

**EVALUATION OF SYNERGISTIC EFFECT OF EFFLUX PUMP INHIBITORS WITH
ANTI-TB DRUGS IN MULTIDRUG-RESISTANT MYCOBACTERIUM
TUBERCULOSIS STRAINS**

PROJECT THESIS

Submitted in partial fulfillment of the requirements for the award of

Bachelor of Technology degree in Biotechnology

By

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ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to **the Board of Management of Sathyabama Institute of Science and Technology** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. Ramesh Kumar**, Head of the Department, Department of Biotechnology for providing me with the necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my Internal Project Guide **Dr. R. Thyagarajan**, for his constant support and encouragement during the advancement of my project work.

I am extremely grateful to my External Guide, **Dr. V.N. Azger Dusthacker**, for enabling me to work on my dissertation at ICMR-NIRT and for his valuable guidance, suggestions, and constant encouragement which actually paved way for the successful completion of my Dissertation/Project work.

I also express my deep gratitude to **Dr. R. Dinakaran Michael**, former Dean of Life Sciences, Vels University and **Dr. Ganesh Munuswamy-Ramanujam**, Assistant Professor at SRMIST Kattankulathur for helping me acquire essentials that were used in this project.

I wish to express my thanks to all **Teaching and Non-teaching staff members** of the Department of Biotechnology and Department of Bacteriology, ICMR-NIRT, who were helpful in many ways for the completion of my project.

ABSTRACT

Tuberculosis (TB) remains to be a health challenge that has been the root cause of millions of deaths each year. Even though an immense amount of research has been done towards eradicating the disease, the formation of multi-drug resistant (MDR) strains of *Mycobacterium tuberculosis* (M.TB) stands as a major hurdle in the action of first-line drugs on said M.TB strains.

One method through which MDR strains resist against TB drugs is using efflux pumps. These pumps are used to eject the drugs before they can have a therapeutic effect on M.TB. Thus, the recent identification of efflux pump inhibitors (EPI) is a major breakthrough that needs to be further analyzed. Hence, this project has been undertaken to experimentally analyze the clinical advantages of using EPI and first-line TB drugs in combination in infected THP-1 monocyte cells.

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i. ABBREVIATIONS

M.TB	<i>Mycobacterium tuberculosis</i>
NTM	Non-Tuberculosis Mycobacterium
TB	Tuberculosis
EP	Efflux Pump(s)
EPI	Efflux Pump Inhibitor(s)
MIC	Minimum Inhibitory Concentration
ADME	Adsorption, Distribution, Metabolism, Excretion
Mi	Myoinositol
RIF	Rifampicin
MS	Methyl Stearate
PA	Palmitic Acid
DA	Dodecanoic Acid
MFS	Major Facilitator Superfamily
ABC	ATP-Binding Cassette
SMR	Small Multidrug Resistance
RND	Resistance Nodulation Division
MATE	Multi-Antimicrobial Extrusion
OADC	Oleic Albumin Dextrose Catalase
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
LJ	Lowenstein Jensen
MF	McFarland
PMA	Phorbol 12-myristate 13-acetate

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CHAPTER 1

INTRODUCTION

1.1 History of Tuberculosis

The emergence and widespread mayhem caused by bacteria poses a formidable challenge to modern medicine, threatening the effectiveness of our most potent antimicrobial agents. In 2016, one-fourth of the world population remained infected with TB. The annual incidence was 10.4 million, with an annual mortality rate of 1.3 million. Ten percent of the annual incidence, be it dormant, or latent, TB group will develop acute TB.[2] In contrast, in the United States in 2016, there were only 9287 new TB cases and a 2.9 in 100,000 case rate. The history of TB has been traced to the Stone Age Paleolithic period, circa 3.3 million years ago.[55]

It reached epidemic levels in Europe and North America in the eighteenth and nineteenth centuries. TB has decreased during the twentieth century in developed countries but remains a serious threat in low-middle income countries (LMICs) emerging economies (EEs) especially with the emergence of drug-resistant strains and the increasing association with human immunodeficiency virus (HIV). It is important to understand the underlying social and economic aspects of human development. Epidemiologic transition serves as a basis to understand the evolution of diseases, especially TB. Researchers highlight the epidemiologic transitions of Omran who identified four stages of human development: stage 1, the age of pestilence and famine; stage 2, the age of receding pandemics; stage 3, the age of degenerative and manmade diseases; and stage 4, the age of delayed degenerative diseases. [1][60] The current age of commerce has created more socialization and human interaction, along with urbanization and the increased population growth and crowding, thus increasing the risk for disease transmission, especially for TB. TB has been involved in all the stages, either in a dormant or latent form, or an active form throughout the ages, yet has been more active and apparent during the periods of industrialization, migration, famine, poverty, and in areas of increased population density.[2][61]

Globally, TB remains the ninth leading cause of global deaths, and the leading cause of a single infectious agent. In addition, in 2016, there were an additional 374,000 deaths among HIV-positive people.[27] An estimated 10.4 million people fell ill with TB in 2016, and 56% of the total were living in five countries: India, Indonesia, China, Philippines, and Pakistan. Associated TB with HIV, as well as TB drug resistance remains a

continuing threat and challenge. There were 600,000 new cases with resistance to rifampicin (RRTB), the most effective first-line drug, of which 490,000 were multidrug-resistant TB (MDR-TB), and almost half of these cases were in India, China, and Russia.[48][3]

