LogP$ ($F = 7.592$; $P < 0.016$)
4. $MIC_{MDR} = 0.563Mpt(K) - 1.721MR – 103.414$ ($F = 6.241$; $P < 0.014$)
5. $MIC_{MDR} = 0.339Mpt(K) – 99.757 – 15.288LogP$ ($F = 4.346$; $P < 0.015$)
6. $MIC_{MDR} = 211.028 – 246Mpt(K)$ ($F = 4.290$; $P < 0.059$)
7. $MIC_{MDR} = 172.481 – 1.356MR + 8.853LogP$ ($F = 4.093$; $P < 0.044$)
8. $MIC_{MDR} = 82.0204 – 7.403LogP$ ($F = 7.59$; $P < 0.016$)
9. $MIC_{MDR} = 81.7229 – 12.567LogP + 0.914LogP^2$ ($F = 4.79$; $P < 0.030$)
10. $MIC_{H37Rv} = 266.488 – 50.561LogMR$ ($F = 5.65$; $P < 0.034$)
11. $MIC_{H37Rv} = 4156.72/MR – 10.493$($F = 6.79$; $P < 0.022$)
12. $MIC_{H37Rv} = 33195.0 – 1.5942^{MR}$ ($F = 7.44$; $P < 0.017$)
13. $MIC_{MDR} = 124.542 – 0.6323MR$ ($F = 8.48$; $P < 0.012$)
14. $MIC_{MDR} = 334.907 – 60.305LogMR$ ($F = 10.70$; $P < 0.006$)
15. $MIC_{MDR} = 7.0240 + 4765.42/MR$ ($F = 11.64$; $P < 0.005$)
16. $MIC_{MDR} = 182.217 – 1.9305MR + 0.0064MR^2$ ($F = 5.14$; $P < 0.024$)

17. $MIC_{H37Rv} = 355.103 – 45.819LogCv$ ($F = 5.76$; $P < 0.032$)

18. $MIC_{H37Rv} = 402337.4/Cv – 5.1620$ ($F = 7.09$; $P < 0.020$)

19. $MIC_{H37Rv} = 487616 – Cv^{1.4290}$ ($F = 7.35$; $P < 0.018$)

20. $MIC_{MDR} = 119.479 – 0.0519Cv$ ($F = 8.41$; $P < 0.012$)

21. $MIC_{MDR} = 439.385 – 54.471LogCv$ ($F = 10.85$; $P < 0.006$)

22. $MIC_{MDR} = 13.8391 + 45542.7/Cv$ ($F = 11.70$; $P < 0.005$)

23. $MIC_{MDR} = 17659.5 – Cv^{0.8350}$ ($F = 8.71$; $P < 0.011$)

24. $MIC_{H37Rv} = 344.494 – 53.071LogRmm$ ($r^2 = 0.292$; $F = 5.36$; $P < 0.038$)

25. $MIC_{H37Rv} = 16096.4/Rmm – 15.14$ ($r^2 = 0.330$; $F = 6.4$; $P < 0.025$)

26. $MIC_{H37Rv} = 414401 – Rmm^{1.6848}$ ($r^2 = 0.355$; $F = 7.17$; $P < 0.019$)

27. $MIC_{MDR} = 124.545 – 0.1804Rmm$ ($r^2 = 0.376$; $F = 7.84$; $P < 0.015$)

28. $MIC_{MDR} = 430.027 – 63.666LogRmm$ ($r^2 = 0.440$; $F = 10.21$; $P < 0.007$)

29. $MIC_{MDR} = 0.4029 + 18811.2/Rmm$ ($r^2 = 0.472$; $F = 11.62$; $P < 0.005$)

30. $MIC_{MDR} = 197.792 – 0.6334Rmm + 0.0006Rmm^2$ ($r^2 = 0.462$; $F = 5.15$; $P < 0.024$)

31. $MIC_{MDR} = 15584.7 – Rmm^{0.9792}$ ($r^2 = 0.391$; $F = 8.33$; $P < 0.013$)

QSARs were grouped according to their common characteristics as shown below.

a) QSARs with statistically relatively high predictive power ($F > 10.0$).

i) $MIC_{MDR} = 0.4029 + 18811.2/Rmm$ ($r^2 = 0.472$; $F = 11.62$; $P < 0.005$)

ii) $MIC_{MDR} = 430.027 – 63.666LogRmm$ ($r^2 = 0.440$; $F = 10.21$; $P < 0.007$)

iii) $MIC_{MDR} = 439.385 – 54.471LogCv$ ($F = 10.85$; $P < 0.006$)

iv) $MIC_{MDR} = 7.0240 + 4765.42/MR$ ($F = 11.64$; $P < 0.005$)

v) $MIC_{MDR} = 334.907 – 60.305LogMR$ ($F = 10.70$; $P < 0.006$)

All the five QSARs were generated for compound activity against MDR strain and relate benzoquinone MICs to size related parameters.

b) MR based QSARs.

1. $MIC_{MDR} = 124.542 – 0.632MR$ ($F = 8.481$; $P < 0.012$)
4. $MIC_{MDR} = 0.563Mpt(K) - 1.721MR – 103.414$ ($F = 6.241$; $P < 0.014$)
7. $MIC_{MDR} = 172.481 – 1.356MR + 8.853LogP$ ($F = 4.093$; $P < 0.044$)
10. $MIC_{H37Rv} = 266.488 – 50.561LogMR$ ($F = 5.65$; $P < 0.034$)
11. $MIC_{H37Rv} = 4156.72/MR – 10.493$($F = 6.79$; $P < 0.022$)
12. $MIC_{H37Rv} = 33195.0 – 1.5942^{MR}$ ($F = 7.44$; $P < 0.017$)
13. $MIC_{MDR} = 124.542 – 0.6323MR$ ($F = 8.48$; $P < 0.012$)
14. $MIC_{MDR} = 334.907 – 60.305LogMR$ ($F = 10.70$; $P < 0.006$)
15. $MIC_{MDR} = 7.0240 + 4765.42/MR$ ($F = 11.64$; $P < 0.005$)
16. $MIC_{MDR} = 182.217 – 1.9305MR + 0.0064MR^2$ ($F = 5.14$; $P < 0.024$)

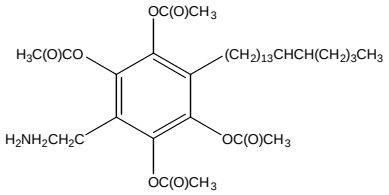
QSARs representing a practically and physically significant activity model were used to calculate the expected MR value of a benzoquinone or benzoquinone derivative with an MIC of 0.2 µg/ml. The MIC value was chosen arbitrary with the aim of predicting a structure with potency similar to that of Isoniazid, the most potent conventional drug used in the study. It was felt that such structures would form very promising leads against MTB. Table 85 below gives the MR values calculated from the MR based QSARs for compounds expected to have an MDR MIC of 0.2 µg/ml.

Table 85: MR values calculated from different QSARs for a benzoquinone with MIC = 0.2 µg/ml

QSAR	1	4	7	10	11	12	13	14	15	16
MIC(MDR) (µg/ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
MR	197.26			295615.9	20794.0	22.3	197.1	566960.8	23820.1	

Since the MR values of the MGIT analysed compounds range from 44.5 to 164.6, the QSARs that generated MR values close to this were chosen as the most practical in predicting and modeling of structures of high potency. The QSAR chosen on this criterion was QSAR number **13** (MR =197.1).

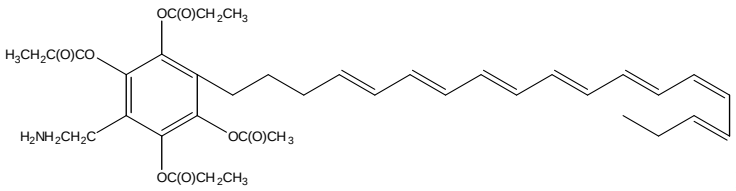
In an effort to come up with structures of benzoquinones or benzoquinone derivatives with MR values close to 197.1 (the MR value corresponding to an MIC value of 0.2 µg/ml) the following structures were generated:-



160

Acetic acid 2,4,5-triacetoxy-6-(2-amino-ethyl)-3-nonadec-14-enyl-phenyl ester

LogP =7.38, MR = 172.79, Mpt = 820.23, Cv = 2000.50, Rmm = 617.39 calculated MGIT MIC_{MDR} = 15.28688

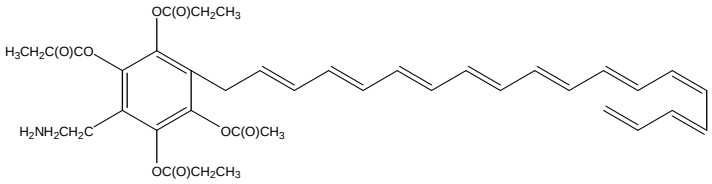


161

Propionic acid 2-acetoxy-6-(2-amino-ethyl)-3-nonadeca-4,6,8,10,12,14,16-heptaenyl-4,5-bis-propionyloxy-phenyl ester

LogP = 7.43, MR = 193.37, Mpt = 823.56, Cv = 2058.50, Rmm = 647.35

Calculated MGIT MIC_{MDR} = 2.274

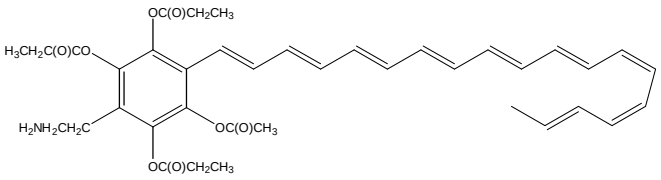


162

Propionic acid 3-acetoxy-5-(2-amino-ethyl)-2-nonadeca-2,4,6,8,10,12,14,16,18-nonaenyl-4,6-bis-propio nyloxy-phenyl ester

LogP = 6.84, MR = 194.53, Mpt = 816.72, Cv= 2009.50, Rmm = 643.77 calculated

MGIT MIC_{MDR} = 1.28776

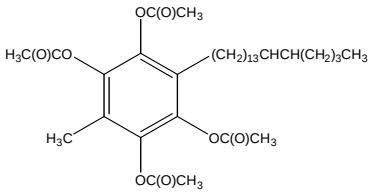


163

Propionic acid 3-acetoxy-5-(2-amino-ethyl)-2-nonadeca-1,3,5,7,9,11,13,15,17-nonaenyl-4,6-bis-propion yloxy-phenyl ester

LogP = 6.79, MR = 195.60, Cv = 2008.50, Mpt = 813.40 , Rmm = 643.77, calculated

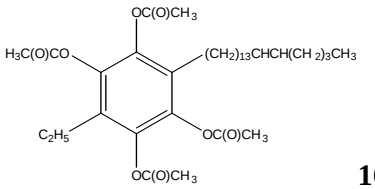
MGIT MIC_{MDR} = 0.86412



164

Acetic acid 2,4,5-triacetoxy-3-methyl-6-nonadec-14-enyl-phenyl ester

LogP = 8.54, MR = 164.60, Mpt = 725.70, Cv = 1915.5, Rmm = 588.77, calculated MGIT MIC_{MDR} = 20.46542

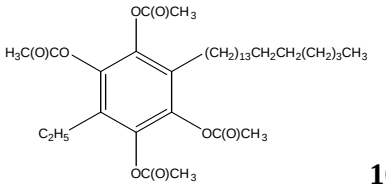


165

Acetic acid 2,4,5-triacetoxy-3-ethyl-6-nonadec-14-enyl-phenyl ester

LogP =8.96, MR = 169.21, Mpt = 736.97, Cv = 1971.5, Rmm = 602.80,

calculated MGIT MIC_{MDR} = 17.55052

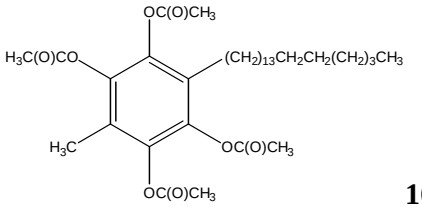


166

Acetic acid 2,4,5-triacetoxy-3-ethyl-6-nonadecyl-phenyl ester

logP = 9.28, MR = 168.09, Mpt = 742.05, Cv = 1991.5, Rmm = 604.81,

calculated MGIT $MIC_{MDR = 18.30912}$



167

Acetic acid 2,4,5-triacetoxy-3-methyl-6-nonadecyl-phenyl ester

LogP = 8.86, MR = 163.49, Mpt = 730.78, Cv = 1935.50, Rmm = 590.79 calculated MGIT $MIC_{MDR =}$

21.21632

b) LogP based QSARs.

3. $MIC_{MDR} = 82.02 – 7.401LogP$ (F = 7.592 ; P < 0.016)

5. $MIC_{MDR} = 0.339Mpt(K) – 99.757 – 15.288LogP$ (F = 4.346 ; P < 0.015)

8. $MIC_{MDR} = 82.0204 – 7.403LogP$ (F = 7.59 ; P < 0.016)

9. $MIC_{MDR} = 81.7229 – 12.567LogP + 0.914LogP^2$ (F = 4.79 ; P < 0.030)

Table 86 below shows the expected LogP values for a benzoquinone with MIC = 0.2 µg/ml as calculated using various LogP based QSARs.

Table 86. Expected LogP values for a benzoquinone whose MIC = 0.2 µg/ml

QSAR	3	5	8	9
MIC	0.2	0.2	0.2	0.2
LogP	11.07958		11.07713	

d) The following are the statistically significant Rmm based QSARs:-

2. $MIC_{MDR} = 124.545 – 0.180Rmm$ (F = 7.838 ; P < 0.015)

24. $MIC_{H37Rv} = 344.494 – 53.071LogRmm$ ($r^2 = 0.292$; F = 5.36 ; P < 0.038)

25. $MIC_{H37Rv} = 16096.4/Rmm – 15.14$ ($r^2 = 0.330$; F = 6.4 ; P < 0.025)

26. $MIC_{H37Rv} = 414401 – Rmm^{1.6848}$ ($r^2 = 0.355$; F = 7.17 ; P < 0.019)

27. $MIC_{MDR} = 124.545 – 0.1804Rmm$ ($r^2 = 0.376$; F = 7.84 ; P < 0.015)

28. $MIC_{MDR} = 430.027 – 63.666LogRmm$ ($r^2 = 0.440$; F = 10.21 ; P < 0.007)

29. $MIC_{MDR} = 0.4029 + 18811.2/Rmm$ ($r^2 = 0.472$; F = 11.62 ; P < 0.005)

30. $MIC_{MDR} = 197.792 – 0.6334Rmm + 0.0006Rmm^2$ ($r^2 = 0.462$; F = 5.15 ; P < 0.024)

31. $MIC_{MDR} = 15584.7 – Rmm^{0.9792}$ ($r^2 = 0.391$; F = 8.33 ; P < 0.013)

Table 87 below gives the Rmm calculated using the QSARs for structures with an MIC values of 0.2.

Table 87: Expected Rmm values for a benzoquinone whose MIC = 0.2 µg/ml

QSAR	2	24	25	26	27	28	29	30	31
MIC	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Rmm	123.43	30719442	1049.309		689.2738	5640012	311.9545	-	

The Rmm of the benzoquinones screened in the MGIT media range from 168.15 to 588.77. The Rmm based QSARs that cover this chemical space are QSARs number 27 and 29.

e) Mpt based QSARs:-

4. $MIC_{MDR} = 0.563Mpt(K) - 1.721MR – 103.414$ (F = 6.241 ; P < 0.014)

5. $MIC_{MDR} = 0.339Mpt(K) – 99.757 – 15.288LogP$ (F = 4.346 ; P < 0.015)

6. $MIC_{MDR} = 211.028 – 246Mpt(K)$ (F = 4.290 ; P < 0.059)

Table 88 below gives the Mpts calculated using the QSARs for structures with an MIC values of 0.2.

Table 88: Expected Mpt values for a benzoquinone whose MIC = 0.2 µg/ml

QSAR	4	5	6
MIC			
Mpt			0.85704

f) Cv based QSARs:-

17. $MIC_{H37Rv} = 355.103 – 45.819LogCv$ ($F = 5.76$; $P < 0.032$)
18. $MIC_{H37Rv} = 402337.4/Cv – 5.1620$ ($F = 7.09$; $P < 0.020$)
19. $MIC_{H37Rv} = 487616 – Cv^{1.4290}$ ($F = 7.35$; $P < 0.018$)
20. $MIC_{MDR} = 119.479 – 0.0519Cv$ ($F = 8.41$; $P < 0.012$)
21. $MIC_{MDR} = 439.385 – 54.471LogCv$ ($F = 10.85$; $P < 0.006$)
22. $MIC_{MDR} = 13.8391 + 45542.7/Cv$ ($F = 11.70$; $P < 0.005$)
23. $MIC_{MDR} = 17659.5 – Cv^{0.8350}$ ($F = 8.71$; $P < 0.011$)

Table 89 below gives the Cv values calculated using the Cv based QSARs for structures with MIC values of 0.2.

Table 89: Expected Cv values for a benzoquinone whose MIC = 0.2 µg/ml

QSAR	17	18	19	20	21	22	23
MIC	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Cv	55657855	75034.88		2298.247	1.16E + 08	-	

The Cv values of the compounds screened in the MGIT media range from 441 to 1915.5. QSAR number 20 was the only one that generated a value close to this range for a structure with MIC value of about 0.2 µg/ml. QSARs that generated acceptable parameter values were used to calculate expected MIC values for structures earlier on identified using MR based QSARs. Table 90 below is a summary of the calculated MIC values.

Table 90: MIC values of some unscreened benzoquinones as calculated using different QSARs

Compound	160	161	162	163	164	165	166	167
Calculated LogP MIC(3,8)	27.4	27.03	31.76	18.8154	15.70704	13.33872	16.447	31.39716
MR MIC(1,13)	15.286	21.274	0.86412	20.4654	17.55	18.309	21.2163	1.28776
Rmm MIC (27)	13.168	7.763	8.409	18.331	15.799	15.437	17.966	8.409
Rmm MIC(30)	30.872	29.462	29.623	32.35	31.61	31.506	32.243	29.623
Cv MIC (20)	15.653	12.643	15.238	20.065	17.158	16.120	19.027	15.186
QSAR 7 MIC	3.513	-23.951	-32.641	24.888	22.355	26.701	29.226	-30.747
QSAr 16 MIC	39.727	48.225	49.471	37.853	38.802	38.546	37.665	48.865
QSAR 5	65.476	65.85	72.180	15.696	13.095	9.939	12.526	72.541
QSAR 9	38.759	38.807	38.532	41.06	42.500	43.813	42.128	38.527
QSAR 30	35.439	39.198	38.692	32.855	33.999	34.182	33.005	38.692
QSAR 4	61.001	27.461	17.903	21.871	20.29	25.1	26.649	21.613

The relative treads of the MIC values calculated from different QSARs for eight untested compounds is shown more clearly in Fig. 76 below.