1.2 Ancient Methodologies of Identifying and Treating Tuberculosis

There is a paucity of credible documented scientific literature sources regarding the discovery and description of TB during the ancient period, Middle Ages, and Renaissance that has given factual insight into the causation or natural history of TB. Fear and acceptance dominated this period. The recorded literature concentrated on the bizarre and lethal occurrences that affected all levels of society. Many lingering disagreements and debates centered around hereditary and acquired contagion causation. [53]

Prevailing medical evaluation and treatment cures were still influenced by the Hippocrates and Galen humors theories. Both believed that TB (phthisis) was the most widespread of all the diseases of that time. Hippocrates believed that tuberculosis was hereditary, whereas Galen believed the disease was essentially contagious. Aretaeus of Cappadocia [40] defined the cause of the disease in which phthisis was a pulmonary abscess that produced cough, fever, increased expectoration, and emaciation. A number of terms reflected the effects of TB. Consumption was the translation of a Sanskrit word (1000 BC) for a wasting disease. Hippocrates coined the word phthisis (decaying) to describe the disease. Scars from TB cervical lymph nodes were called scrofula. They were called “Kings Evil” by the king, and it was widely believed that the kings of England and France could cure scrofula by touching those affected, as well as relieving symptoms like hacking cough, bloody cough, miasmas, and fatigue. Another example of folk theory was advanced in 1530 by Paracelsus. He believed that tuberculosis was caused by a failure of an internal organ to accomplish its Alchemical duties. When this occurred in the lungs, stony precipitates would develop causing tuberculosis in what he called the tartaric process.

Folk remedies were widespread. Galen recommended fresh air, human breast milk, wolf livers, and phlebotomy, as well as diet, exercise for children, rest for adults, and sunlight (ultraviolet light or heliotherapy). A bevy of preventive methods with plausible effects included the following: no spitting, the use of spittoons (pinger was the sound of wet tobacco when it hit the spittoon); house cleaning with antiseptic carbolic acid; avoiding women's trailing skirts; and

not sweeping dry floors, thus avoiding contaminated dust inhalation. Other preventive or palliative measures without proven efficacy included foods like plantain, nuts, honey, and pomegranates; cod liver oil; and medicinal, like hyssop, eucalyptus, opioids, and moxibustion mugwort burned on the skin. During the eighteenth and nineteenth centuries, the tuberculosis debate between hereditary versus contagious causation continued.

By the end of the nineteenth century, with the increasing population growth in urban areas, 70% to 90% of the populations of Europe and North America were infected with TB, and more than 80% of those with active tuberculosis died.[44] In Europe, the annual mortality rate for TB was 800 to 1000 per 100,000 people. The poorly ventilated and congested urban dwellings, along with primitive sanitation, hygiene, and poverty, all contributed to the increased TB morbidity and mortality. In addition, increasing migration, expanding trade routes, and the onset of the industrial revolution prolonged the phenomenon of this great white plague. Other concentrated areas for increased TB activity included shelters and prisons.

The feelings of the victims were expressed in their writings and works. Ralph Waldo Emerson, the American essayist called his consumptive partner "too lovely to live long." The paintings of Dante Gabriel Rossetti were filled with pale, thin girls with their hair cascading over their shoulders. Even now, in the late twentieth century, the singer Van Morrison dedicated a beautiful song, "TB Sheets" to a dying friend. In the early twentieth century, notable thoracic surgeons were also infected with TB. Examples included Edward Archibald and Norman Bethune from Canada, and John Alexander and Alfred Blalock from the United States. Not surprisingly, they all played an active role in developing TB surgical procedures, even as they dealt with their own TB illness. Specific scientific progress slowly evolved over the ensuing years, especially in Europe. In 1546, Hieronymus Fracastorius described contagion theory. This increased the search for the cause and spread of this dreadful illness. In 1679, Franciscus Sylvius described the progress of the disease in his *Opera Medica*. Using chemical principles, he highlighted the TB nodules as tubercula. In 1790, Benjamin Martin believed the disease was caused by heredity but was possibly acquired and infectious. In 1720, Benjamin Martin, in *A New Theory of Consumption*, hypothesized that TB resulted from the actions of "wonderfully Minute living creatures." Once established in the body, they would generate symptoms of consumption. In 1816, Rene Theophile Laennec invented the stethoscope, as well as increasing the understanding of the pathogenesis of TB in *Traite du Diagnostic des Maladies*. He studied

the pathogenesis of tuberculosis in detail. In 1819, he combined the pulmonary and extrapulmonary manifestations of TB into one common understanding and pathway. This improved the diagnostic capability. In 1839, Johann Lucas Schoenlein declared the TB tubercle was the basis of the disease. In 1865, Jeon-Antoine VilleMin showed that TB was both infectious and transmittable; that is, contagion. He demonstrated that one could infect a healthy rabbit with tuberculosis from infected cadaver tissue. This was a breakthrough, because medical theory at that time held that each case of consumption arose spontaneously in predisposed people. [39]

1.3 The Beginning of The End of TB

The real battle against TB began with Robert Koch's infamous staining technique which is still in use today. In 1882, Robert Koch used a new staining method of the sputum from TB patients. This established *M. tuberculosis* bacteria (tubercle bacillus) as the etiology and cause of clinical disease. The Ziehl-Nielsen Microscopic acid-fast stain was described by the bacteriologist Franz Ziehl (1859–1926) and the pathologist Friedrich Nelsen (1854–1898).

Using the microscope, Koch showed that the thick cover of the organism's protein made it difficult to visualize unless a specific spot of oxide of the Zeihl-Neelson oxide stain was removed. The bacteria were termed the Koch bacillus and because it took red acid dye, the red rods were called acid-fast bacilli or red snappers. Later investigations proved that air and secretions expelled from consumptive lungs contained live bacteria. These were the defining moments in the history of TB. Koch was awarded the Nobel Prize in 1905 for this seminal accomplishment.[4] Koch also developed a glycerin extract of the tubercle bacilli as a remedy for tuberculosis in 1890, calling it "tuberculin," yet it was not effective, especially for children, and was terminated. In 1895, Wilhelm Konrad Roentgen developed the radiograph, which advanced the diagnoses of TB. The chest radiograph became a standard diagnostic tool that revealed tubercular pulmonary lesions, even before any clinical symptoms.

1.4 Evolution of Treatment of Tuberculosis

1.4.1 Sanatorium

In Europe, the first sanatorium was founded in 1854 by Hermann Brehmer in Germany, in Goebersdorf in Silesia, a village on the border between Poland and the Czech Republic. Brehmer stressed the therapeutic effect of the climate in the treatment of phthisis. His method of treatment spread throughout Germany, France, and Switzerland, especially in the region of Davos, in the Engadin Valley, where the first high-altitude sanatoria were opened for paying patients.

The new sanatoria were designed in such a way that patients could stay in single rooms or rooms with a few beds, which usually gave onto large terraces where inmates could take so-called “sun baths.” The buildings were surrounded by spacious meadows and tree-lined gardens, where guests could go for long healthy walks. Patients stayed in the institution for very long periods – from a minimum of six months to seven, eight or even 16 years.

In Italy, Biagio Castaldi, who was himself affected by pulmonary tuberculosis, personally experienced the positive effects of a balanced diet and a stay in the mountains at the beginning of the 20th century, supporting the theories widely held in the rest of Europe at that time. In an 1858 publication, Castaldi had first reported that the incidence of phthisis declined as altitude increased, and that the disease was very rare at altitudes above 1000 m.

1.4.2 Surgery

Thoracic surgery advanced with the discovery of the radiograph in 1895. This allowed an anatomic view of the TB pathology within the chest cavity. Then the development and severity of TB could be objectively evaluated. [5][6] However, thoracic surgery at the end of the nineteenth century was somewhat limited because surgical entry to the chest cavity beyond the chest pleura was not advised or advocated for fear that opening the pleura would compromise the patient’s negative lung pressure. This changed with the advances in anesthesia and positive airway control and management. [7][28] It is ironic that the goal changed from external fresh air to the lungs in the sanatorium era to relaxing the lung and suppressing oxygen entry with surgical collapse and resection techniques. Both the sanatorium and surgery approach complemented each other in the early stages, because many sanatorium patients went

on to have surgery with increasing successful outcomes.

In 1821, the concept of collapse was originally advanced by Dr. James Carson. There was a long lapse before the collapse concept was further developed. Collapse surgery with the artificial pneumothorax procedure using a pneumothorax apparatus, was initiated by Carlo Forlanini in 1882. [8][30] The rationale was that the affected lung would collapse, thus allowing the lung to “rest” and heal. However, the pneumothorax was limited to the upper lung TB cavities, yet it remained popular for many years. [9][42] The procedure involved inserting a long needle between the patient’s ribs and pumping the chest cavity with filtered air to a goal of zero pressure in the created space. This allowed the diseased lung to rest because the lung could not heal under the constant irritation of inflation and deflation. This technique required “multiple refills” over several years. [10][32] In addition, incomplete collapse, secondary to residual adhesions, restricted total collapse.

Jacobaeus, in 1912, introduced the thoroscopic closed intrapleural pneumolysis procedure utilizing a galvanocautery. This was the early forerunner of the current video-assisted thoracoscopic surgery procedure that decreased the blind finger method. [11] This approach was well received because it was less invasive and more effective in patients in whom adhesions prevented a satisfactory result by the open technique. As noted, artificial pneumothorax was used primarily in patients with TB cavitory disease. It was postulated that collapse would limit disease spread by inducing fibrosis and encapsulation of the infected area. More likely, by impairing ventilation to the treated lung, and collapsing the cavity within, it reduced oxygen levels and inhibited growth of the oxygen-dependent tubercle bacillus. [12][45] Because air injected during pneumothorax therapy was resorbed after 1 to 2 weeks, recurrent injections were required (usually repeated over 1–2 years). The risk of pleuritis often prevented maintenance of the collapse. Other complications included infection and bleeding into the pleural space. Further advances with collapse therapy continued, as these early approaches were not very effective over the long term, especially with lower lung and bilateral cavities. For lower-lobe cavities, other less invasive methods were performed. Pneumoperitoneum, in which air was injected into the abdominal cavity, resulting in diaphragmatic elevation or, with phrenic nerve crush, where the diaphragm it innervated was temporarily paralyzed, were both marginally effective, but not difficult to perform, and often used together.[13] [14]

1.4.3 Medical Treatment

The major therapeutic modality in the early to middle twentieth century continued to be the sanatorium and surgical treatment. Thereafter, with the development of antibiotics, between 1944 and the early 1950s, surgery declined, as well as sanatoriums. Adequate treatment of TB required a reliable correct diagnosis, as noted with a positive skin test (PPD), positive sputum acid-fast stain (Ziehl-Neelsen) test (number of red snappers per HPF), or the auramine fluorescein stain, and a positive Lowenstein-Jenson medium culture. Other current diagnostic tests included IGRAS-Quanti FERON TB Gold test, and T-Spot TB test. Following the principles of Louis Pasteur, in 1880, with attenuating the virulence of a living microbe, a vaccine produced with *Mycobacteria bovis* was developed in 1908 by Albert Calmette and Camille Guérin. They grew the Koch bacillus in several media to decrease its virulence and to increase immunity. The Bacille Calmette Guérin (BCG) vaccine was first used in humans in 1921 and became widely used primarily for children. Currently, there remains no universal effective vaccine for TB. [41][49]