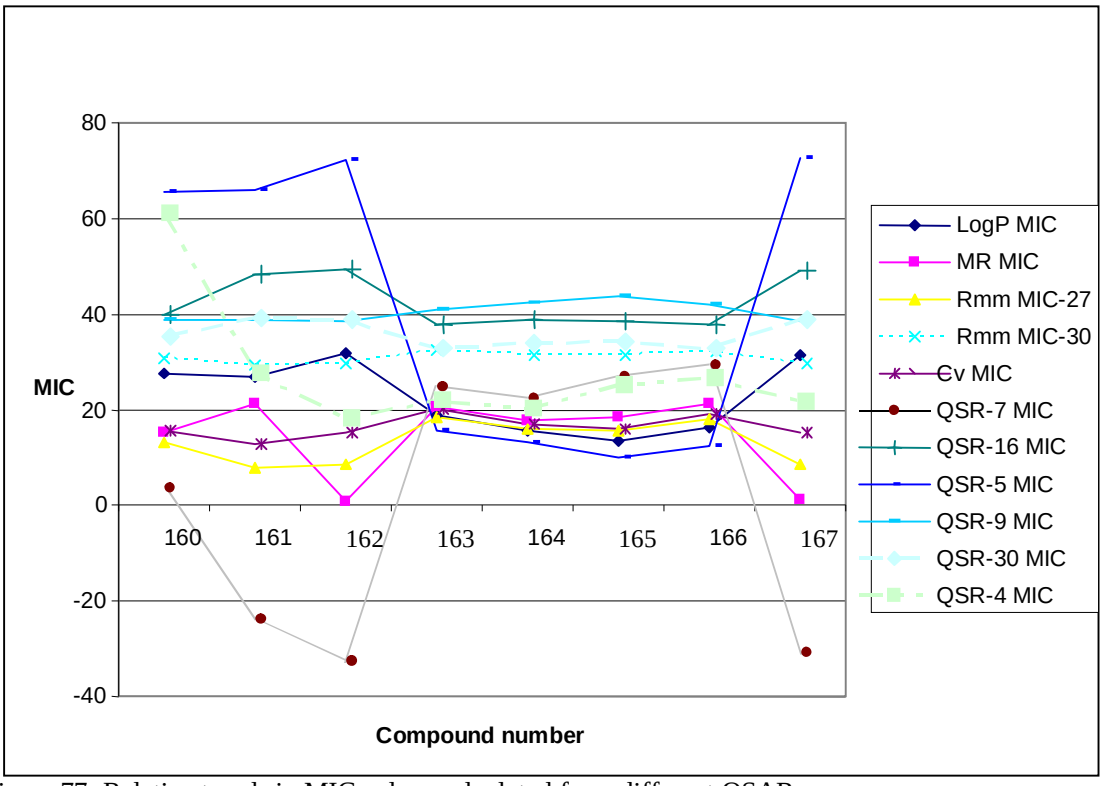


Figure 77: Relative trends in MIC values calculated from different QSARs

Fig. 78 below shows a less clouded figure for trends in MIC values calculated from five different QSARs.

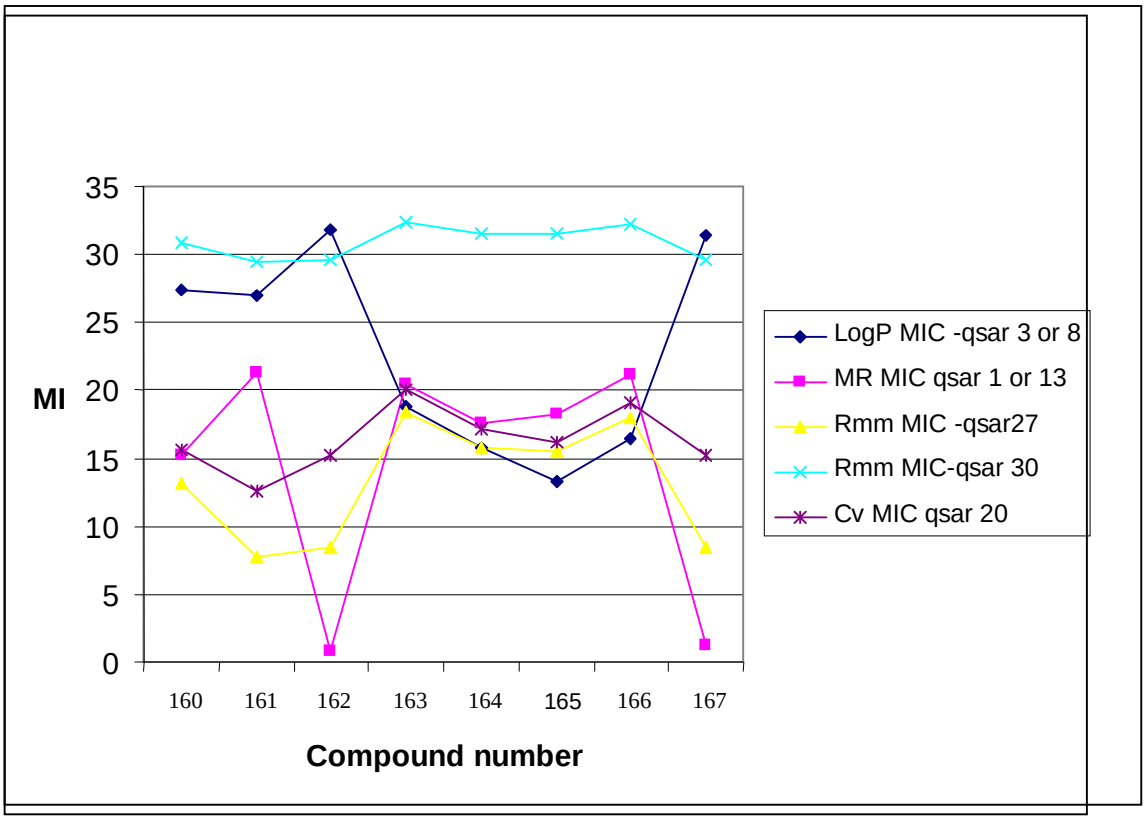


Figure. 78: Relative trends in various QSARs generated MIC values for 8 compounds

The patterns in MIC values calculated from size related parameters (MR, Rmm and Cv) are very similar except for values calculated from QSAR number 30. The LogP calculated MIC values are similar to the other MICs for most of the non amine compounds (**163, 164, 165**, and **166**) but differ for most of the other compounds (**160, 161,162** and **167**). This indicates that the change in chemical space for different physicochemical parameters may be different for an identical change in compound structure (amine to non amine). Size related parameters are less sensitive to functional group change than LogP values.

The overall correlations among the MIC values calculated from QSARs based on different parameters (see MIC values in table 90) are however strong and significant as shown by the SPSS output in table 91 in appendices IX.

Examples of QSARs that correlate strongly and significantly with almost all the other QSARs are:-

QSAR 5 ($MIC_{MDR} = 0.339Mpt(K) – 99.757 – 15.288LogP$ ($F = 4.346$; $P < 0.015$)) ,

QSAR 7 ($MIC_{MDR} = 172.481 – 1.356MR + 8.853LogP$ ($F = 4.093$; $P < 0.044$)), QSAR

QSAR 16 ($MIC_{MDR} = 182.217 – 1.9305MR + 0.0064MR^2$ ($F = 5.14$; $P < 0.024$)), and

QSAR 20 ($MIC_{MDR} = 119.479 – 0.0519Cv$ ($F = 8.41$; $P < 0.012$)).

The precision of such QSARs in predicting activity of untested compounds may be low. The QSARs are however precise enough in predicting trends in activity within a given series of compounds. They can hence be useful in predicting desirable structural modification for optimization of drug properties of a compound. The predictive power of QSARs in predicting activity or trends in activity of unscreened compounds will depend on the chemical space covered by members of the training set used in the QSARs generation.

4.3 Conclusion

The study has identified six highly promising Leads for TB drug development (**114, 116, 121, 141, 145** and **151**) from only fourty ethnobotanically selected compounds. The null hypothesis must then be true. Structure activity relationships (SARs) and Quantitative Structure Activity Relationships (QSARs) have been generated for some congeric series. Structural modifications necessary for improved activity have been

suggested based on activity prediction using QSARs. The isolation, characterization and screening of phytocompounds from plant sources with a history of use in ethnomedicine is hence a relatively efficient method in the search for potentially promising Leads in TB drug development. Where congeric series exist, it is possible to generate SARs and QSARs that can be useful in guiding structure modifications aimed at activity optimization.

4.4 Recommendations

From the study results the researcher made the following recommendations:-

i) The data generated by the MGIT liquid method is more quantitative, less subjective and more enabling in classifying anti-mycobacterial activity as either bacteriostatic or bactericidal. Future screening and MIC determination of test compounds should hence be conducted using the MGIT liquid media method as opposed to the LJ solid media method.

ii) Further in vitro studies on the Benzoquinone series to be guided by carefully selected MGIT generated QSARs. Existing phytocompounds with good QSAR-predicted activity should be screened against both H37RV and MDR MTB strains. Structure modification through chemical synthesis can be used to optimize activity. QSARs on cytotoxicity of the test compounds on human cell-lines should be determined so as to guide synthesis of low toxic compounds with potential use as anti TB drugs.

iii) Alkaloid phytocompounds showed encouraging activity. These were however very few and were only screened on LJ solid media. Further studies on the compounds should be done using the MGIT liquid media.

iv) Although the MGIT liquid media screen is relatively better than the LJ solid media method, it is still a slow labour intensive and low throughput method. An automated higher throughput method would be faster in searching for promising Leads.

v) TB treatment involves administration of several drugs simultaneously (combination therapy). Future studies involving the identified promising compounds should also aim at generating data on the effects of various combinations of the promising compounds on *Mycobacterium tuberculosis*. This could start with various combinations of the six compounds (three *benzoquinones* **114, 121** and **116**, a *flavone* **141** an *isoflavone* **145** and an *alkaloid* **151**) that were identified as highly promising Leads in this study.

vi) The promising Leads from this study (**114, 116, 121, 141, 145** and **151**) should be subjected to further *in vitro* and *in vivo* animal model studies to establish their therapeutic index and their pharmacokinetic profiles.

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Appendices

Appendix I: Tables on various LJ media contents

Table 9: Media preparation for DMSO solubilised drugs

Volume of 1mg/ml Drug solution (ml)	Media Volume (ml)	Total Volume(ml)	Drug conc. µg/ml	DMSO conc. In % v/v
0.5	4.5	5	100	1.0
0.25	4.75	5	50	0.5
0.15	4.85	5	30	0.3
0.1	4.9	5	20	0.2
0.05	4.8	5	10	0.1

Table 10: DMSO solvent controls preparation

Volume of DMSO	Volume of media ml	Total volume ml	DMSO concentration (% v/v)
0.5	4.5	5.0	10.0
0.25	4.75	5.0	5.0
0.15ml	4.85	5.0	3.0
0.1ml	4.9	5.0	2.0
0.05ml	4.95	5.0	1.0
0.025	4.975	5.0	0.5

Table 11: Aqueous media preparation

Volume of 1mg/ml Drug solution (ml)	Media Volume (ml)	Total Volume(ml)	Drug conc. µg/ml
0.5	4.5	5	100
0.25	4.75	5	50
0.15	4.85	5	30
0.1	4.9	5	20
0.05	4.8	5	10

Table 12: Ethanol/Methanol media preparation for more soluble compounds

Set	A	b	C	d	E	F	G	H	I
Volume of stock solution (ml)	0.2 of (i)	0.1 of (i)	0.05 of (i)	0.25 of (ii)	0.15 of (ii)	0.05 of (ii)	0.25 of (iii)	0.15 of (iii)	0.05 of (iii)
Volume of media (ml)	4.8	4.9	4.95	4.75	4.85	4.95	4.75	4.85	4.95
Concentration of drug in Media µg/ml	40	20	10	5	3	1	0.5	0.3	0.1
Solvent con.(% v/v of media)	0.8	0.4	0.2	0.1	0.06	0.02	0.01	0.006	0.002

Table 13: Ethanol/Methanol media preparation for less soluble compounds

Set	A	b	C	D	E	f	G	h	I
Volume of stock solution (ml)	0.4 (i)	0.2 (i)	0.1 (i)	0.05 (i)	0.3 (ii)	0.1 (ii)	0.05 (ii)	0.3 (iii)	0.1 (iii)
Volume of media (ml)	4.6	4.8	4.9	4.95	4.7	4.9	4.95	4.7	4.9
Dug concentration In media µg/ml	40	20	10	5	3	1	0.5	0.3	0.1
Solvent con.(% v/v of media)	1.6	0.8	0.4	0.2	0.12	0.04	0.02	.012	0.004

Table 14 (a): Preparation of controls for Ethanol/Methanol media

Set	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13
Volume of stock solution (ml)	0.05 (c)	0.1 (c)	0.15 (c)	0.25 (c)	0.3 (c)	0.05 (b)	0.1 (b)	0.15 (b)	0.25 (b)	0.3 (b)	0.05 (a)	0.1 (a)	0.125 (a)
Volume of media (ml)	4.95	4.9	4.85	4.75	4.7	4.95	4.9	4.85	4.75	4.7	4.95	4.9	4.875
Solvent concentration(% v/v)	.01	.02	.03	.05	.06	.1	.2	.3	.4	.6	1.0	2.0	2.5

Table 14 (b): Preparation of controls for Ethanol/Methanol media

Set	C14	C15	C16	C17	C18	C19	C20
Vol. of Stock sol.	0.15 (a)	0.175 (a)	0.2 (a)	0.225 (a)	0.25 (a)	0.275 (a)	0.4 (a)
Vol. of media	4.85	4.825	4.8	4.775	4.75	4.725	4.6
Solvent conc.(% v/v)	3.0	3.5	4.0	4.5	5.0	5.5	8.0

Table 15: Preparation of Isoniazid containing LJ media

Set	1	2	3	4	5	6	7	8
Volume of stock solution (ml)	0.2 (c)	0.175 (c)	0.15 (c)	0.125 (c)	0.1 (c)	0.075 (c)	0.05 (c)	0.25 (d)
Volume of media (ml)	4.8	4.825	4.85	4.875	4.9	4.925	4.95	4.75
Drug concentration in media µg/ml	0.40	0.35	0.30	0.25	0.20	0.15	0.10	0.05

Table 16: Preparation of Rifampicin containing LJ media

Set	1	2	3	4	5	6	7	8
Volume of stock Solution (a) (ml)	0.275	0.25	0.225	0.2	0.175	0.15	0.125	0.100
Volume of media (ml)	4.725	4.750	4.775	4.800	4.825	4.850	4.875	4.900
Drug concentration in media µg/ml	55	50	45	40	35	30	25	20
Solvent Con. (% v/v of media)	5.5	5.0	4.5	4.0	3.5	3.0	2.5	2.0

Table 17: Preparation of streptomycin containing LJ media

Set	1	2	3	4	5	6	7	8
Volume of stock Solution (b) (ml)	0.275	0.25	0.225	0.20	0.175	0.150	0.125	0.100
Volume of media (ml)	4.725	4.75	4.775	4.8	4.825	4.85	4.875	4.900
Drug concentration in media µg/ml	5.5	5.0	4.5	4.0	3.5	3.0	2.5	2.0

Table 18: Preparation of Ethambutol containing LJ media

Set	1	2	3	4	5	6	7	8
Volume of stock Solution (ml)	0.2 (b)	0.175 (b)	0.15 (b)	0.125 (b)	0.1 (b)	0.075 (b)	0.05 (b)	0.25 (c)
Volume of media (ml)	4.8	4.825	4.85	4.875	4.9	4.925	4.95	4.75
Drug concentration in media µg/ml	4.0	3.5	3.0	2.5	2.0	1.5	1.0	0.5

Appendix II: MGIT tube media contents in the preliminary liquid media screen

Table19: Isoniazid (**2**) containing tubes

Tube set No.	Drug stock solution concentration (µg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	1	415	0.5	7.0	136	249	0.05
2	10	83	0.5	7.0	136	581	0.1
3	10	166	0.5	7.0	136	498	0.2
4	20	166	0.5	7.0	136	498	0.4
5	20	332	0.5	7.0	136	332	0.8
6	20	664	0.5	7.0	136	-	1.6

Table20: rifampicin (**3**) containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.1	415	0.5	7.0	136	249	5
2	1	83	0.5	7.0	136	581	10
3	1	166	0.5	7.0	136	498	20
4	2	166	0.5	7.0	136	498	40
5	2	332	0.5	7.0	136	332	80
6	2	664	0.5	7.0	136	-	160

Table 21: Ethambutol (**21**) containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.01	415	0.5	7.0	136	249	0.5
2	0.1	83	0.5	7.0	136	681	1
3	0.1	166	0.5	7.0	136	547	2
4	02	166	0.5	7.0	136	498	4
5	0.2	332	0.5	7.0	136	332	8
6	0.2	664	0.5	7.0	136	-	16

Table 22: Streptomycin (**1**) containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.01	415	0.5	7.0	136	249	0.5
2	0.1	83	0.5	7.0	136	681	1
3	0.1	166	0.5	7.0	136	547	2
4	02	166	0.5	7.0	136	498	4
5	0.2	332	0.5	7.0	136	332	8
6	0.2	664	0.5	7.0	136	-	16

Table 23: test compounds containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
A	1	265	0.5	7.0	136	664	30.9
B	1	190	0.5	7.0	136	664	22.4
C	0.5	190	0.5	7.0	136	664	11.2
D	0.1	440	0.5	7.0	136	664	5.0

Table 24: Positive control tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of 0.5/100 mF inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
P_a	-	-	0.5	-	7.0	136	664	0
P_b	-	-	-	0.5	7.0	136	664	0

Table 25: DMSO containing tubes

Tube set no.	Vol. of DMSO (µl)	Vol. of supplement (µl)	Vol. of Water (µl)	Vol. of 0.5mF Inoculum (ml)	Concentration Of DMSO (v/v%)
1	8.5	136	1164	0.5	0.1
2	33.5	136	1164	0.5	0.4
3	58.5	136	1164	0.5	0.7
4	439.0	136	1164	0.5	5.0
5	1240.5	136	1164	0.5	13.0

Table 26: blank control tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
B	-	-	-	7.0	-	-	0
Bc	-	-	-	7.0	136	664	0
Bw	-	-	-	7.0	-	664	0
Bs	-	-	-	7.0	265	-	0
Bfd	Highest	265	-	7.0	136	-	highest
Bufd	Highest	265	-	7.0	136	-	highest

Appendix III: MGIT tube media contents in the Growth units monitoring screening

Table 27: tubes containing drugs with MIC<30.9 (by MGIT)

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
A	0.2	622.5	0.5	7.0	136	41.5	15
B	2	103.5	0.5	7.0	136	560.0	25
C	2	145.0	0.5	7.0	136	518.5	35

Table 28: tubes containing drugs with MIC<30.9(by visual inspection)

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
G	0.1	83.0	0.5	7.0	136	581	1
H	0.1	166	0.5	7.0	136	581	1.98
I	0.1	332	0.5	7.0	136	581	3.88

Table 29: Tubes for compounds with MIC<5<30.9 by visual inspection

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
J	0.2	207.5	0.5	7.0	136	456.5	5
K	0.2	415	0.5	7.0	136	249	10
L	1.0	166	0.5	7.0	136	498	20

Table 30: tubes for compounds with MIC>30.9(visual)

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv and MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
P	2	145	0.5	7.0	136	518	35
Q	2	270	0.5	7.0	136	394	65
R	2	436	0.5	7.0	136	228	105

Table 31: tubes for compounds with MIC≈ 30.9 (visual)

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv and MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
M	2	104	0.5	7.0	136	560	25
N	2	124.5	0.5	7.0	136	539.5	30
O	2	145	0.5	7.0	136	519	35

Table 32: tubes for compounds with MIC>30.9(by MGIT)

Tube No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv / MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
S	2	145	0.5	7.0	136	518	35
T	2	270	0.5	7.0	136	394	65
U	2	436	0.5	7.0	136	228	105

Table 33: Isoniazid containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv / MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.1	75	0.5	7.0	136	589	0.904
2	0.1	149.5	0.5	7.0	136	514.5	1.801
3	0.1	299	0.5	7.0	136	365	3.602

Table 34: Streptomycin containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv / MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.1	207.5	0.5	7.0	136	456.5	2.5
2	0.1	249	0.5	7.0	136	415	3.0
3	0.1	290.5	0.5	7.0	136	373.5	3.5

Table 35: Ethambuto containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv / MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.5	83	0.5	7.0	136	445	5
2	0.5	166	0.5	7.0	136	498	10
3	0.5	332	0.5	7.0	136	332	20