Casadevall in 2017, discussed the role of vaccines in cell-mediated responses to TB. He postulated that latent TB is more effective in protection and inhibition of the growth of *M tuberculosis* in vitro than active TB. This implies that newer vaccines that promote latency may be more beneficial in infection control.[24] The timeline for the antibiotic development for bronchopleural fistula TB started in 1935 when Gerhard Domagk published a report on the use of "Prontosil," an organic compound containing sulfur (Sulfanilamide), yet it was deemed too toxic for humans. In 1944, a major advance occurred with the discovery of streptomycin (*Streptomyces griseus*) by Schatz, Bugie, and Waksman. It was limited by the early development of resistance. In 1945, para-amino salicylic acid (PAS) was discovered. When combined with streptomycin, it decreased drug resistance. [59]

Following streptomycin, isoniazid (1952), pyrazinamide (1954), ethambutol (1962), and rifampin (1963) were introduced. Treatment strategies beyond the drugs depended on prevention, education, social aspects, and compliance, along with access to care, availability, and surveillance. In the 1970s, the directly observed treatment, short-course (DOTS) strategy was recommended by the World Health Organization (WHO). In 1995, the WHO adopted the DOTS that focused on cure rates and limiting the spread of drug resistance. Subsequent

trials found significant differences in cure rates or treatment completion between DOTS.

Efflux pump inhibitors (EPIs) have also emerged as promising adjunctive therapies in the treatment of multidrug-resistant tuberculosis (MDR-TB). These compounds work by inhibiting the efflux pumps present in TB bacteria, thereby preventing the extrusion of antibiotics from the bacterial cell and increasing their intracellular concentration. By restoring the susceptibility of MDR-TB strains to conventional antibiotics, EPIs offer a potential strategy to enhance the efficacy of existing TB treatment regimens.[23][57]

In recent years, there has been growing interest in the development and evaluation of EPIs as adjuvants to standard TB therapy. Early preclinical studies have shown Promising results, demonstrating the ability of EPIs to potentiate the activity of first line and second-line TB drugs against drug-resistant strains. However, further research is needed to optimize EPIs' pharmacokinetic properties, assess their safety profile, and evaluate their efficacy in clinical settings.[25][26]

The integration of EPIs into TB treatment regimens holds the potential to improve treatment outcomes, shorten the duration of therapy, and reduce the risk of treatment failure and relapse. Additionally, EPIs may play a crucial role in combating the emergence and spread of drug resistance by enhancing the effectiveness of existing antibiotics against MDR-TB strains. [15] [16]

CHAPTER 2

LITERATURE SURVEY

2.1 Advent of Multidrug Resistance in TB

The emergence of multidrug-resistant *Mycobacterium tuberculosis* (MDR-TB) stands as a somber testament to the tenacity and adaptability of infectious diseases. Tuberculosis, one of the oldest known human diseases, has afflicted civilizations for millennia. Ancient skeletal remains bear witness to the devastating impact of TB, showing signs of the disease's presence in human populations throughout history.[43]

Historically, TB was often referred to as the "white plague" due to its debilitating effects and the pallor it cast upon its victims. The advent of antibiotics in the mid- 20th century was a ray of hope, promising an end to the era where TB was synonymous with a death sentence. Isoniazid, rifampicin, and other first-line drugs became the cornerstone of TB treatment, significantly reducing mortality rates and bringing hope for eventual eradication.[22]

However, the jubilation was short-lived. The emergence of MDR-TB in the 1980s threw a wrench into the gears of progress. It was not merely the failure of a specific drug but a profound reminder of the evolutionary process of bacteria. M.TB, the causative agent of TB, possesses an innate ability to adapt, mutate, and develop resistance mechanisms in response to the selective pressure exerted by antibiotics.

The rise of multidrug-resistant strains was not an abrupt event, but a gradual process facilitated by various factors. One of the primary contributors was the widespread and often indiscriminate use of antibiotics. Incomplete treatment regimens, fueled by patient non-compliance or the lack of access to healthcare, created an environment where drug-resistant strains had a distinct survival advantage. Moreover, the rise of HIV/AIDS, which weakens the immune system, further exacerbated the TB epidemic, and provided an ideal breeding ground for MDR-TB.

Understanding the genetic basis of drug resistance in M. TB has been a critical area of research. Specific mutations in genes related to drug targets, such as *katG* for isoniazid and *rpoB* for rifampicin, have been identified as primary culprits. These mutations alter the structure and function of the target proteins, rendering antibiotics ineffective. Additionally, efflux pumps, which actively pump out drugs from bacterial cells, and enzymatic modifications contribute to the complex tapestry of drug resistance mechanisms.[48]

The clinical ramifications of MDR-TB are profound. Patients afflicted with MDR-TB face a grueling and protracted treatment regimen. Second-line drugs, which are often less effective, more toxic, and significantly more expensive than their first-line counterparts, are used. The prolonged duration of treatment increases the risk of adverse effects, compromises the patient's quality of life, and poses significant challenges in terms of treatment adherence. This scenario not only jeopardizes individual patient outcomes but also fuels the transmission of MDR-TB within communities.[21][50]

Socioeconomic determinants play a pivotal role in the persistence and spread of MDR-TB. Poverty, malnutrition, overcrowded living conditions, and limited access to education are factors that fuel the TB epidemic, particularly in developing countries. The lack of awareness and education about TB, coupled with social stigma, hampers early diagnosis and treatment initiation. Moreover, marginalized communities, including refugees and migrants, face additional challenges in accessing healthcare services, making them particularly vulnerable to MDR-TB.

The epidemiological landscape of MDR-TB is diverse, with varying prevalence rates across regions. Eastern Europe, parts of Asia, and sub-Saharan Africa bear a disproportionate burden of MDR-TB cases. In these regions, limited healthcare infrastructure, inadequate diagnostic facilities, and socioeconomic disparities create a conducive environment for the transmission of drug-resistant strains. However, MDR-TB is not limited to these regions; globalization and international travel have facilitated the global spread of drug-resistant TB.

Efforts to combat MDR-TB have spurred significant advancements in diagnostic technologies. Molecular assays, such as PCR and line probe assays, enable rapid and accurate detection of drug-resistant strains. These methods not only facilitate early diagnosis but also guide clinicians in tailoring appropriate treatment regimens. Furthermore, whole-genome sequencing has revolutionized our understanding of TB epidemiology, allowing scientists to track the transmission patterns of MDR-TB strains with unprecedented precision.

In recent years, researchers and pharmaceutical companies have been exploring novel therapeutic approaches to tackle MDR-TB. Host-directed therapies, which modulate the host immune response to enhance the clearance of bacteria, show promise in improving treatment outcomes. Drug repurposing, the practice of identifying new therapeutic uses for existing drugs, offers a cost-effective and expedited path to finding effective treatments. Immunomodulatory

agents, which enhance the immune system's ability to fight off infections, are also under investigation as potential adjunct therapies for MDR-TB. [55]

Despite these advancements, the fight against MDR-TB is far from over. Addressing the socioeconomic determinants of TB, such as poverty, malnutrition, and lack of access to education, is crucial. Strengthening healthcare infrastructures, particularly in resource-limited settings, is imperative for ensuring timely diagnosis and treatment initiation. Investment in research and development of new antibiotics, as well as vaccines, is essential to stay ahead of the evolving *M. TB* strains. Furthermore, international collaboration and knowledge sharing are indispensable in the battle against MDR-TB. The sharing of research findings, best practices, and successful interventions can inform policies and strategies on a global scale. Additionally, advocacy and awareness campaigns are essential in dispelling myths and reducing the stigma associated with TB, encouraging individuals to seek healthcare services promptly.

The emergence of multidrug-resistant *Mycobacterium tuberculosis* presents a formidable challenge to global public health. The intricate interplay of biological, clinical, and socioeconomic factors underscores the complexity of the problem. However, the collective resolve of the international community, coupled with sustained investments in research, healthcare infrastructure, and education, can pave the way for effective strategies to combat MDR-TB. This alarming phenomenon has brought focus on the need for exploration of innovative approaches to combat bacterial resistance. One such strategy, which has gained significant attention in recent years, is the use of efflux pump inhibitors (EPIs) as adjunct therapy to enhance the efficiency of existing antibiotics.[56]

2.2 Efflux Pump Inhibitors

Antibiotic-specific efflux pumps, bedded within the bacterial cell membranes, play a vital part in the development of multi-drug resistance by laboriously expelling antibiotics from bacterial cells. These efflux pumps, when overexpressed or hyperactive due to elaboration or environmental factors, contribute significantly to the survival and continuity of antibiotic-resistant microorganisms.

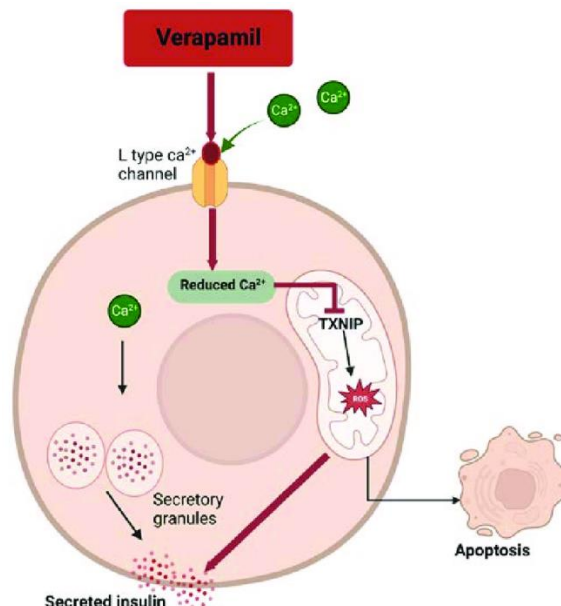


Figure 2.1: Mechanism of Action of Verapamil, a known Efflux Pump Inhibitor (EPI)

To overcome this challenge, experimenters have turned their focus towards relating and developing composites known as efflux pump inhibitors (EPIs), which could restore antibiotic vulnerability to the bacteria by inhibiting these efflux pumps. The logic behind EPI's is their capability to enhance the exertion of antibiotics by precluding their exodus from bacterial cells, thereby adding the intracellular attention of the medicine at the target point.