Table 36: Rifampicin containing tubes

Tube set No.	Drug stock solution concentration (mg/ml)	Volume of stock solution (µl)	Volume Of 0.5mF H37Rv / MDR Inoculum (ml)	Volume of Media (ml)	Volume of BACTEC MGIT SIRE supplement(µl)	Volume Of water (µl)	Final drug Concentration (µg/ml)
1	0.05	83	0.5	7.0	136	445	0.5
2	0.05	166	0.5	7.0	136	362	1.0
3	0.05	415	0.5	7.0	136	77	2.5
4	0.5	83	0.5	7.0	136	445	5
5	0.5	166	0.5	7.0	136	362	10
6	0.5	332	0.5	7.0	136	196	20

Table 37: Contents of positive control tubes

Inoculum(H37Rv or MDR) volume in tube set (ml)	Pa (0.5mF)	500	-	-	-
	Pb (0.5/100mF)	-	500	-	-
	Pc (0.5/200mF)	-	-	500	-
	Pd (0.5/500mF)	-	-	-	500
Media		7000	7000	7000	7000
Supplement		136	136	136	136
Water		665	665	665	665

Appendix IV: RAW DATA FROM THE LJ SOLID MEDIA SCREEN

Table IV (a): Data for JM1, JM4, JM5, JM 24 and JM 102

Expt.	Set.	Compound number, stock solution solvent and corresponding MIC in µg/ml										
		jm1	jm1 dms	jm1 eth/ms	jm4	jm4dms	jm5a	jm5dms	jm24a	jm24dms	jm102a	jm102d
1	A	50	35	15	50	40	44	40	65	75	50	50
	B	55	35	10	40	35	35	45	60	70	45	60
	C	45	40	15	55	45	50	55	50	70	55	60
2	A	50	35	10	60	40	45	35	70	65	50	45
	B	50	35	20	45	40	40	40	60	60	60	50
	C	50	30	20	45	30	40	45	65	75	40	50
3	A	45	40	15	50	50	45	30	65	65	55	45
	B	45	35	10	50	50	35	50	70	75	50	55
	C	60	40	15	50	45	40	40	55	65	50	50
4	A	40	30	20	45	35	45	40	65	70	45	55
	B	45	35	15	55	40	35	40	70	75	60	45
	C	50	35	15	50	40	40	35	55	75	50	50
5	A	55	30	15	50	35	50	45	60	65	45	60
	B	45	40	10	55	45	30	30	60	70	50	55
	C	50	35	15	50	40	40	50	65	75	50	45

Table IV (b): Data for JM104, JM114, JM201, JM 202 and JM 204

Expt.	Set.	Compound number, stock solution solvent and corresponding MIC in µg/ml									
		jm104a	jm104d	jm114a	jm114d	jm201a	jm201d	jm202a	jm202d	jm204a	jm204d
1	A	85	40	40	40	40	60	50	50	50	40
	B	80	45	45	45	45	65	55	55	50	40
	C	70	35	45	40	40	60	50	50	55	35
2	A	80	40	35	35	40	55	60	45	40	30
	B	95	45	30	50	35	50	55	50	45	35
	C	90	40	40	55	30	55	50	45	45	40
3	A	90	35	40	40	4	60	50	50	50	55
	B	85	35	45	35	40	60	60	50	50	50
	C	90	40	35	35	50	65	55	60	55	50
4	A	85	45	30	40	40	56	50	60	45	40
	B	75	50	45	40	35	50	50	55	45	50
	C	80	30	50	40	40	60	65	45	50	35
5	A	90	45	45	45	55	65	50	50	50	45
	B	85	40	40	45	30	60	55	40	45	40
	C	90	40	40	50	45	55	50	40	50	30

Table IV (c): Data for JM208, JM303, JM313, JM402 and JM405

Expt.	Set.	Compound number, stock solution solvent and corresponding MIC in µg/ml										
		jm208a	jm208d	jm303a	jm303d	jm313a	jm313d	jm402a	jm402d	jm405a	jm405d	jm405
1	A	50	35	65	75	60	50	50	60	60	30	15
	B	45	30	60	70	65	50	50	65	65	35	10
	C	40	30	70	65	50	55	45	70	60	40	10
2	A	40	40	70	65	50	65	40	60	55	45	15
	B	50	45	75	75	55	60	60	60	50	30	15
	C	50	35	65	70	65	60	60	55	55	30	10
3	A	55	35	50	75	70	50	55	50	60	30	25
	B	60	40	55	60	60	50	50	50	65	35	20
	C	55	30	60	60	60	65	50	55	70	45	20
4	A	60	35	65	75	70	40	45	60	65	40	15
	B	50	40	70	80	65	40	50	60	60	45	15
	C	45	25	65	85	50	45	60	60	55	40	10
5	A	40	30	55	85	55	50	55	55	50	30	5
	B	50	30	60	80	60	45	45	60	60	45	30
	C	55	35	65	75	55	70	50	65	65	45	15

Table IV (d): Data for JM414, JM501, JM521, and JM524

Expt. No.	Set. No.	Compound number, stock solution solvent and corresponding MIC in µg/ml								
		jm414aq	jm414dm	jm501aq	jm501dm	jm521aq	jm521dm	jm521eth	jm524aq	jm524dm
1	A	25	30	35	40	37	75	40	85	75
	B	25	35	35	40	42	75	50	85	70
	C	20	35	30	45	44	70	45	80	75
2	A	30	25	30	35	35	70	35	75	80
	B	35	20	40	30	34	75	35	70	85
	C	30	20	35	35	35	80	40	75	85
3	A	25	30	30	40	34	85	45	70	75
	B	30	30	40	40	37	80	40	75	80
	C	15	45	45	45	38	75	35	90	85
4	A	20	40	35	30	37	80	40	95	75
	B	20	40	35	35	42	70	40	90	80
	C	25	30	30	30	43	65	35	85	60
5	A	35	35	45	30	37	65	40	90	60
	B	30	30	45	40	37	70	45	75	65
	C	25	45	35	40	37	85	40	85	65

Table IV (e): Data for JM604, JM611 and JM620

Expt. No.	Set. No.	Compound number, stock solution solvent and corresponding MIC in µg/ml								
		jm604aq	jm604dm	jm604et	jm611aq	jm611dm	jm611et	jm620aq	jm620dm	jm620et
1	A	35	37	30	67	37	40	25	40	40
	B	35	37	30	67	37	40	25	40	35
	C	30	40	35	60	35	35	30	35	40
2	A	30	42	30	55	34	35	15	53	40
	B	40	35	25	58	37	40	15	35	35
	C	45	35	20	65	43	45	20	40	40
3	A	40	37	25	69	42	50	20	45	55
	B	45	37	25	72	43	50	25	50	50
	C	30	42	35	70	38	40	25	45	55
4	A	35	33	45	79	38	35	25	45	50
	B	35	35	45	70	34	35	20	50	40
	C	35	37	30	67	35	40	25	40	40
5	A	40	38	30	66	37	40	25	40	35
	B	45	39	35	69	37	40	30	45	35
	C	30	37	25	68	37	30	25	40	45

Table IV (f): Data for JM701, JM702, JM703, and JM704

Expt. No.	Set. No.	Compound number, stock solution solvent and corresponding MIC in µg/ml									
		jm701et	jm702aq	jm702dm	jm702et	jm703aq	jm703dm	jm703eth	jm704aq	jm704dm	
1	A	15	50	30	15	50	40	30	37	40	
	B	15	50	30	15	50	45	30	43	40	
	C	10	45	35	20	40	45	35	35	35	
2	A	12	45	25	20	40	50	35	35	35	
	B	10	40	25	10	45	45	40	37	40	
	C	20	55	30	10	45	40	40	43	45	
3	A	25	60	45	9	50	35	30	37	45	
	B	20	55	40	10	55	35	25	40	40	
	C	15	45	45	15	55	30	20	42	40	
4	A	20	50	30	18	60	35	25	42	35	
	B	15	50	30	20	56	40	24	37	40	
	C	10	50	35	25	50	55	30	37	35	
5	A	10	45	25	23	50	50	30	41	30	
	B	15	60	20	15	45	40	30	42	3	
	C	25	55	25	15	48	45	25	33	40	

Table IV (g): Data for conventional drugs

Expt. No.	Set. No.	Compound number, stock solution solvent and corresponding MIC in µg/ml			
		RF	ST	EMB	IHZ
1	A	43	7.1	2.5	0.4
	B	46	4.6	2.5	0.6
	C	50	3.3	2.2	0.2
2	A	45	5.4	3.4	0.4
	B	48	5.2	3.6	0.3
	C	46	5.1	2.5	0.5
3	A	40	4.8	2.4	0.3
	B	35	2.6	2.6	0.5
	C	55	3.4	2.7	0.6
4	A	50	5.5	3.2	1.3
	B	43	5.2	1.9	1
	C	42	5.6	1.8	1.3
5	A	52	5.6	3.4	0.4
	B	45	5.4	2.5	0.5
	C	46	5.5	2.5	0.6

Appendix V: Statistical data of the LJ solid media results

Table 38: Mean MIC values of the benzoquinones in LJ media

Compound number		Jm1(**107**)		Jm 4(**109**)		Jm5(**110**)		Jm 24(**108**)	
	Aq	Dm	Eth	Aq	dm	aq	dm	aq	Dm
Mean MIC in µg/ml	49	35.3	14.7	50	40.7	40.9	41.3	62.3	70
Standard Error	1.3	0.9	0.9	1.3	1.5	1.5	1.9	1.5	1.3
Standard Deviation	5.1	3.5	3.5	5	5.6	5.7	7.2	5.9	5
Confidence Level(95.0%)	2.8	1.9	1.9	2.768908	3.116303	3.1	4	3.3	2.8

Table 39: Mean MIC values of Chalcones in LJ media

Compound number	JM 201(**133**)		JM202(**134**)		JM204(**135**)		JM208(**136**)	
	aq	dm	Aq	dm	Aq	dm	Aq	dm
Mean MIC (µg/ml)	44.3	59	51.3	21.3	48.3	41	50	34.3
Standard Error	1.5	1.7	1.6	2	1.1	2	1.7	1.4
Confidence Level(95.0%)	3.3	3.7	3.4	4.2	2.3	4.2	3.7	2.9

Table 40: Mean MIC values of the Flavanones in LJ media

Compound number	JM303 (**137**)		JM402(**138**)		Jm404(**139**)			JM405(**140**)			JM414 (**141**)	
	aq	dm	Aq	dm	aq	dm	eth	aq	dm	eth	aq	dm
Mean MIC (µg/ml)	63.3	73	51	59	48	15	7.4	59.7	37.7	15.3	26	32.7
Standard Error	1.7	2.06	1.6	1.4	1.5	1.6	0.5	1.5	1.7	1.7	1.5	2.0
Confidence Level (95.0%)	3.7	4.4	3.3	3.	3.3	3.5	1.1	3.2	3.6	3.5	3.2	4.3

Table 41 (a): Mean MIC values of non series compounds **125-143**

	125		132		131		124		142		143		
Compound number	Aq	dm	aq	dm	aq	dm	aq	dm	aq	dm	aq	dm	Eth /meth
Mean MIC (µg/ml)	50.3	51.7	84.7	40.3	40.3	42.3	59.3	53	36.3	37	37.9	74.7	40.3
Standard Error	1.4	1.4	1.7	1.3	1.5	1.5	1.5	1.4	1.4	1.4	0.8	1.7	1.1
Confidence Level(95.0%)	3.0	3.1	3.7	2.9	3.2	3.3	3.1	3.0	3.0	2.9	1.8	3.5	2.4

Table 41 (b) Mean MIC values of non series compounds **144-147**

	144		145			146			147	
Compound number	Aq	dm	aq	dm	Eth/meth	aq	dm	Eth/meth	aq	dm

Mean MIC (µg/ml)	81.7	74.3	36.7	37.4	31	66.8	37.6	39.7	23.3	72.5
standard E	2.1	2.2	1.4	0.7	1.8	1.5	0.8	1.4	1.2	32.8
Confidence	4.4	4.8	3.1	1.4	3.9	3.2	1.6	3.0	2.5	70.3

Table 41 (c) Mean MIC values of non series compounds **147-151**

Compound Number	**147**	**148**	**149**			**150**			**151**	
	Eth/meth	Eth/meth	aq	dm	Eth/meth	Aq	dm	Eth/meth	aq	dm
Mean MIC (µg/ml)	42.3	15.8	50.3	31.3	16	49.3	73.2	29.9	38.7	36.2
Standard E	1.8	1.3	1.5	1.9	1.3	1.5	33.1	1.5	0.8	2.6
Confidence	3.9	2.9	3.2	4.1	2.7	3.2	71.2	3.2	1.8	5.5

Appendix VI: Results for preliminary liquid media screening

a) BACTEC MGIT 960 instrument readings

The results in this section are for MGIT tubes that were initially incubated in the normal walk-in incubator and latter on entered in the BACTEC MGIT 960 instrument for monitoring of growth as mentioned in the methodology section. The results were of a qualitative nature indicated by positive (+) reading for substantial growth or no reading for insignificant or no growth in the MGIT tubes. Although the results were not used in determination of compound MIC values they were a useful guide in the choice of concentrations to be used in further screening. Lack of growth was an indicator of inhibitory effect by the compound in the MGIT tube.

b) Tubes containing conventional drugs

The concentrations of conventional drugs in various MGIT tubes are shown in table 45 below.

Table 45: Concentration of conventional drugs in MGIT tubes

Tube no.	Isoniazid(2) µg/ml	Streptomycin(1) µg/ml	Ethambuto(4) µg/ml	Rifampicin(3) µg/ml
1	0.05	0.5	0.5	5
2	0.1	1	1	10
3	0.2	2	2	20
4	0.4	4	4	40
5	0.8	8	8	80
6	1.6	16	16	160

Table 46 MGIT readings for tubes containing conventional drugs and $H_{37}Rv$ MTB strains inoculum.

Drug	Tube number and MGIT READING					
	1	**2**	**3**	**4**	**5**	**6**
Isoniazid	+	+	+	+	+	+
Rifampicin						
Ethambuto	+	+	+	+	+	+
Streptomycin	+	+	+			

Table 47 MGIT readings for tubes containing conventional drugs and MDR MTB strains inoculum.

Drug	Tube number and MGIT reading					
	1	**2**	**3**	**4**	**5**	**6**
Isoniazid	+	+	+	+	+	+
Rifampicin	+	+				
Ethambuto	+	+	+	+	+	+
Streptomycin	+	+	+	+	+	+

d) Test Compounds Tubes

The concentration of the test compounds in the MGIT tubes were as shown in table 48 below.

Table 48 Concentration of the test compounds in the MGIT tubes

Tube number	A and BL	B	C	D and BH
Compound concentration in µg/ml	30.9	22.4	11.2	5.0

MIGIT readings for the test compounds containing tubes are shown in table 49 and 50 below.

Table 49 MGIT readings for test compounds in H37Rv inoculated tubes

Compound No.	Code no.	Tube number and results (+ growth; - no growth; nd no drug)					
		A	**B**	**C**	**D**	**BH**	**BL**
107	JM 1	+	+	+	+	+	+
111	JM 2	+	+	+	+	-	+
118	JM 25	-	+	+	+	+	+
108	JM 24	+	+	+	+	+	+
116	JM 14	+	+	+	+	+	+
122	JM 29	+	+	+	+	+	+
119	JM 26	+	+	+	+	+	+
112	JM 06	-	-	+	+	-	+
117	JM 23	+	+	+	+	+	+
120	JM 27	+	+	+	+	+	+
110	JM 05	+	+	+	+	+	+
114	JM 12	+	+	+	+	+	+
115	JM 13	+	+	+	+	+	+
121	JM 28	+	+	+	+	+	+
123	JM 36	-	+	+	+		+

Table 50 MGIT readings for test compounds in MDR inoculated tubes

Compound Number	Code no.	Tube number and results				BH	BL
		A	**B**	**C**	**D**		
107	**JM 1**	+	+	+	+	+	+
111	**JM 2**	+	+	+	+	+	+
118	**JM 25**	+	+	+	+	+	+
108	**JM 24**	+	+	+	+	+	+
116	**JM 14**	+	+	+	+	+	+
122	**JM 29**		+	+	+	+	+
119	**JM 26**	+	+	+	+	+	+
112	**JM 06**	+	+	+	+	+	+
117	**JM 23**		+	+	+	+	+
120	**JM 27**	+	+	+	+	+	+
110	**JM 05**	+	+	+	+	+	+
114	**JM 12**	+	+	+	+	+	+
115	**JM 13**	+	+	+	+	+	+
121	**JM 28**	+	+	+	+	+	+
123	**JM 36**	+	+	+	+	+	+
107	**JM 1**	+	+	+	+	+	+

Control Tubes

MGIT readings for DMSO containing tubes are shown in table 51 below.

Table 51 MGIT readings for DMSO controls tubes

	Tube number				
	1	**2**	**3**	**4**	**5**
Results	+	+	+	-	-

Table 52 below shows the MGIT readings for tubes used as positive controls for growth of H37Rv and MDR MTB trains.

Table 52 MGIT readings for positive control Tubes

		Tube no.					
		1	**2**	**3**	**4**	**5**	**6**
Results(H37Rv)	Pa	+	+	+	+	+	+
	Pb	+	+	+	+	+	+
Results(MDR)	Pa	+	+	+	+	+	+
	Pb	+	+	+	+	+	+

Readings for blank controls are shown in table 53 below.

Table 53 MGIT readings for Blank control

	Tube set no.		
	1 plain	2 water	3 supplement
Results(H37Rv)		+	
Results(MDR)			

Appendix VII: Growth Unit curves from MGIT liquid media screening

(A) Growth patterns in H37Rv inoculated MGIT tubes

a) *M. tuberculosis* H_{37}Rv growth patterns in drug free tubes

The inoculum concentrations used are shown in table 54 below:-

Table 54: Inoculum concentrations in positive controls

Tube set	Pa	Pb	Pc	Pd
Inoculum concentration	0.5mF	0.5/100 mF	0.5/200 mF	0.5/500 mF

Fig. 18 below shows Log of growth units plotted against time in days for various drug – free tubes:-

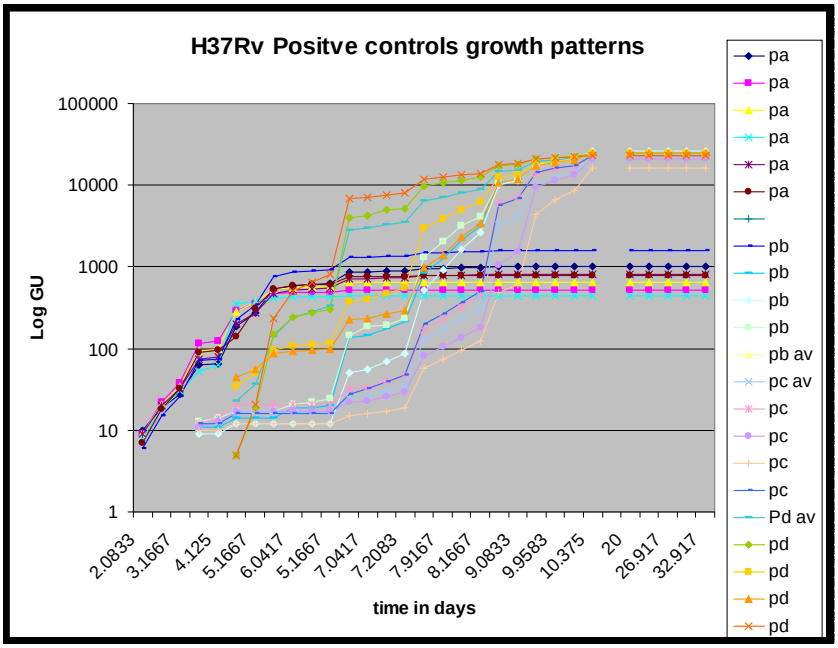

Figure 18: *M. tuberculosis* H_{37}Rv growth patterns in drug free tubes

Growth in tubes with undiluted inoculum (set pa) started early and maximum Log growth units (GU) was attained early. Growth for the diluted inoculum was registered after three days but was more rapid than that of the undiluted one. The diluted inoculum

attained higher maximum growth units than the undiluted one. Growth patterns for tubes with similar inoculation were very similar and it was found appropriate to take the average of each set for a clearer picture of the trends in growth. This is shown in Fig. 19 below.