This approach holds the pledge of invigorating the clinical viability of existing antibiotics and extending the lifetime of our antibiotic magazine. The field of efflux pump impediments has witnessed a swell of exploration in recent times, with a growing body of literature pressing their eventuality as adjuvants in antibiotic remedy. These impediments not only offer a means to combat resistance but also give a platform for the development of new remedial strategies that could revise the treatment of bacterial infections.

Efflux pump inhibitors (EPIs) are compounds that play a critical role in combating antibiotic resistance by targeting bacterial efflux pumps. To understand their mechanism, it is essential to delve into the intricate interplay between efflux pumps, antibiotics, and how EPIs disrupt this process. Efflux pumps are specialized membrane proteins found in bacterial cells, and their primary function is to expel toxic substances, including antibiotics, from the bacterial cell, rendering the antibiotics ineffective. Here, we will provide a detailed explanation of the mechanism of EPI action, including exceptions and variations in this process.

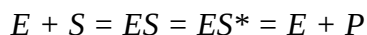
In this context, the efflux pump is viewed as a sophisticated protein complex that contributes to bacterial virulence and drug resistance. Efflux pumps consist of three major parts:

- Transmembrane transporter - Allows transfer of substances across the membrane.
- Membrane fusion protein – Acts as communicator between outer membrane component and transporter
- Outer membrane factor – Helps in the final extrusion of the substance.

Another important factor involved in efflux mechanism is Adenosine Triphosphate aka ATP which provides the efflux pump with the necessary energy required for the process. For example, the NorA efflux pump identified in *S. aureus* utilizes the energy from the hydrolysis of ATP to extrude fluoroquinolone, thus lessening its effects. Primarily, it employs energy to transport these substances out of the cell. The energy gathered often originates from ATP. The steps involved in efflux pump mechanism are:

- Binding: Efflux pumps are specific to certain types of substances. Different pumps recognize different classes of compounds based on their chemical structure. Within the structure of efflux pumps, there are binding sites that are complementary to the structure of the substances they pump out. The binding between the efflux pump and the harmful substance occurs due to chemical interactions, such as hydrogen bonding, van der Waals forces, and electrostatic interactions. They can also bind to the efflux pump site by causing conformational changes in the pump protein.[20]
- Activation: Efflux pumps utilize cellular energy to actively transport the bound substance against its concentration gradient. Binding of the harmful substance triggers the pump to hydrolyze ATP, leading to a change in the pump's conformation. This change allows the substance to be moved across the cell membrane and expelled from the cell.
- Transport: Efflux pumps continuously work to prevent the accumulation of toxic substances within the cell. Bacteria with overactive efflux pumps can pump out antibiotics, rendering the antibiotics ineffective and contributing to bacterial survival.
- Ejection: And finally, the harmful substance is ejected out.

The efflux pump employs this active transport to expel unfavorable substances from the bacterial cell. The efflux pump remains inactive until a harmful substance binds to it. This action triggers the use of ATP as energy to eject the substance, a mechanism mathematically represented as:



Here, E represents the efflux pump, S symbolizes the harmful substance, ES denotes the non-covalent complex of the pump and substance, ES* illustrates the activated state of the complex, and P stands for the ejected substance.

The hydrolysis of ATP, a process that breaks down ATP into ADP (Adenosine Diphosphate) and inorganic phosphate, provides the necessary energy for the expulsion. After the expulsion, the efflux pump reverts to its impurities state, primed for another round of ejection when required. This ingenious mechanism, thus, enables bacteria to flourish amidst adversity. Diverse bacteria utilize divergent versions of the pump, each demonstrating unique structural features and performing specific functions. This wealth of variation justifies the need to classify efflux pump types.

Efflux pumps have been broadly categorized into five main families, based on distinct sequences and structural features. Each family is further sub-divided into various types of efflux pumps - offering a rich diversity that aligns with the manifold demands of bacterial existence.

2.2.1 Major Facilitator Superfamily (MFS)

The MFS is one of the largest families, with pumps showing broad substrate specificity. MFS pumps are single-component systems, meaning they solely rely on a membrane transporter. A prominent example is the NorA pump in *Staphylococcus aureus*. MFS proteins are involved in the transport of a wide variety of substrates across biological membranes, including sugars, ions, Amino acids, peptides, drugs, and other small molecules. They are essential for various physiological processes, including nutrient uptake, drug resistance, and solute transport.

2.2.1.1 Structural Features of MFS Proteins

Transmembrane Domains (TMDs): MFS proteins typically consist of 12-14 alpha-helical transmembrane segments that span the lipid bilayer. These transmembrane segments form the core structure of the protein, creating a channel for substrate transport across the membrane.

Cytoplasmic Loops: MFS proteins have cytoplasmic loops connecting the transmembrane segments. These loops often contain functional domains or motifs that are involved in substrate binding, transport regulation, or interactions with cellular signaling pathways.

2.2.2 ATP-Binding Cassette (ABC) family

ABC efflux pumps use ATP as a direct energy source. These are multi-component systems, involving ATP-binding proteins, transporter proteins, and often accessory proteins, working in harmony. The ATP-Binding Cassette (ABC) transporters constitute one of the largest and most widespread protein families, found in all living organisms. These transporters are involved in the active transport of a diverse range of substrates across cellular membranes, including ions, sugars, amino acids, lipids, peptides, and drugs. ABC transporters play crucial roles in various physiological processes, such as nutrient uptake, cellular detoxification, and the regulation of intracellular concentrations of ions and metabolites.

2.2.2.1 Structural Features of ABC Transporters

Transmembrane Domains (TMDs): ABC transporters consist of multiple transmembrane domains that form a channel through the lipid bilayer. These TMDs are responsible for substrate recognition and transport across the membrane.