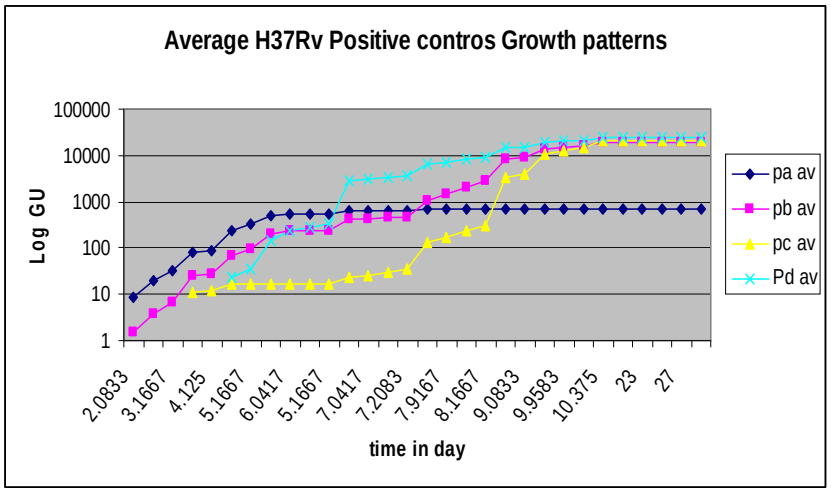

Figure.19: Average growth of *M. tuberculosis* H_{37}Rv in drug free tubes

From the growth patterns observed with the various positive control dilutions, inhibition of growth by compounds was associated with:-

iii) GU values that are equal or lower than those of the 1:100 diluted inoculum between the 2nd and the 5th day of incubation.

iv) GU values that did not rise above those of the undiluted inoculum before the 9th day of incubation.

b) ***M. tuberculosis* H_{37}Rv growth patterns in MGIT tubes containing test compounds**

The concentrations of the test compounds in various sets of MGIT tubes inoculated with *M. tuberculosis* H_{37}Rv are shown in tables (a) to (d) below.

Table (a): concentration of test compounds in sets **a** to **g**

Tube set	a	b	c	d	e	f	g
[Compound] µg/ml	15	25	35	15	25	35	05

Table (b): concentration of test compounds in sets **h** to **n**

Tube set	h	I	j	k	l	m	n
[Compound] µg/ml	10	20	05	10	20	25	30

Table (c): concentration of test compounds in **o** to **u**

Tube set	o	p	q	r	s	t	u
[Compound] µg/ml	35	35	65	105	35	65	105

Table (d): concentration of test compounds in sets **9;1** to **9;7**

Tube set	9;1	9;2	9;3	9;4	9;5	9;6	9;7
[Compound] µg/ml	1	5	10	20	40	80	105

Fig. 20 to Fig. 38 below show the growth patterns of *M. tuberculosis* H37Rv in media containing test compounds at different concentrations as monitored through growth units (GU) generated by the BACTEC MGIT 960 instrument.

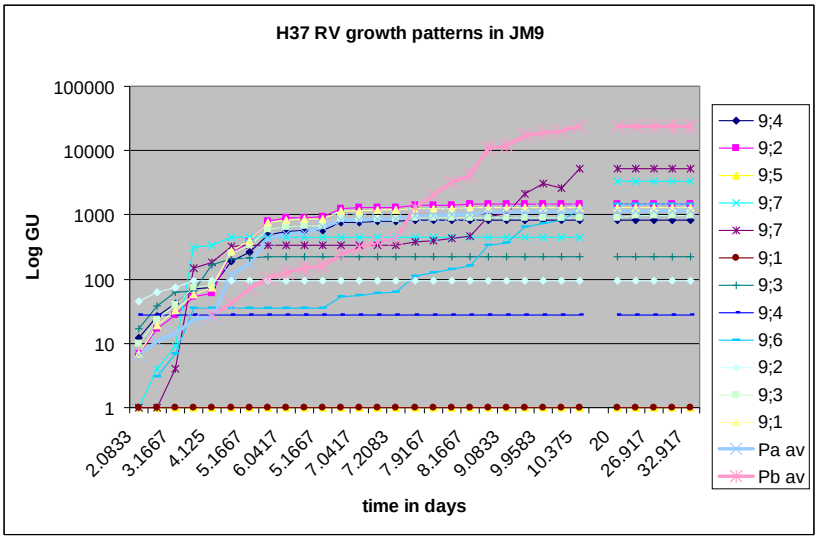

Figure. 20: H37Rv growth in tubes containing **113 (JM9)**

The growth patterns indicate that 40<MIC<80 and hence MIC = 60µg/ml. Activity could be bactericidal

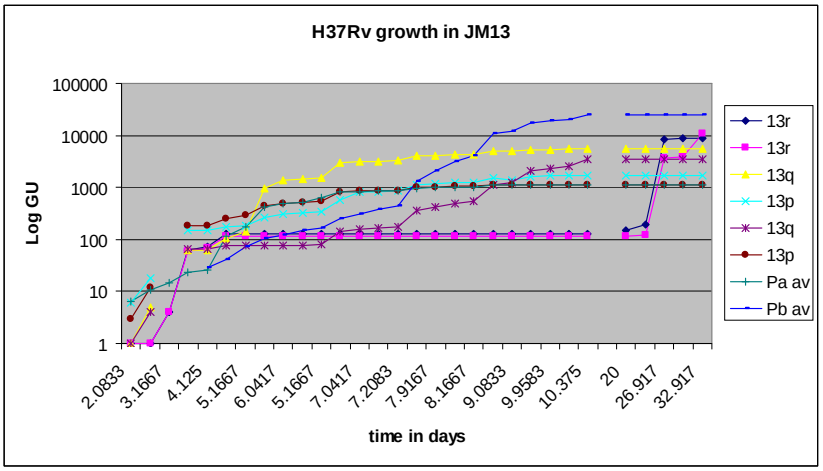

Figure. 21: H37Rv growth in tubes containing JM13 (**115**)

From the growth patterns, 35 < MIC < 65. The MIC value is closer to 35 than to 65 and was calculated as MIC= 42.5 µg/ml. The patterns also suggest a bacteriostatic action.

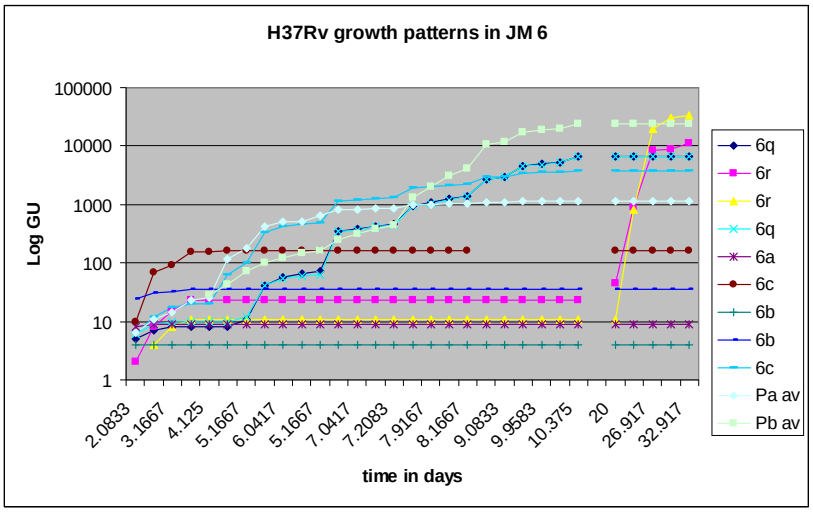

Figure. 22: H37Rv growth in tubes containing JM6 (**112**).

The patterns indicate that the MIC< 65. The value could be as low as 15 µg/ml and a value was deduced from earlier study considerations (see table 49 in appendix V). MIC = 15 µg/ml. Patterns suggest a bacteriostatic activity.

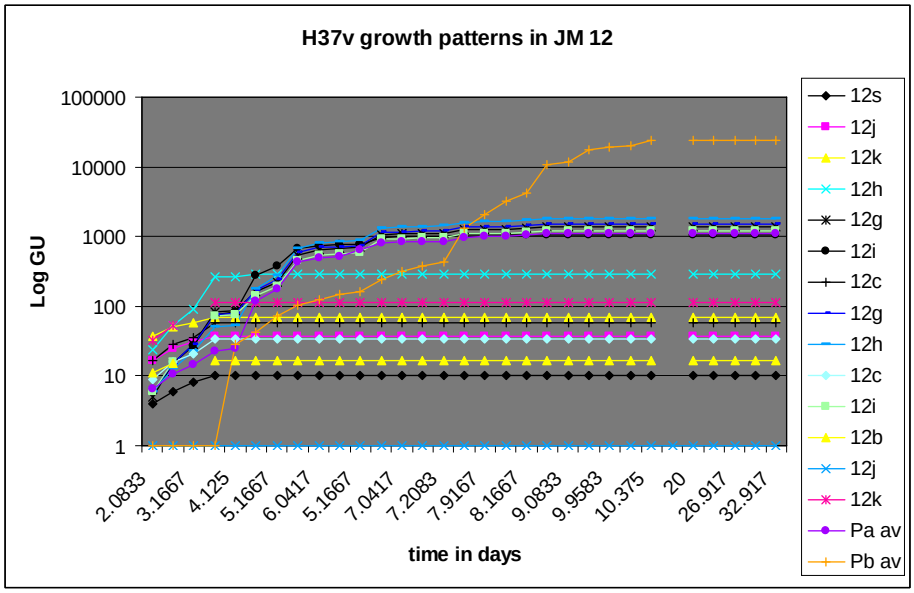

Figure. 23: H37Rv growth in tubes containing JM 12(**114**)
The growth patterns indicate that MIC=5 µg/ml. Activity appears to be bactericidal.

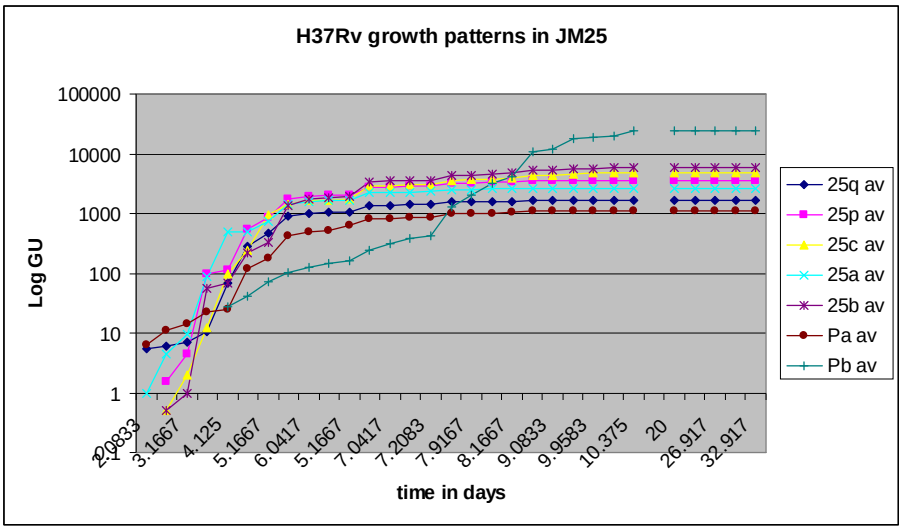

Figure. 24: H37Rv growth in tubes containing JM 25(**118**)

From the growth patterns, 105>MIC>65 and the value assigned is MIC = 85 µg/ml

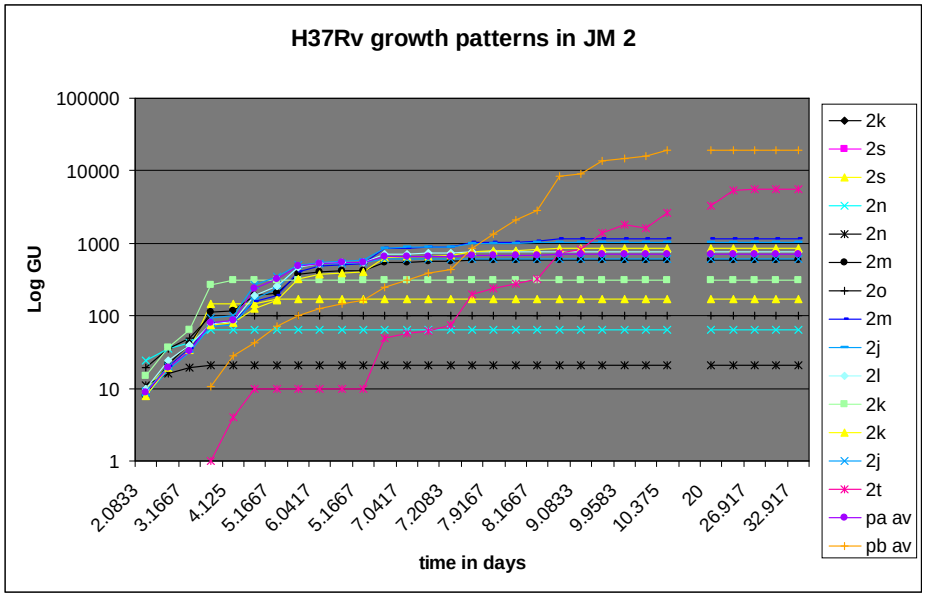

Figure. 25: H37Rv growth in tubes containing JM 2(**111**).

The growth patterns indicate that MIC=35 µg/ml. The activity appears to be bactericidal.

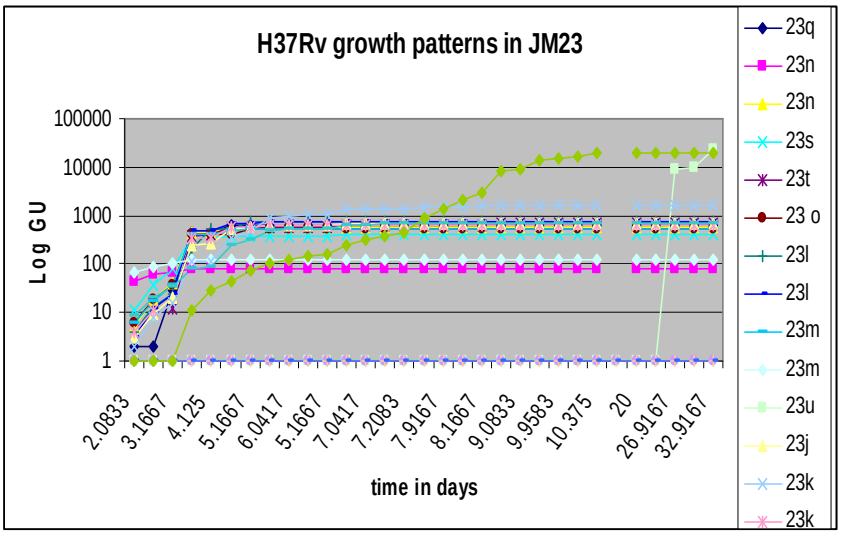

Figure. 26: H37Rv growth in tubes containing JM 23(**117**)

The growth patterns indicate that 35<MIC<65. The MIC value was hence calculated as MIC = 50 µg/ml

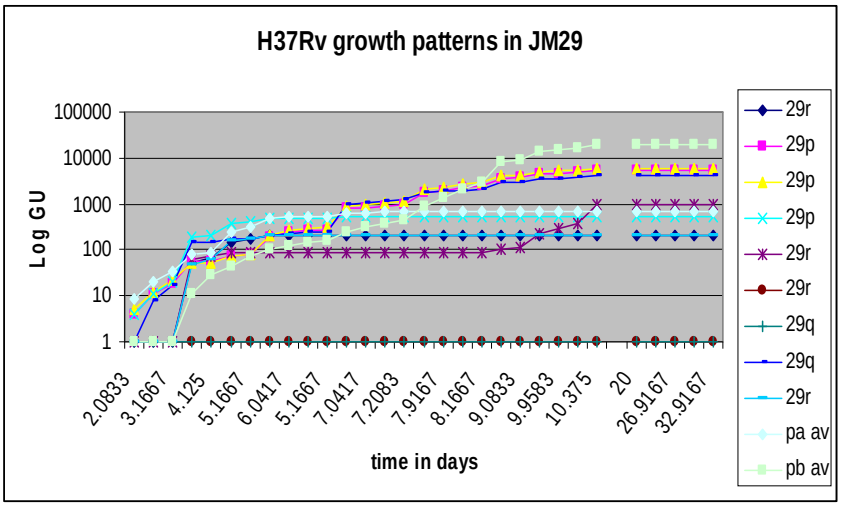

Figure. 27: H37Rv growth in tubes containing JM 29(**122**)

The patterns indicate that MIC>105 µg/ml. The actual value was estimated as MIC = 107 µg/ml (closer to 29r because one set of 29r inhibited growth for a while).

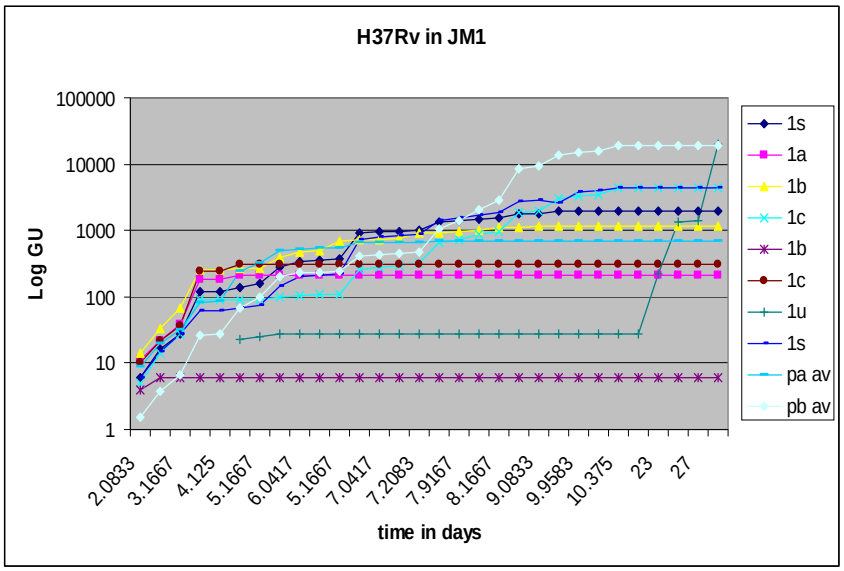

Figure. 28: H37Rv growth in tubes containing JM1 (**107**).

The patterns indicate that MJC=15 µg/ml.

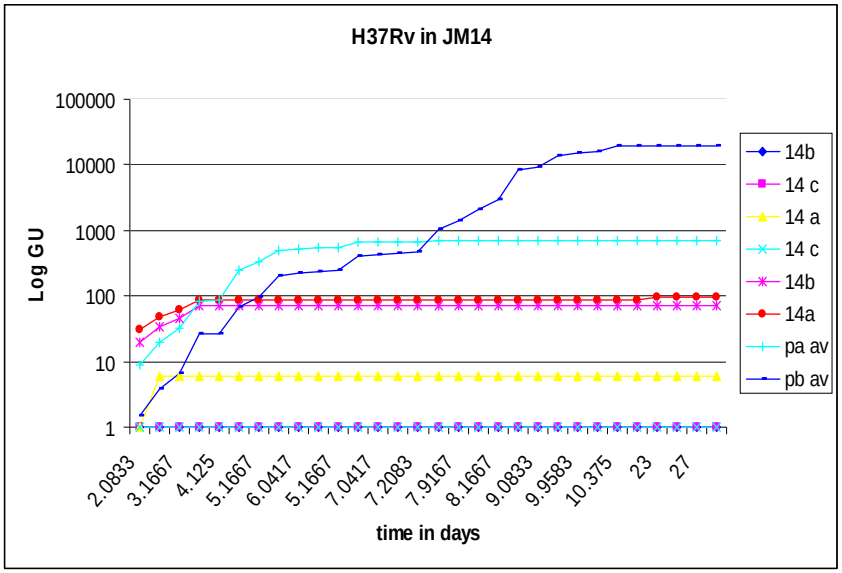

Figure. 29: H37Rv growth in tubes containing JM14 (**116**).

The growth patterns indicate that MIC = 15 µg/ml. Activity suggested is bactericidal.

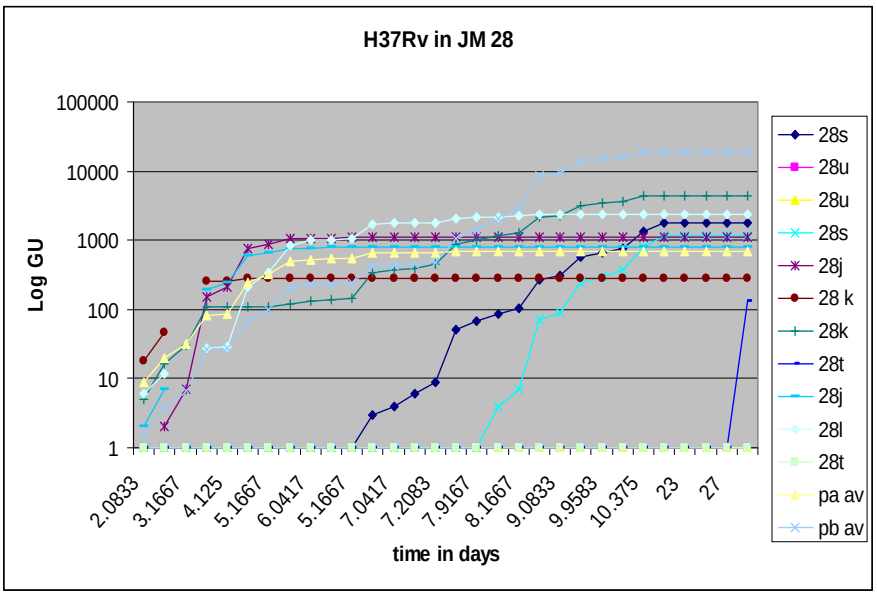

Figure. 30: H37Rv growth in tubes containing JM28(**121**)

The patterns indicate that 5<MIC<10. The Calculated MIC = 7.5 µg/ml. The patterns also indicate a bacteriostatic characteristic since inhibition is only temporary.

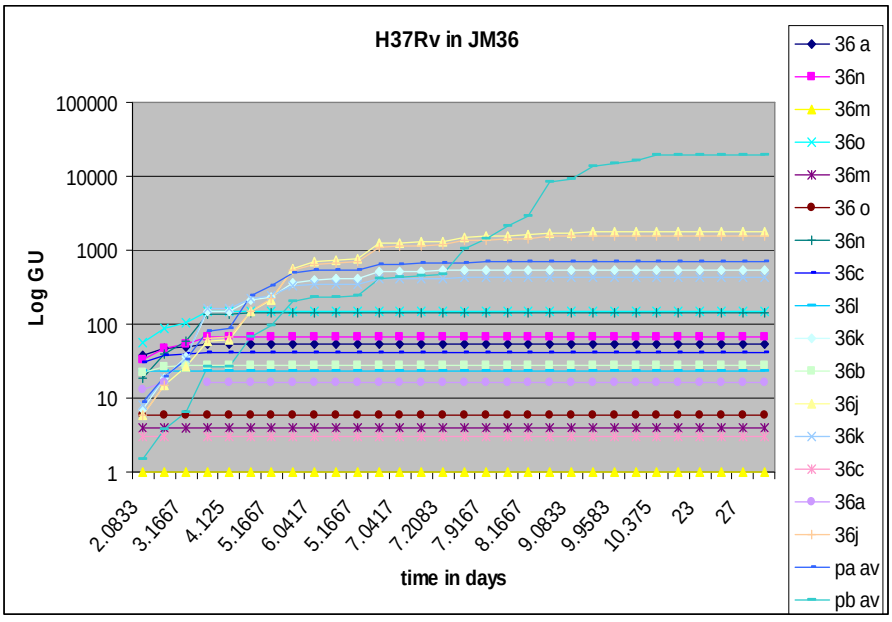

Figure. 31: H37Rv growth in tubes containing JM36 (**123**)

The patterns indicate that 5<MJC<15. The assigned value was calculated as MIC = 10 µg/ml. A bactericidal activity is evident.

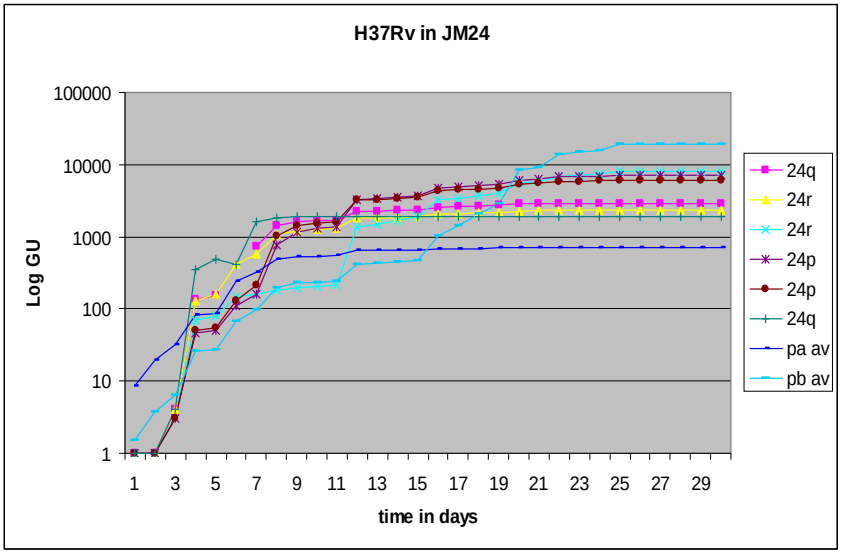

Figure. 32: H37Rv growth in tubes containing JM24 (**108**).

The patterns indicate that MIC > 105 but very close to 105 µg/ml. Calculated MIC = 109 µg/ml

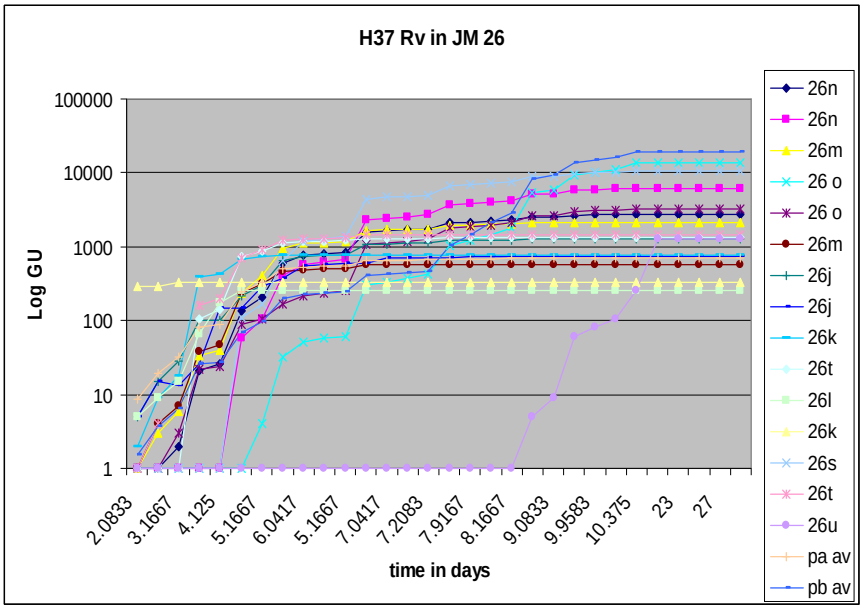

Figure.33: H37Rv growth in tubes containing JM26 (**119**).

The patterns indicate that 30< MIC< 35. Deduced MIC = 32 µg/ml. The patterns also indicate a bacteriostatic compound with a very short half life.

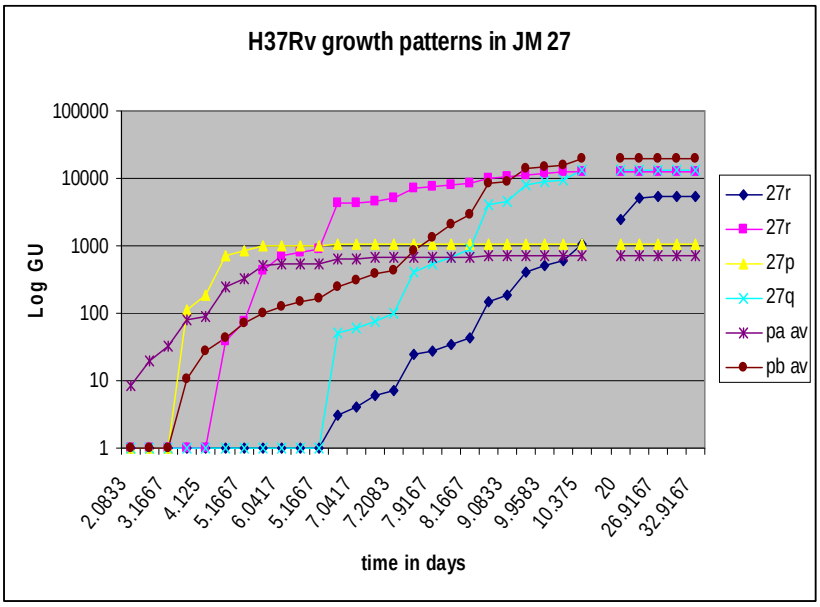

Figure. 34: H37Rv growth in tubes containing JM27 (**120).**

The patterns indicate 35<MIC<65 µg/ml and the deduced value is MIC = 50 µg/ml. The patterns also suggest a bacteriostatic activity.

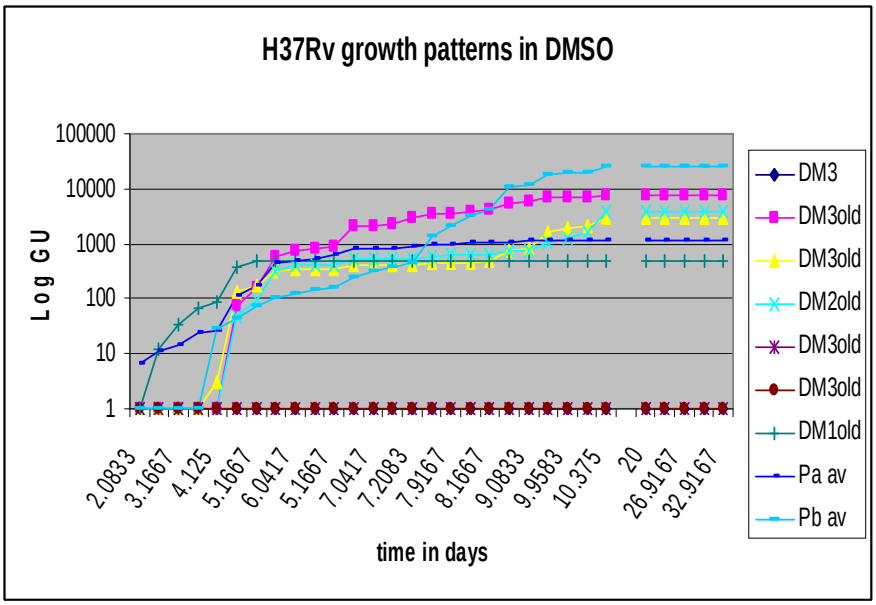

Figure. 35: H37Rv growth in tubes containing DMSO

The patterns show the percentage minimum inhibitory volume of DMSO as

0.7%v/v<MIC<2%v/v. The actual value was calculated as MIC = 1.35% v/v.

c) **H37Rv growth patterns in conventional drugs**

The conventional drugs used and their concentrations are shown in table 59 below.

Table 59: concentrations of the conventional drugs in H37Rv inoculated tubes.

Drug set and concentration	Isoniazid(2) µg/ml	Streptomycin(1) µg/ml	Ethambuto(4) Mg/ml	Rifampicin(3) µg/ml
1	0.9	2.5	5	0.5
2	1.8	3.0	10	1.0
3	3.6	3.5	20	2.5
4			25	5
5				10
6				20

The figures below show the growth patterns of H37Rv in MGIT tubes containing conventional drugs.

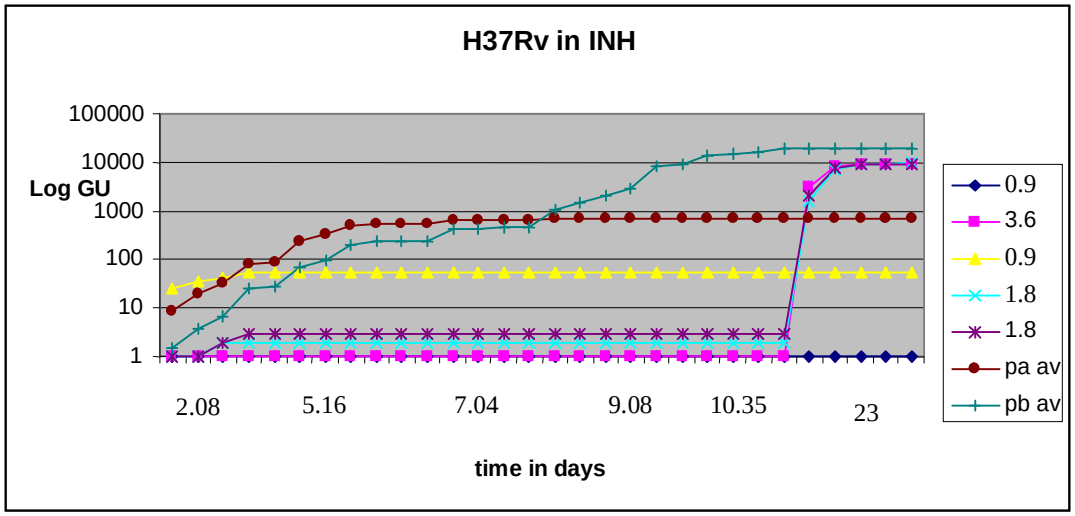

Figure. 36: H37Rv growth patterns in Isoniazid (**2**) containing MGIT tubes

The patterns indicate that MIC< 0.9 µg/ml. The deduced MIC = 0.8 µg/ml

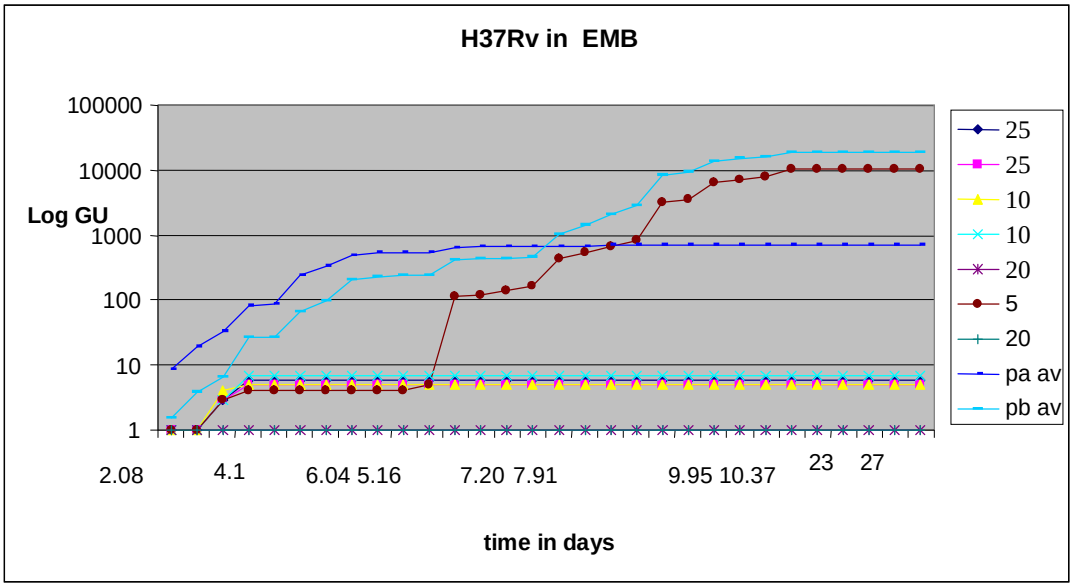

Figure. 37: H37Rv growth patterns in Ethambuto (**4**) containing MGIT tubes

The patterns indicate that 2 µg/ml <MIC< 5 µg/ml. The value calculated is hence MIC = 2.5 µg/ml

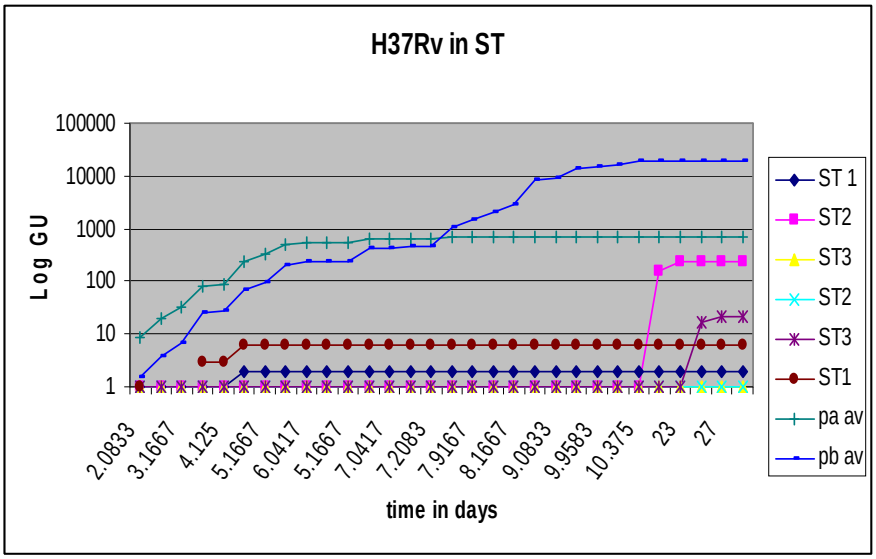

Figure. 38: H37Rv growth patterns in Streptomycin (**1**) containing MGIT tubes

The trends indicate a MIC< 2.5 µg/ml. The value deduced from considerations of preliminary screening results is 2.5 µg/ml < MIC< 4 µg/ml. the value was hence assigned as MIC = 3.25 µg/ml

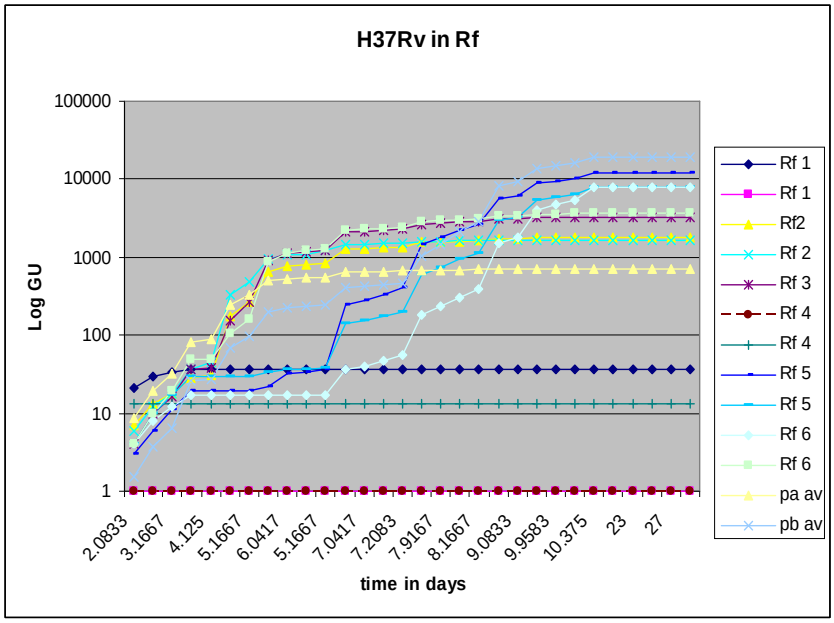

Figure. 39: H37Rv growth patterns in Rifampicin (**3**) containing MGIT tubes

The patterns indicate that 5 µg/ml < MJC < 10 µg/ml. The MIC calculated = 7.5 µg/ml

(B) Growth patterns in tubes inoculated with MDR strain

Tubes were labeled like those used for the $H_{37}Rv$ strain except but in a different colour. The processes of drug addition, inoculation and incubation were done same time as for the $H_{37}Rv$ tubes.

a) Growth patterns in drug free MDR inoculated tubes

Fig. 40 below shows the plot of logarithm of growth units against time in days for tubes inoculated with various concentrations of MDR with no drugs added.