Nucleotide-Binding Domains (NBDs): ABC transporters also contain cytoplasmic NBDs that bind and hydrolyze ATP. ATP binding and hydrolysis provide the energy required for substrate transport. NBDs are highly conserved across ABC transporters.

Periplasmic Binding Proteins (in some cases): In certain ABC transport systems, especially in bacteria, periplasmic binding proteins are present. These proteins capture substrates from the external environment and deliver them to the transmembrane domains for transport.[54]

2.2.3 Small Multidrug Resistance (SMR) Family

SMR pumps are ubiquitous in bacteria, handling a variety of small, hydrophobic cationic molecules. These pumps are quite small, composed of merely around 100-120 Amino acids. SMR proteins are involved in the efflux of various toxic compounds, including drugs and antimicrobial agents, from the interior of the cell to the extracellular environment. Unlike the ATP-binding cassette (ABC) transporters, SMR proteins do not require ATP hydrolysis for their

function. Instead, they utilize the proton motive force (PMF) across the cell membrane as an energy source for drug efflux.

2.2.3.1 Structural Features of SMR Family

Hydrophobic Transmembrane Domains (TMDs): SMR proteins typically consist of four transmembrane helices. These helices form a channel that spans the lipid bilayer of the cell membrane.

Cytosolic Loops: SMR proteins have short cytosolic loops connecting the transmembrane helices. These loops are involved in substrate recognition and transport.

2.2.4 Resistance-Nodulation-Division (RND) Family

RND pumps are known for their high-level antibiotic resistance, especially in Gram-negative bacteria. These tripartite systems can extrude a vast array of substrates, aided by their unique periplasmic 'funnel'. RND transporters are involved in the efflux of a wide range of substrates, including antibiotics, heavy metals, detergents, and other toxic compounds, from the bacterial cell to the extracellular environment. These transporters play a crucial role in bacterial resistance to various antimicrobial agents and are a significant concern in the context of antibiotic resistance.

2.2.4.1 Structural Features of RND Family

Transmembrane Domains (TMDs): RND proteins typically consist of 10 to 12 transmembrane helices. These helices form a tunnel-like structure that spans the inner and outer membranes of gram-negative bacteria, allowing substrates to be transported from the cytoplasm to the external environment.

Periplasmic Domain: RND transporters have a periplasmic domain located between the inner and outer membranes. This domain captures substrates from the periplasmic space and delivers them to the transmembrane channel for efflux.

Outer Membrane Channel (Outer Membrane Factor, OMF): RND transporters form complexes with outer membrane proteins, such as OMF, which create a channel in the outer membrane. This channel allows substrates to be released into the extracellular space.[45]

2.2.5 Multi-Antimicrobial Extrusion (MATE) Family

The MATE pumps exude a more varied range of substrates, including drugs and metabolites, often using a gradient of Na⁺ or H⁺ ions. MATE transporters play a crucial role in the efflux of various substrates, including antibiotics, organic ions, and other toxic compounds, from the cell to the extracellular environment. Like other efflux transporter families, MATE transporters are involved in multidrug resistance and contribute to the ability of Microorganisms to survive in the presence of antimicrobial agents. Here are the key features and functions of the MATE family efflux pumps:

2.2.5.1 Structural Features of MATE Family

Transmembrane Domains (TMDs): MATE transporters typically have twelve transmembrane helices that form a channel spanning the lipid bilayer. These helices are responsible for substrate recognition and transport.

Cytosolic Loops: MATE transporters contain cytosolic loops connecting the transmembrane helices. These loops often have conserved motifs involved in substrate binding and transport.

It is essential to understand that the above families are not rigid classes - overlapping instances may occur due to the rapid evolution and diverse functionality of bacterial efflux pumps. Additionally, the same bacterium may utilize pumps from different families to guarantee survival in varied environments. [17] [18][19]

CHAPTER 3

AIM AND OBJECTIVES

Aim: To Analyze and Evaluate the Synergistic Effect of Efflux Pump Inhibitors with Anti-TB Drugs in Multidrug-Resistant *Mycobacterium Tuberculosis* Strains.

Objectives:

- Analyze the Drugability of Efflux Pump Inhibitors
- Analyze the Stability and Permeability of Efflux Pump Inhibitors
- Suggest Potent Efflux Pump Inhibitors with Positive ADME Properties
- State The Anti-TB Effect of Said Efflux Pump Inhibitors on MDR Strains
- Clinically Analyze the Synergistic Effect of Efflux Pump Inhibitors with First-Line Drugs like Rifampicin and Isoniazid

CHAPTER 4

MATERIALS AND METHODS

4.1 Preparation of 7H9 Broth Medium:

To prepare 7H9 broth media for a total volume of 200ml.

- Measure and add 0.94g of Middlebrook 7H9 broth powder to a media flask.
- Add 400µl glycerol.
- Add 180ml distilled water and agitate the mixture to avoid clumps.
- Autoclave at 121°C and 15psi pressure for 30-60 minutes.
- Once the broth has cooled down completely, place the media in the biosafety cabinet and add 10% OADC growth supplement (20ml for 200ml media).
- Store at 4°C

4.2 Preparation of 7H11 Agar + PANTA Medium:

To prepare 7H11 Agar + PANTA medium for a total volume of 300ml.