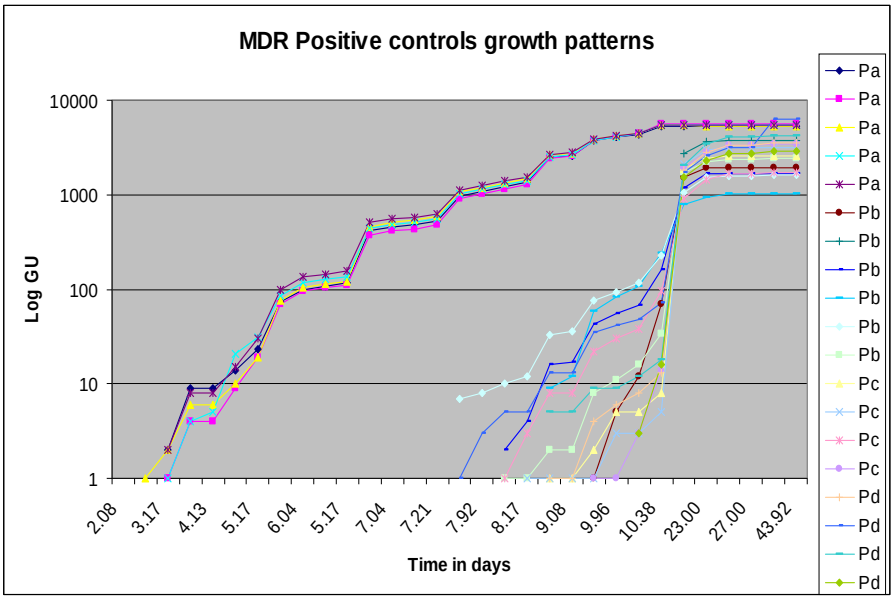

Figure. 40: MDR growth in drug free MGIT tubes

The average growth units were calculated for each set of inoculum concentration and logarithms of the averages plotted against time in days as shown in Fig. 41 below:

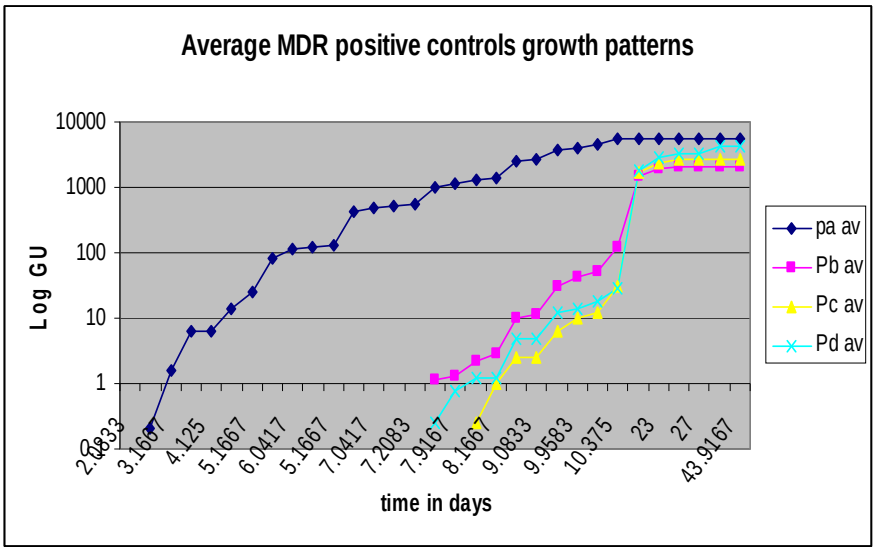

Figure. 41: Average MDR growth in drug free MGIT tubes

Growth inhibition was associated with:-

iii. GU registered latter than those of the undiluted inoculum

iv. GU values equal to or less than those of the 1:100 diluted inoculum between the 7th and the 20th day of incubation.

b) MDR growth patterns in test compound tubes

Tube labeling and processing was similar to that of the H37Rv tubes except that a different colour was used. Figures below show the plots of logarithms of growth units against time in days for the compounds screened against *Mycobacterium tuberculosis* MDR strain.

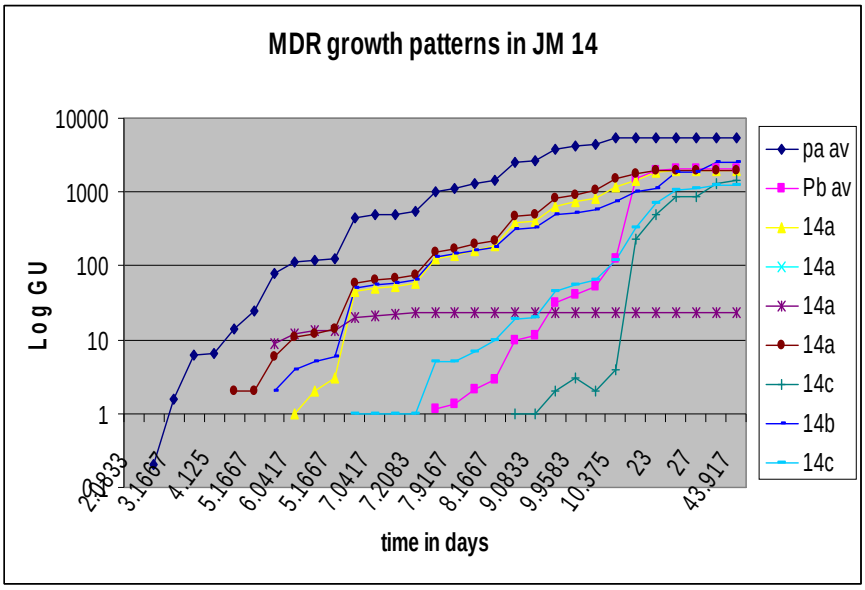

Figure. 42: MDR growth in MGIT tubes containing JM14 (**116**)

The patterns indicate that, 15 µg/ml <MIC<25 µg/ml. The activity of the compound was hence calculated as the average value; MIC = 20 µg/ml.

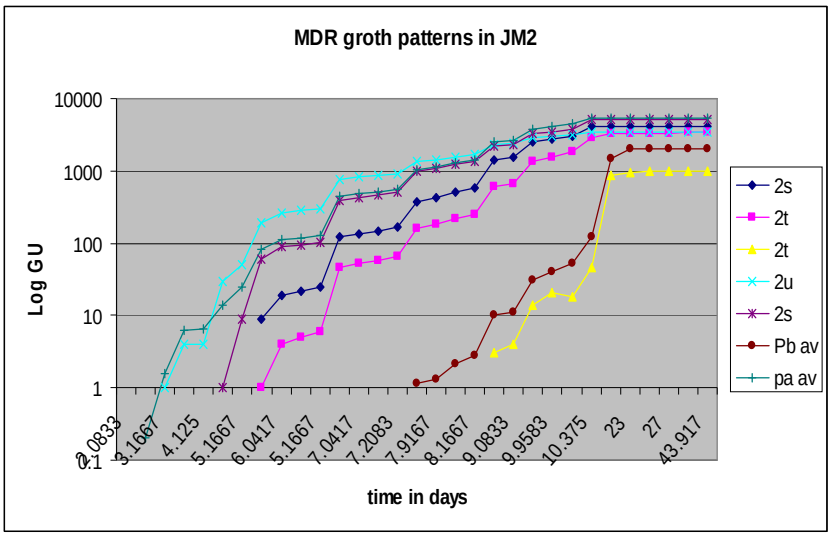

Figure. 43: MDR growth in MGIT tubes containing JM2 (**111**).

The patterns indicate that MIC = 65 µg/ml

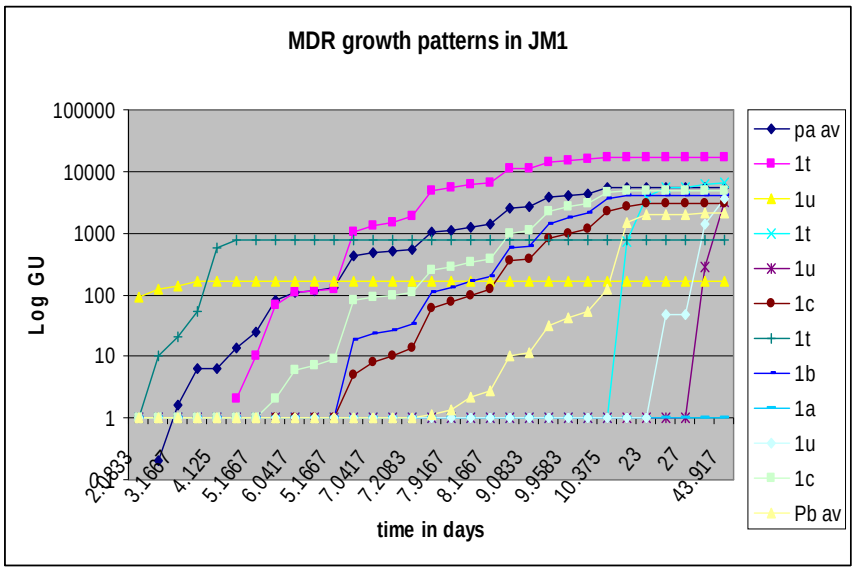

Figure. 44: MDR growth in MGIT tubes containing JM1 (**107**)

The activity indicated by the growth patterns is 35 µg/ml <MIC<65 µg/ml. The average was calculated as MIC = 50 µg/ml.

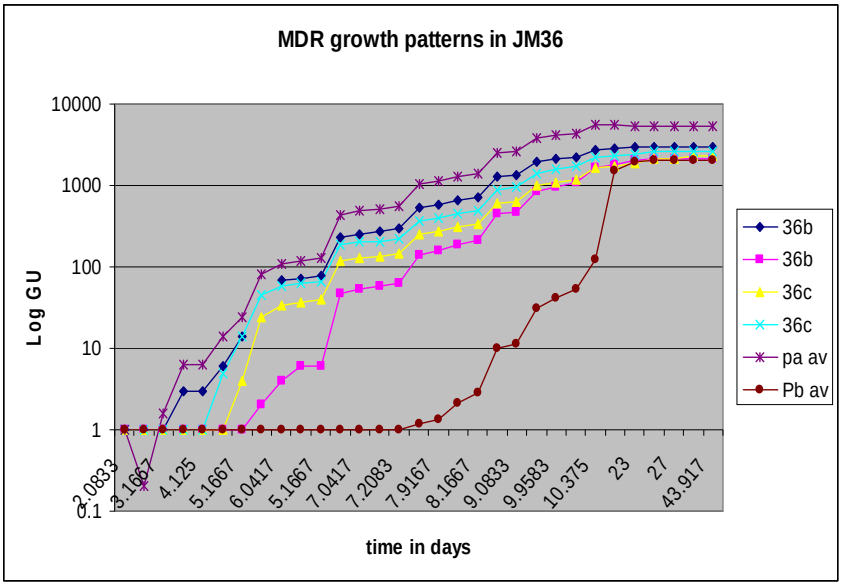

Figure. 45: MDR growth in MGIT tubes containing JM36 (**123**)

The patterns suggest that MIC> 35 µg/ml. A value of 40 µg/ml was assigned from curve closeness

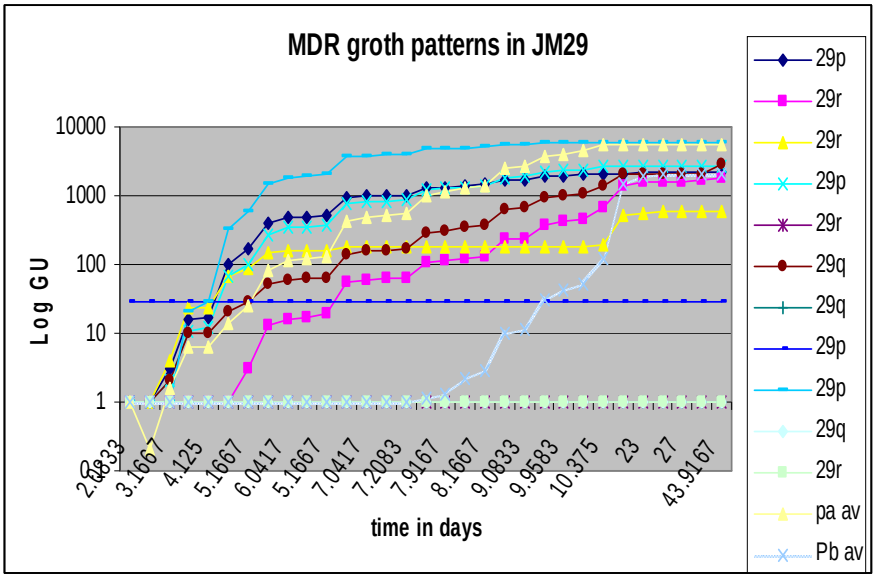

Figure. 46: MDR growth in MGIT tubes containing JM29 (**122**).

The activity deduced from the growth patterns is MIC = 105 µg/ml characterized by low solubility. Activity could be bactericidal.

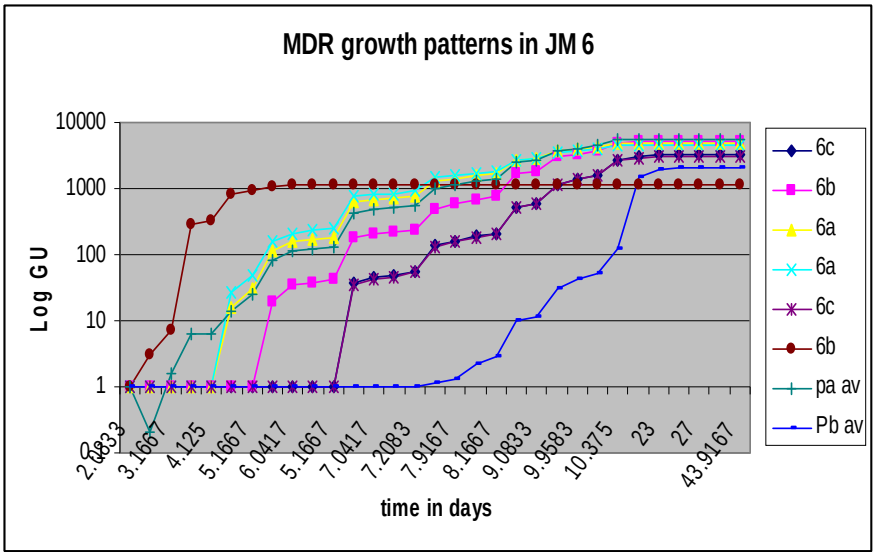

Figure. 47: MDR growth in MGIT tubes containing JM6 **(112**)

The activity implied by the patterns is 30.9 µg/ml >MIC>25 µg/ml. The average MIC = 32.9 µg/ml

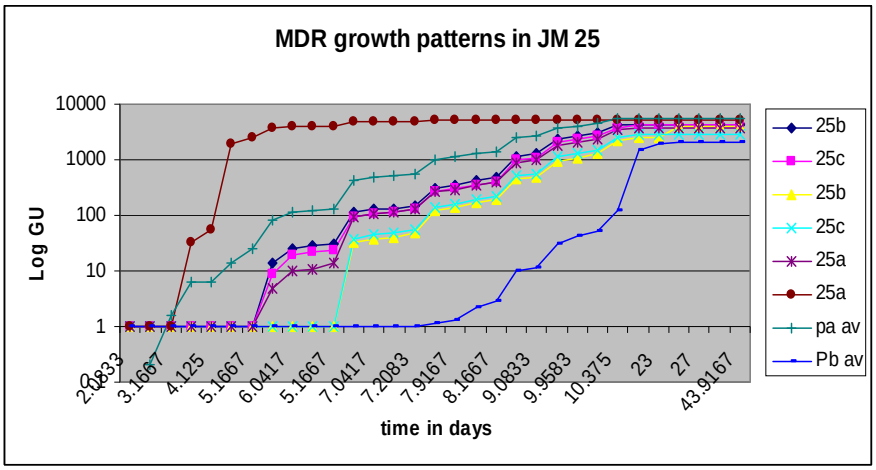

Figure. 48: MDR growth in MGIT tubes containing JM25(**118**)

The patterns suggest that 65 µg/ml >MIC>35 µg/ml and hence assigned average value is

MIC = 50 µg/ml

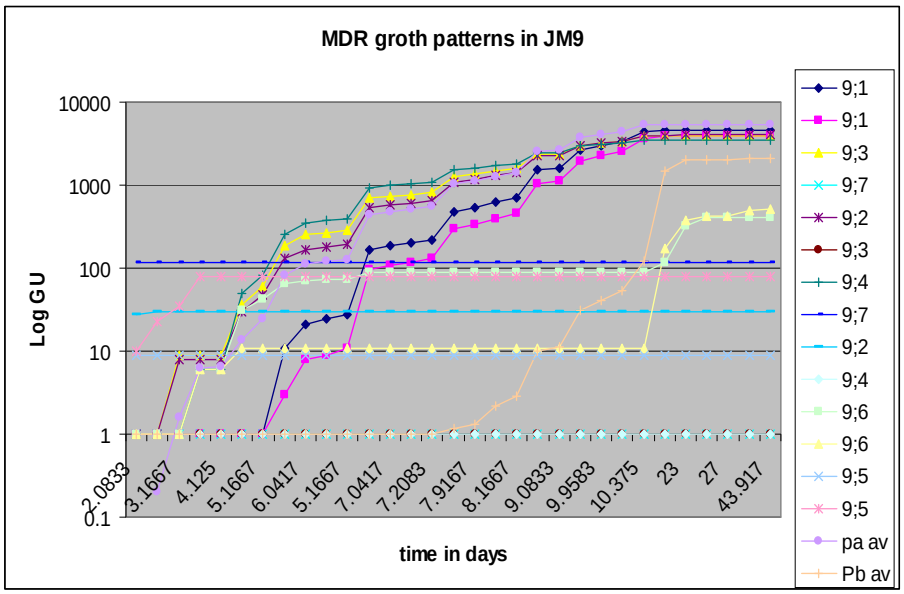

Figure. 49: MDR growth in MGIT tubes containing JM9 (**113**)

The activity suggested by the growth patterns is 40 µg/ml <MIC<80 µg/ml. The value is however very close to 80 µg/ml and a value of MIC = 70 µg/ml was assigned. The patterns also suggest that the compound is bacteriostatic. Possible contamination with aerobic organisms or over inoculation was also evident in 9;2(blue), 9;4(blue), 9;5(blue and pink) and 9;7(blue).

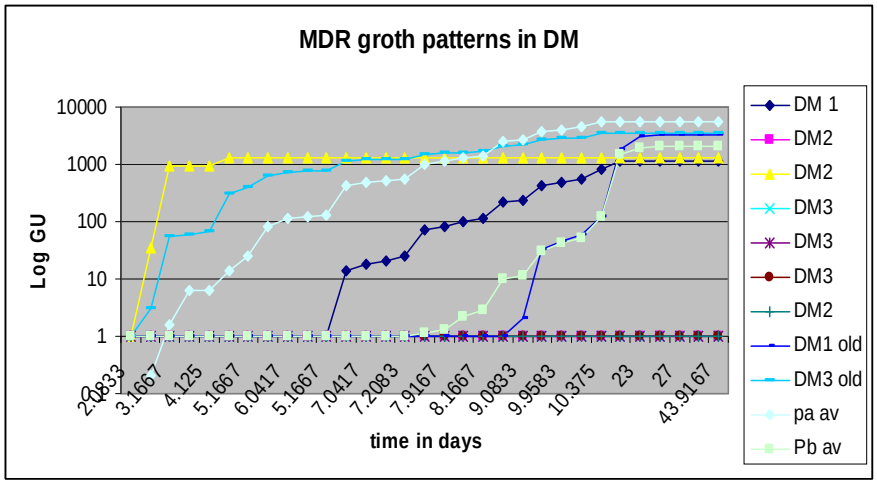

Figure. 50: MDR growth in MGIT tubes containing DMSO

The patterns suggest a solvent inhibitory activity of 1%v/v < MIC < 2 %v/v. An average MIC = 1.5 %v/v was hence assigned. Activity is bactericidal.

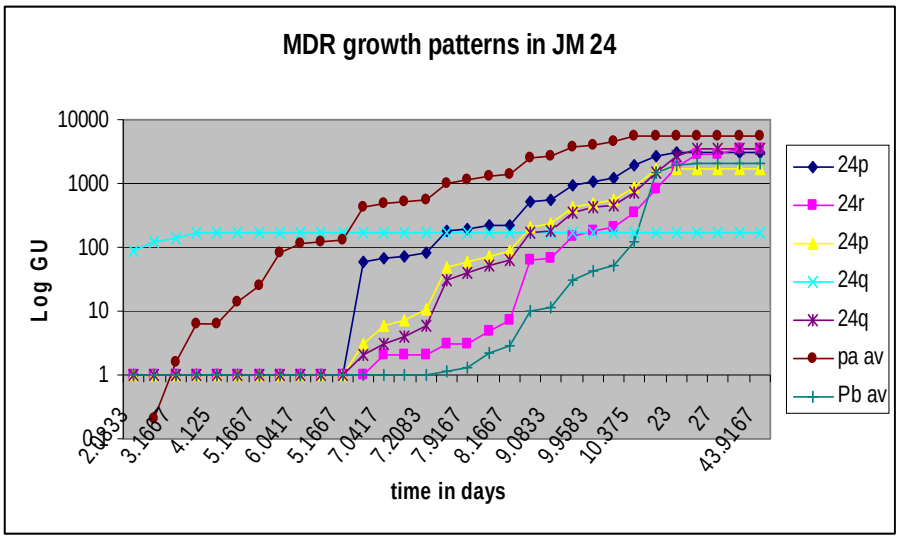

Figure. 51: MDR growth in MGIT tubes containing 24(**108)**

The activity suggested from the patterns is MIC>105 µg/ml. The value assigned is MIC = 110 µg/ml from curve closeness.

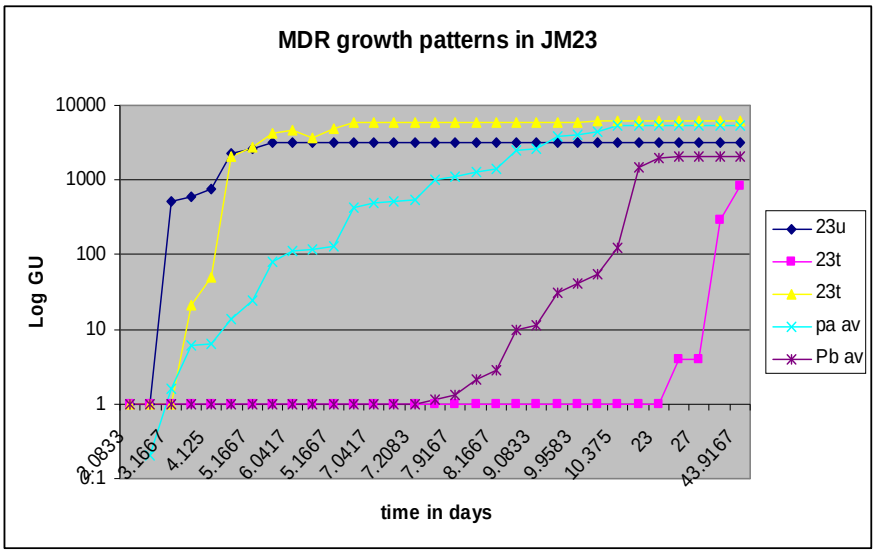

Figure. 52: MDR growth in MGIT tubes containing 23(**117**)

The patterns suggest that MIC<65 µg/ml. The value assigned is MIC = 55 µg/ml from curve closeness

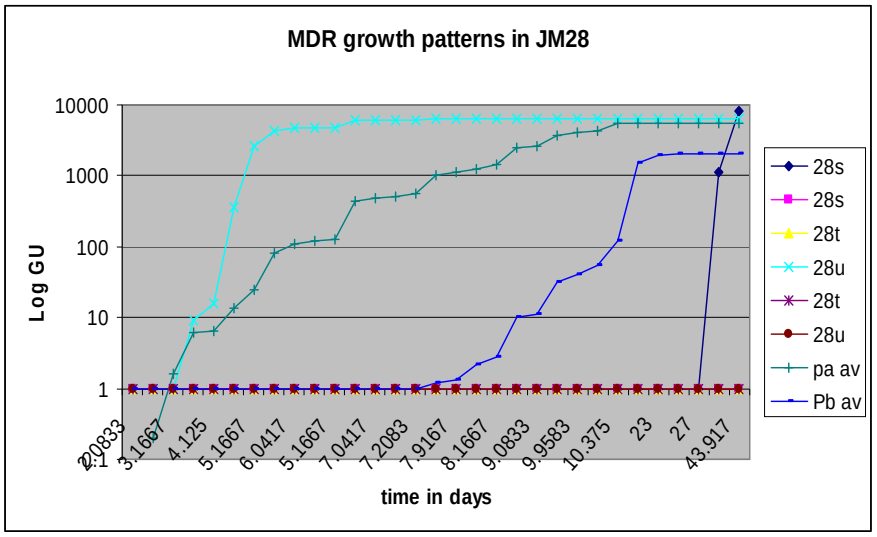

Figure. 53: MDR growth in MGIT tubes containing 28(**121**)
The growth patterns indicate that MIC<35 µg/ml. Consideration of earlier preliminary screening results indicates an activity of 30.9 µg/ml <MIC<35 µg/ml. The value assigned was MIC = 32.9 µg/ml

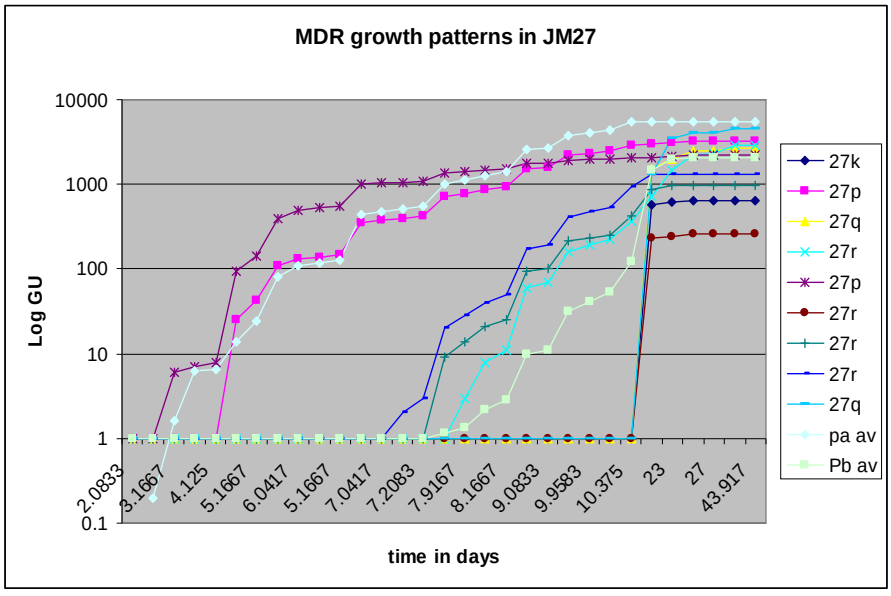

Figure. 54: MDR growth in MGIT tubes containing JM27(**120**).

The patterns indicate that 35 µg/ml <MIC<65 µg/ml. The average activity was calculated as MIC = 50 µg/ml. The patterns also indicate a bacteriostatic characteristic

of the compound on MDR. A similar bacteriostatic activity was observed with the H37Rv strain.

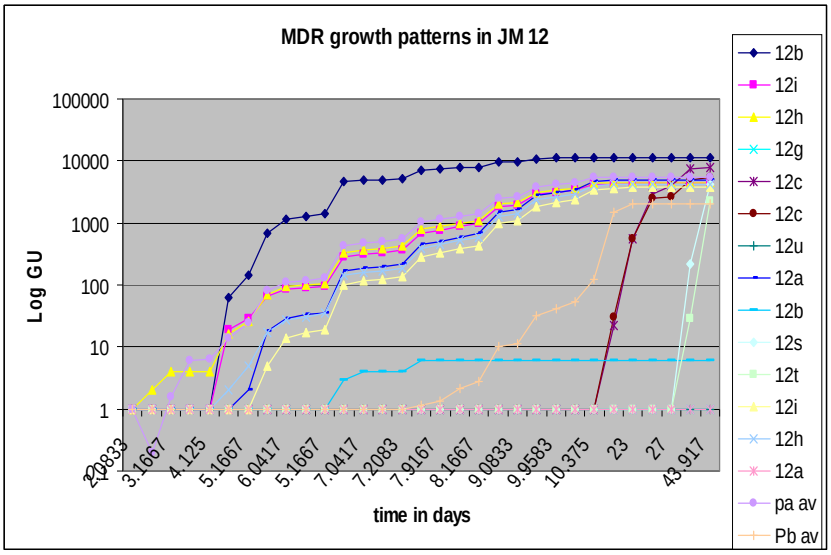

Figure. 55: MDR growth in MGIT tubes containing JM12 (**114**)

The activity suggested by the patterns is 20 µg/ml <MIC<35 µg/ml. The average value was hence calculated as MIC = 27.5 µg/ml

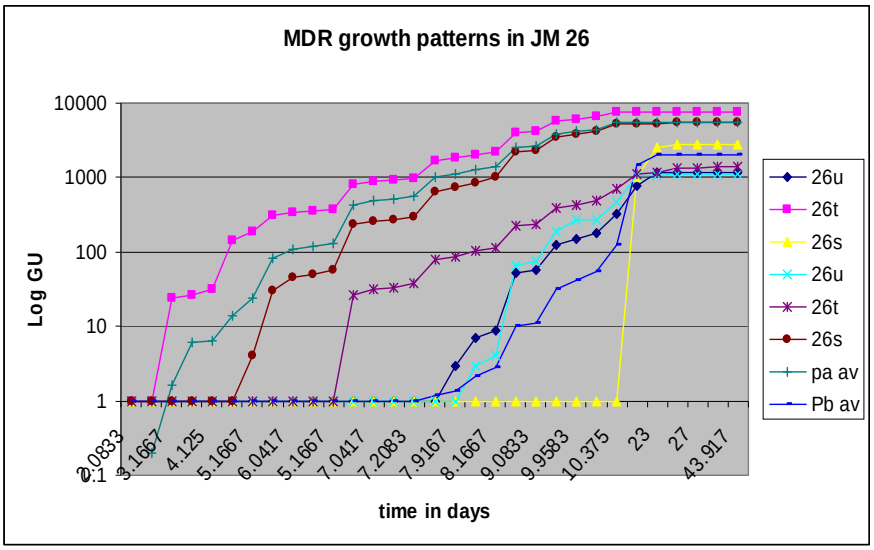

Figure. 56: MDR growth in MGIT tubes containing JM26 (**119**)

The activity suggested by the patterns is MIC>105 µg/ml. The value assigned was

MIC = 110 µg/ml (from relative closeness of curves). A bacteriostatic action on MDR is also suggested by the patterns, just like with the H37Rv strain

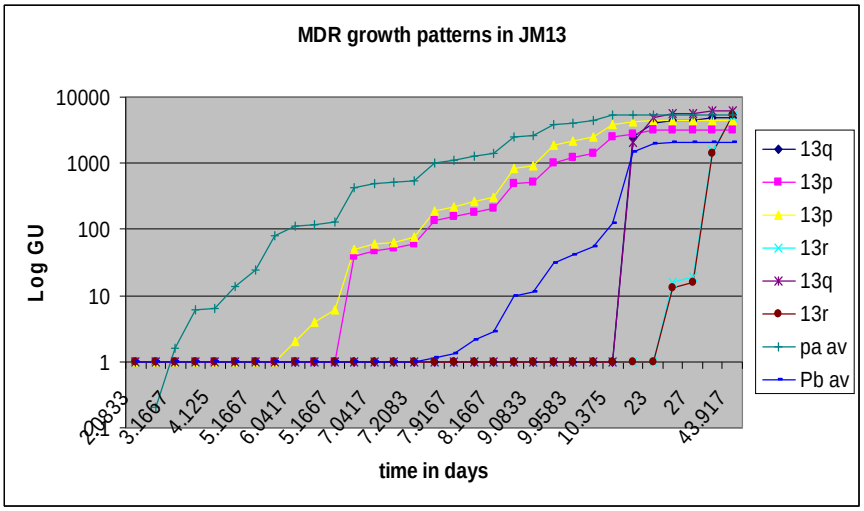

Figure. 57: MDR growth in MGIT tubes containing JM13(**115**)

The patterns indicate that 35 µg/ml <MIC<65 µg/ml. The average activity was calculated as MIC = 50 µg/ml. The patterns also suggest a bacteriostatic activity.

c) **MDR growth patterns in tubes containing conventional drugs**

The labeling and processing of the tubes was done in a similar manner as with the $H_{37}Rv$ inoculated tubes as shown in table 58 below.

Table 60: concentrations of the conventional drugs used in MDR inoculated tubes

Drug set and concentration	Isoniazid(**2**) µg/ml	Streptomycin(**1**) µg/ml	Ethambuto(**4**) µg/ml	Rifampicin(**3**) µg/ml
1	0.9	2.5	5	0.5
2	1.8	3.0	10	1.0
3	3.6	3.5	20	2.5
4			25	5
5				10
6				20

The concentrations used for each drug were guided by the results of the preliminary screening in liquid media. The observed growth patterns are shown in the following figures:-

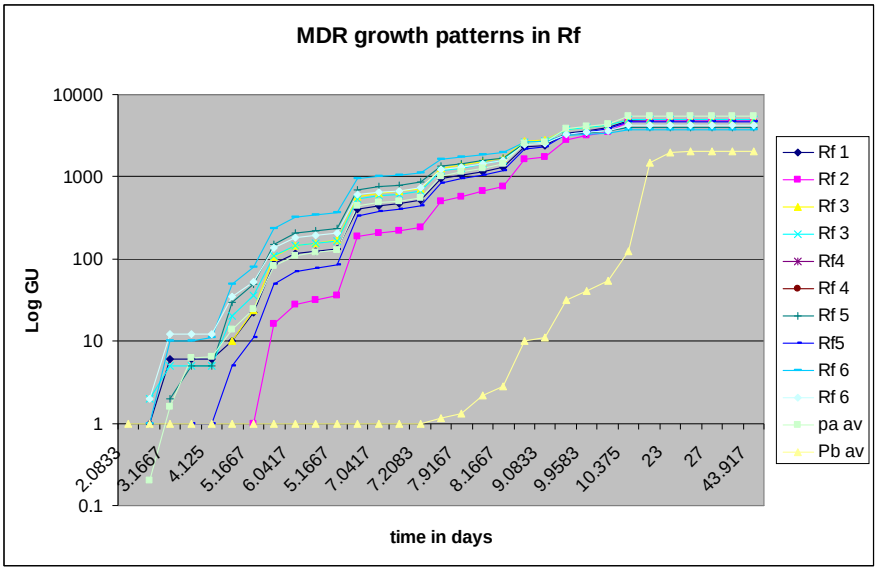

Figure.58: MDR growth patterns in Rifampicin (**3**) containing tubes.

The patterns suggest that MIC>20 µg/ml. Deduced MIC = 30 µg/ml from previous work

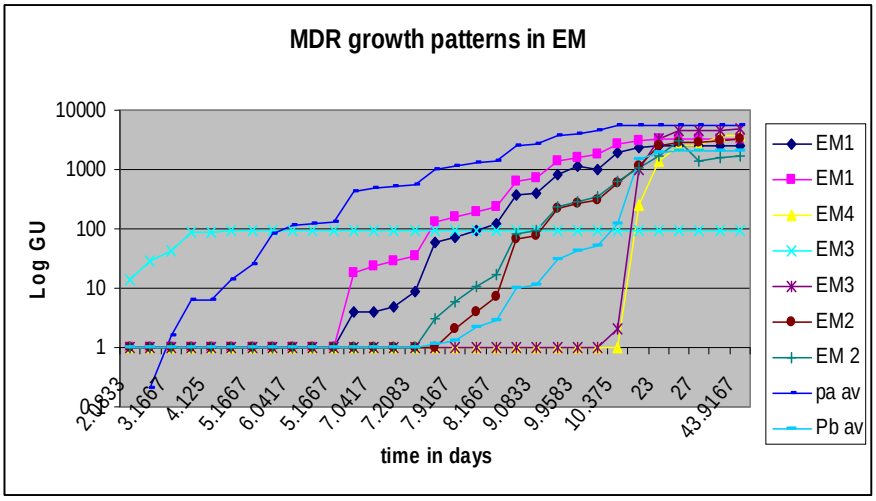

Figure. 59: MDR growth patterns in Ethambutol (**4**) containing tubes

The patterns suggest an activity defined by 10 µg/ml <MIC<15 µg/ml and a value of 12.5 µg/ml was assigned. Bacteriostatic action is evident. One set of tubes (blue EM3) could have been contaminated with aerobic organisms.

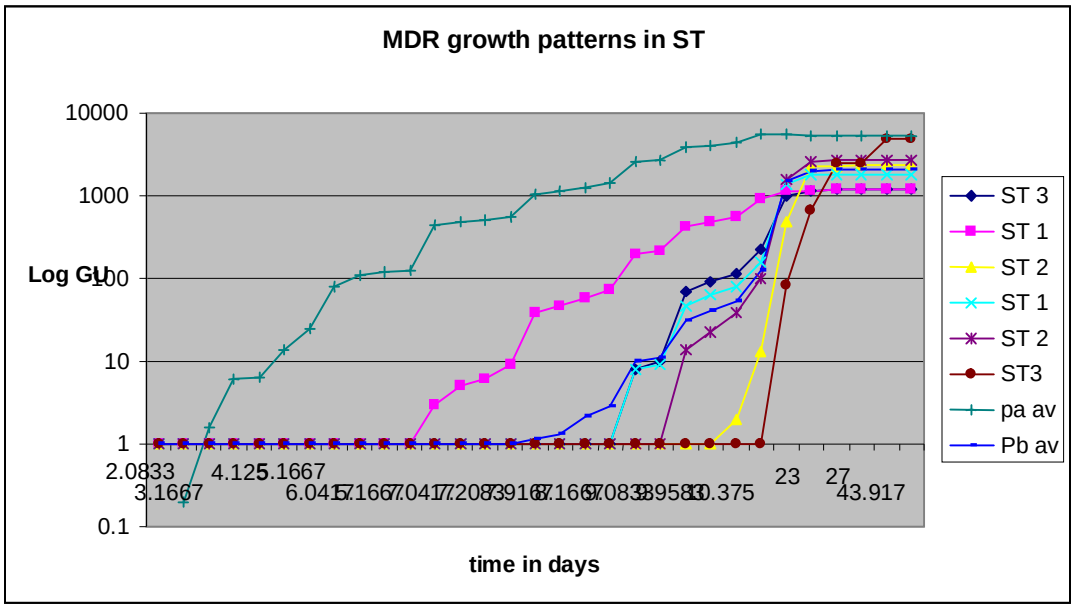

Figure. 60: MDR growth patterns in Streptomycin (**1**) containing tubes.

The patterns suggest an activity of MIC=2.5 µg/ml. Bacteriostatic activity is evident.

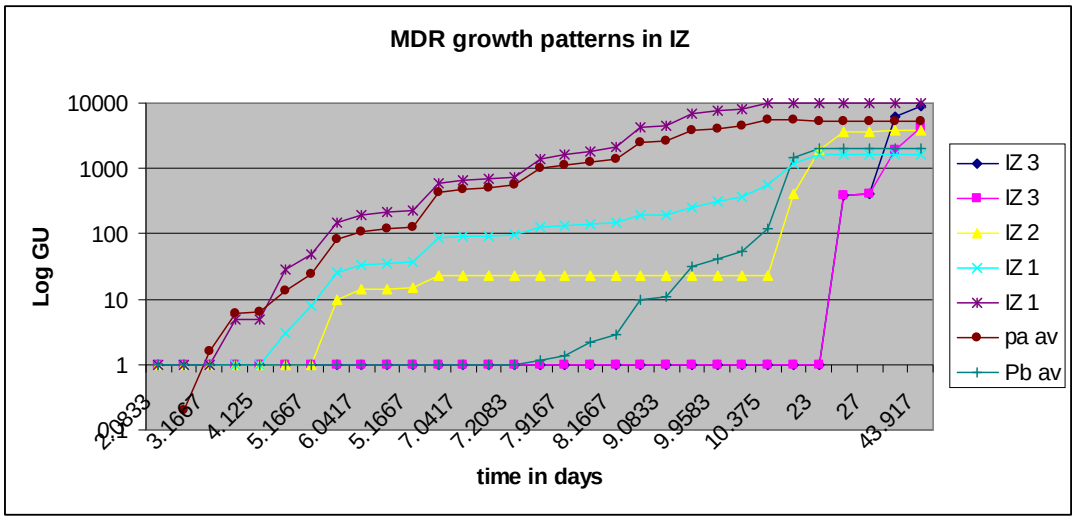

Figure. 61: MDR growth patterns in Isoniazid (**2**) containing tubes

The patterns suggest an activity defined by; 1.8 µg/ml <MIC<3.6 µg/ml. An average value of 2.7µg/ml was hence assigned. Bacteriostatic activity is evident.

Appendices VIII: QSARs generation

A) Benzoquinones series

i) SPSS linear regression analysis for QSAR modeling of MGIT liquid media activity of the benzoquinones generated the following QSARs:-

1. **MIC_{MDR} = 124.542 – 0.632MR (F = 8.481 ; P < 0.012)**
2. **MIC_{MDR} = 124.545 – 0.180Rmm (F = 7.838 ; P < 0.015)**
3. **MIC_{MDR} = 82.02 – 7.401LogP (F = 7.592 ; P < 0.016)**
4. **MIC_{MDR} = 0.563Mpt(K) - 1.721MR – 103.414 (F = 6.241 ; P < 0.014)**
5. **MIC_{MDR} = 0.339Mpt(K) – 99.757 – 15.288LogP (F = 4.346 ; P < 0.015)**
6. **MIC_{MDR} = 211.028 – 246Mpt(K) (F = 4.290 ; P < 0.059)**
7. **MIC_{MDR} = 172.481 – 1.356MR + 8.853LogP (F = 4.093 ; P < 0.044)**

ii) The following SPSS outputs were generated from curve regression analysis

a) LogP based QSARs

```
Independent Variable:   LogP Minimum value:     -1.97
The independent variable contains non-positive values.  Models
LOGARITHMIC
and POWER cannot be calculated.
```

Independent: LogP									
	Dependent	Mth	Rsq	d.f.	F	Sigf	b0	b1	b2	b3
	H37RV MIC	LIN	.247	13	4.27	.059	54.4627	-6.2047		
12	H37RV MIC	LOG								
	H37RV MIC	INV	.014	13	.18	.675	44.2022	-1.8557		
	H37RV MIC	QUA	.288	12	2.42	.131	54.2397	-10.075	.6848	
	H37RV MIC	CUB	.449	11	2.99	.077	37.9656	-14.757	6.6007	-.6294
12	H37RV MIC	POW								
	MDR MIC	LIN	.369	13	7.59	.016	82.0204	-7.4013		
12	MDR MIC	LOG								
	MDR MIC	INV	.019	13	.25	.626	67.1383	2.1055		
	MDR MIC	QUA	.444	12	4.79	.030	81.7229	-12.567	.9140	
	MDR MIC	CUB	.452	11	3.03	.075	78.1458	-13.596	2.2143	-.1383
12	MDR MIC	POW								

Figure. 65 SPSS output on curve regression analysis between LogP and MGIT MICs

Notes:

The only significant QSARs are:-

8. **MIC_{MDR} = 82.0204 – 7.403LogP (F = 7.59 ; P < 0.016)**

9. **MIC_{MDR} = 81.7229 – 12.567LogP + 0.914$LogP^2$ (F = 4.79 ; P < 0.030)**

b) MR based QSARs

Independent: MR									
Dependent	Mth	Rsq	d.f.	F	Sigf	b0	b1	b2	b3
H37RV	MIC	LIN	.258	13	4.51	.053	89.4630	-.5228	
H37RV	MIC	LOG	.303	13	5.65	.034	266.488	-50.561	
H37RV	MIC	INV	.343	13	6.79	.022	-10.493	4156.72	
H37RV	MIC	QUA	.287	12	2.41	.132	128.529	-1.4021	.0044
H37RV	MIC	CUB	.497	11	3.62	.049	530.984	-15.894	.1610 -.0005
H37RV	MIC	POW	.364	13	7.44	.017	33195.0	-1.5942	
MDR	MIC	LIN	.395	13	8.48	.012	124.542	-.6323	
MDR	MIC	LOG	.451	13	10.70	.006	334.907	-60.305	
MDR	MIC	INV	.472	13	11.64	.005	7.0240	4765.42	
MDR	MIC	QUA	.461	12	5.14	.024	182.217	-1.9305	.0064
MDR	MIC	CUB	.472	11	3.28	.062	270.747	-5.1184	.0409 -.0001
MDR	MIC	POW	.402	13	8.73	.011	3625.57	-.9285	

Figure. 66 SPSS output on curve regression analysis between MR and MGIT MICs of benzoquinones

The QSARs that are significant at 95% confidence interval are:-

10. **MIC_{H37Rv} = 266.488 – 50.561LogMR (F = 5.65 ; P < 0.034)**

11. **MIC_{H37Rv} = 4156.72/MR – 10.493(F = 6.79 ; P < 0.022)**

12. **MIC_{H37Rv} = 33195.0 – 1.5942^{MR} (F = 7.44 ; P < 0.017)**

13. **MIC_{MDR} = 124.542 – 0.6323MR (F = 8.48 ; P < 0.012)**

14. **MIC_{MDR} = 334.907 – 60.305LogMR (F = 10.70 ; P < 0.006)**

15. **MIC_{MDR} = 7.0240 + 4765.42/MR (F = 11.64 ; P < 0.005)**

16. **MIC_{MDR} = 182.217 – 1.9305MR + 0.0064MR^2 (F = 5.14 ; P < 0.024)**

c) Cv based QSARs

Independent: Cv								
Dependent	Mth	Rsq	d.f.	F	Sigf	b0	b1	b2	b3
H37RV MIC	LIN	.255	13	4.46	.055	85.2041	-.0428		
H37RV MIC	LOG	.307	13	5.76	.032	355.103	-45.819		
H37RV MIC	INV	.353	13	7.09	.020	-5.1620	40237.4		
H37RV MIC	QUA	.285	12	2.39	.133	117.947	-.1101	3.0E-05	
H37RV MIC	CUB	.483	11	3.42	.056	397.978	-1.0428	.0009	-3.E-07
H37RV MIC	POW	.361	13	7.35	.018	487616	-1.4290		
MDR MIC	LIN	.393	13	8.41	.012	119.479	-.0519		
MDR MIC	LOG	.455	13	10.85	.006	439.385	-54.471		
MDR MIC	INV	.474	13	11.70	.005	13.8391	45542.7		
MDR MIC	QUA	.461	12	5.14	.024	167.873	-.1512	4.4E-05	
MDR MIC	CUB	.472	11	3.28	.063	231.516	-.3632	.0003	-6.E-08
MDR MIC	POW	.401	13	8.71	.011	17659.5	-.8350		

Figure. 67 SPSS output on curve regression analysis between Cv and MGIT MICs of benzoquinones

Significant QSARs from this regression modeling of Cv with activity are:-

17. $\mathbf{MIC_{H37Rv} = 355.103 - 45.819LogCv}$ **(F = 5.76 ; P < 0.032)**

18. $\mathbf{MIC_{H37Rv} = 402337.4/Cv - 5.1620}$ **(F = 7.09 ; P < 0.020)**

19. $\mathbf{MIC_{H37Rv} = 487616 - Cv^{1.4290}}$ **(F = 7.35 ; P < 0.018)**

20. $\mathbf{MIC_{MDR} = 119.479 - 0.0519Cv}$ **(F = 8.41 ; P < 0.012)**

21. $\mathbf{MIC_{MDR} = 439.385 - 54.471LogCv}$ **(F = 10.85 ; P < 0.006)**

22. $\mathbf{MIC_{MDR} = 13.8391 + 45542.7/Cv}$ **(F = 11.70 ; P < 0.005)**

23. $\mathbf{MIC_{MDR} = 17659.5 - Cv^{0.8350}}$ **(F = 8.71 ; P < 0.011)**

d) RMM based QSARs

Independent: RMM									
Dependent	Mth	Rsq	d.f.	F	Sigf	b0	b1	b2	b3
H37RV MIC	LIN	.249	13	4.31	.058	89.7904	-.1502		
H37RV MIC	LOG	.292	13	5.36	.038	344.494	-53.071		
H37RV MIC	INV	.330	13	6.40	.025	-15.140	16096.4		
H37RV MIC	QUA	.281	12	2.34	.139	135.325	-.4318	.0004	
H37RV MIC	CUB	.411	11	2.56	.108	465.564	-3.6779	.0101	-9.E-06
H37RV MIC	POW	.355	13	7.17	.019	414401	-1.6848		
MDR MIC	LIN	.376	13	7.84	.015	124.545	-.1804		
MDR MIC	LOG	.440	13	10.21	.007	430.027	-63.666		
MDR MIC	INV	.472	13	11.62	.005	.4029	18811.2		
MDR MIC	QUA	.462	12	5.15	.024	197.792	-.6334	.0006	
MDR MIC	CUB	.470	11	3.25	.064	277.405	-1.4160	.0030	-2.E-06
MDR MIC	POW	.391	13	8.33	.013	15584.7	-.9792		

Figure. 68 SPSS output on curve regression analysis between RMM and MGIT MICs of benzoquinones.

The significant QSARs ($p \leq 0.05$) are:-

24. $\mathbf{MIC_{H37Rv} = 344.494 - 53.071LogRmm}$ **(r^2 = 0.292 ; F = 5.36 ; P < 0.038)**

25. **$MIC_{H37Rv} = 16096.4/Rmm – 15.14$ ($r^2 = 0.330$; F = 6.4 ; P < 0.025)**

26. **$MIC_{H37Rv} = 414401 – Rmm^{1.6848}$ ($r^2 = 0.355$; F = 7.17 ; P < 0.019)**

27. **$MIC_{MDR} = 124.545 – 0.1804Rmm$ ($r^2 = 0.376$; F = 7.84 ; P < 0.015)**

28. **$MIC_{MDR} = 430.027 – 63.666LogRmm$ ($r^2 = 0.440$; F = 10.21 ; P < 0.007)**

29. **$MIC_{MDR} = 0.4029 + 18811.2/Rmm$ ($r^2 = 0.472$; F = 11.62 ; P < 0.005)**

30. **$MIC_{MDR} = 197.792 – 0.6334Rmm + 0.0006Rmm^2$ ($r^2 = 0.462$; F = 5.15 ; P < 0.024)**

31. **$MIC_{MDR} = 15584.7 – Rmm^{0.9792}$ ($r^2 = 0.391$; F = 8.33 ; P < 0.013)**

B) **Flavanone series**

a) LogP based QSARs

Independent: Log P

Dependent	Mth	Rsq	d.f.	F	Sigf	b0	b1	b2	b3
MIC aq	LIN	.306	3	1.32	.333	26.5424	17.7914		
MIC aq	LOG	.297	3	1.27	.342	44.2454	24.8289		
MIC aq	INV	.285	3	1.20	.354	77.1118	-32.866		
MIC aq	QUA	.311	2	.45	.689	44.9410	-9.5365	9.2276	
9 MIC aq	CUB	.311	2	.45	.689	40.4655	2.7538	1.4093	
MIC dm	LIN	.832	3	14.89	.031	-15.848	45.7776		
MIC dm	LOG	.858	3	18.18	.024	29.2781	65.8527		
MIC dm	INV	.873	3	20.54	.020	118.603	-89.743		
MIC dm	QUA	.873	2	6.90	.127	-96.765	165.966	-40.583	
9 MIC dm	CUB	.873	2	6.90	.127	-96.765	165.966	-40.583	

Notes:
9 Tolerance limits reached; some dependent variables were not entered.

Figure. 71 SPSS output on curve regression analysis between LogP and LJ MICs of flavanones.

The significant QSARs ($p \leq 0.05$) are

32. **MIC dm = 29.278 + 65.853 * Log (Log P) (F = 18.18; P < 0.024)**

33. **MIC dm = 118.603 – 89.743/LogP (F = 20.54; P < 0.020)**

34. **MIC dm = 45.778*LogP – 15.848 (F = 14.89; P < 0.031)**

b) Mpt based QSARs

Independent: Mpt								
Dependent	Mth	Rsq	d.f.		F	Sigf	b0	b1
b2	b3							
MICaq	LIN	.164	3	.59	.499	102.296	-.0671	
MICaq	LOG	.181	3	.66	.475	411.498	-54.336	
MICaq	INV	.197	3	.74	.454	-5.9410	43139.8	
MICaq	QUA	.291	2	.41	.709	477.546	-1.0420	.0006
9 MICaq	CUB	.292	2	.41	.708	353.486	-.5570	
2.7E-07								
MICaq	POW	.113	3	.38	.580	50347.2	-1.0460	
MICdm	LIN	.863	3	18.97	.022	232.399	-.2407	
MICdm	LOG	.857	3	17.95	.024	1273.02	-184.61	
MICdm	INV	.844	3	16.24	.027	-135.99	139398	
MICdm	QUA	.864	2	6.37	.136	187.040	-.1229	-8.E-05
9 MICdm	CUB	.864	2	6.37	.136	187.040	-.1229	-8.E-05
MICdm	POW	.820	3	13.70	.034	4.6E+15	-4.8694	

Notes:
9 Tolerance limits reached; some dependent variables were not entered.

Figure. 72 SPSS output on curve regression analysis between MPt and LJ MICs of flavanones

35. **MIC dm = 139397.793/Mpt – 135.9897 (F=16.24; P< 0.027)**

36. **MIC dm = 1273.021- 184.606*Log(Mpt) (F = 17.95; P < 0.024)**

37. **MIC dm = 232.399 – 0.2407* Mpt (F = 18.97; P< 0.022)**

Appendix IX: Table91: Non parametric correlations of predicted MGIT liquid media MIC values of eight untested benzoquinones

Correlations

			LogP MIC QSAR 3or8	MR MIC-QSAR 1or 13	Rmm MIC QSAR 27	Rmm MIC QSAR 29	Cv MIC QSAR 20	QSR-7 MIC	QSR-16 MIC	QSR - 5 MIC	QSR-9 MIC	QSR 30 MIC	QSR-4 MIC
Spearman's rho	LogP MIC QSAR 3 or 8	Correlation Coefficient	1.000	-.571	-.611	-.611	-.595	**-.857****	**.786***	**.929****	**-.994****	.611	-.167
		Sig. (1-tailed)	.	.069	.054	.054	.060	.003	.010	.000	.000	.054	.347
		N	8	8	8	8	8	8	8	8	8	8	8
	MR MIC QSAR 27	Correlation Coefficient	-.571	1.000	.275	.275	.262	.619	**-.643***	-.500	.563	-.275	.571
		Sig. (1-tailed)	.069	.	.255	.255	.265	.051	.043	.104	.073	.255	.069
		N	8	8	8	8	8	8	8	8	8	8	8
	Rmm MIC QSAR 27	Correlation Coefficient	-.611	.275	1.000	**1.000****	**.994****	**.802****	**-.874****	**-.707***	.614	**-1.000****	-.048
		Sig. (1-tailed)	.054	.255	.	.	.000	.008	.002	.025	.053	.	.455
		N	8	8	8	8	8	8	8	8	8	8	8
	Rmm MIC QSAR 29	Correlation Coefficient	-.611	.275	**1.000****	1.000	**.994****	**.802****	**-.874****	**-.707***	.614	**-1.000****	-.048
		Sig. (1-tailed)	.054	.255	.	.	.000	.008	.002	.025	.053	.	.455
		N	8	8	8	8	8	8	8	8	8	8	8
	Cv MIC QSAR 20	Correlation Coefficient	-.595	.262	**.994****	**.994****	1.000	**.786***	**-.857****	**-.714***	.611	**-.994****	-.071
		Sig. (1-tailed)	.060	.265	.000	.000	.	.010	.003	.023	.054	.000	.433
		N	8	8	8	8	8	8	8	8	8	8	8
	QSR-7 MIC	Correlation Coefficient	**-.857****	.619	**.802****	**.802****	**.786***	1.000	**-.976****	**-.929****	**.850****	**-.802****	.357
		Sig. (1-tailed)	.003	.051	.008	.008	.010	.	.000	.000	.004	.008	.193
		N	8	8	8	8	8	8	8	8	8	8	8
	QSR-16 MIC	Correlation Coefficient	**.786***	**-.643***	**-.874****	**-.874****	**-.857****	**-.976****	1.000	**.857****	**-.778***	**.874****	-.333
		Sig. (1-tailed)	.010	.043	.002	.002	.003	.000	.	.003	.011	.002	.210
		N	8	8	8	8	8	8	8	8	8	8	8

	QSR -5 MIC	Correlation Coefficient	**.929****	-.500	**-.707***	**-.707***	**-.714***	**-.929****	**.857****	1.000	**-.946****	**.707***	-.238
		Sig. (1-tailed)	.000	.104	.025	.025	.023	.000	.003	.	.000	.025	.285
		N	8	8	8	8	8	8	8	8	8	8	8
	QSR-9 MIC	Correlation Coefficient	**-.994****	.563	.614	.614	.611	**.850****	**-.778***	**-.946****	1.000	-.614	.144
		Sig. (1-tailed)	.000	.073	.053	.053	.054	.004	.011	.000	.	.053	.367
		N	8	8	8	8	8	8	8	8	8	8	8
	QSR 30 MIC	Correlation Coefficient	.611	-.275	**-1.000****	**-1.000****	**-.994****	**-.802****	**.874****	**.707***	-.614	1.000	.048
		Sig. (1-tailed)	.054	.255	.	.	.000	.008	.002	.025	.053	.	.455
		N	8	8	8	8	8	8	8	8	8	8	8
	QSR-4 MIC	Correlation Coefficient	-.167	.571	-.048	-.048	-.071	.357	-.333	-.238	.144	.048	1.000
		Sig. (1-tailed)	.347	.069	.455	.455	.433	.193	.210	.285	.367	.455	.
		N	8	8	8	8	8	8	8	8	8	8	8
**. Correlation is significant at the 0.01 level (1-tailed).													
*. Correlation is significant at the 0.05 level (1-tailed).													

Significant correlations are highlighted in bold blue fonts.