- Measure and add 6.15g of Middlebrook 7H11 Agar powder to a media flask.
- Add 1.5ml glycerol.
- Add 270ml distilled water and agitate the mixture to avoid clumps.
- Autoclave at 121°C and 15psi pressure for 30-60 minutes.
- Once the broth has cooled down completely, place the media in the biosafety cabinet and add 10% OADC growth supplement (30ml for 300ml media).
- Pour the agar medium into petri plates (300ml gives approx. twenty plates) and store the sealed petri plates at 4°C.

4.3 Preparation of RPMI Medium:

To prepare Complete RPMI medium for a total volume of 50ml.

- Measure 45 ml of incomplete RPMI medium in a 50 ml falcon tube.
- Add 10% (5 ml) fetal bovine serum (FBS)
- Add 500 µl Pen Strep antibiotic and agitate the mixture.
- Filter the complete medium using 0.45µm syringe filter and a 25 ml syringe.
- Store at 4° C.

4.4 Preparation of Bacterial Suspension:

1. Take 2/3rd loop of fresh culture of mycobacterial strain from LJ medium. Take morphologically similar colonies, to avoid selecting an atypical variant.
2. Mix in a 10 to 15-mL sterile screw-cap glass tube (Biju bottle) containing five to ten sterile glass beads (3 mm)
3. Vortex for at least 2 mins after careful closing of the cap to disperse the clumps. Then add 0.7 ml of sterile, complete 7H9 and vortex again.
4. Allow the tube to settle for 5 mins.
5. Match the turbidity of the supernatant to McFarland 1 by adding complete 7H9, and vortex for 30 s. If the suspension turbidity is more than the McFarland standard (MF) 1 turbidity, add complete 7H9 until it is reached.
6. To obtain 1:10 of inoculum, dilute 1 ml of MF matched culture suspension in to 9 ml of complete 7H9.

Note: This method of preparing bacterial suspension has been used throughout the entirety of this project.

4.5 Micro Broth Dilution Assay Protocol

Aim:

To determine the inhibitory activity of rifampicin against M. TB clinical isolates.

Requirements:

- 96 well plate
- 50 ml Centrifuge tube
- M.TB strains
- Rifampicin Standard Stock
- Inverted Microscope
- Complete Middle Brook 7H9 Broth
- DMSO

Procedure:

Primary stock preparation:

Measure 0.01g rifampicin in a vial and add 1ml DMSO to attain 0.01g/ml concentration of rifampicin.

Working stock preparation:

To attain 2048 µg/ml concentration of rifampicin, dissolve 204.8 µl of standard stock 795.2 µl of complete 7H9.

Assay Procedure:

1. Label the 96 well plate with target compound and strain.
2. Add 0.1 ml of complete 7H9 broth into all wells except the 1st column.
3. Add 0.2 ml of rifampicin to 1st column and serially dilute (2-fold dilution) to the first 11 columns. The last column acts as control for each strain.
4. Finally add 0.1 ml of 1:10 culture inoculum to all the wells and seal the plate with parafilm or plate sealer.
5. Incubate the plate for 7-14 days at 37°C with 5% CO₂.



Fig 4.1: Rif MIC Plate for R7, R8 and R9 XDR Strains

4.6 Combination Assay Protocol

Aim:

To determine the combinational effect of efflux pump inhibitors against MDR M. Tb strains along with rifampicin.

Materials Required:

- Isolated M. Tb bacterial colonies.
- Myoinositol powder stock
- Rifampicin stock
- Dist. H₂O

- DMSO
- 96 well plate
- Pipettes

Preparation of Myoinositol and Rifampicin Stock from Powder:

- Weigh 0.01g of powder of rifampicin and myoinositol dissolve in 1ml DMSO and Dist. H₂O respectively to prepare 10,000µg/ml stock for both compounds.
- For myoinositol working stocks: Prepare 4x and 8x concentrations of myoinositol working concentration (800µg/ml)
- For rifampicin working stocks: Prepare 4x MIC of rifampicin for each M. Tb strain.

Assay Procedure:

- Add 0.1 ml of complete 7H9 medium in all wells of 96 well plate.
- Add 0.1 ml of 4x concentration stock of myoinositol from row A (column 1 to 11) and 8x stock in row A (12th Column)
- Serially dilute myoinositol using a multichannel pipette (0.1ml) from row A to G in all columns. Row H acts as myoinositol-free control.
- Add 0.1 ml 4x concentration stock of rifampicin in column 12 (Row A to H) and serially dilute (2-fold) using a multichannel pipette from column 12 to 2 (row A to H). Column 1 acts as rifampicin-free control.
- Add 0.1 ml of M. TB (1:10 dilution) culture inoculum in all wells.
- Place the sealed 96 well plate with its cover into a static incubator at 35±2°C for 7 to 21 days at 5% CO₂.



Fig 4.2: Mi + Rif Combination Assay Plate for Rres 2 Mono-resistant Strain

4.7 Infection Assay Protocol

Aim:

To culture and maintain THP-1 cell lines and to find if the MDR M. TB strains are sensitive to the myoinositol and rifampicin drug combinations in ex vivo conditions.

Materials Required:

- THP-1 Monocyte Cell Lines
- T25 Cell Culture Flasks
- Complete RPMI

- Serological Pipettes and Aider
- PMA
- 7H11 Agar + PANTA Plates
- 24 Well Plate

Preparation of Complete RPMI (CRPMI):

- Take 45 ml incomplete RPMI (IRPMI) with L-Glutamine in a 50 ml falcon tube.
- Measure 5 ml FBS (10% of total CRPMI volume) and add to the falcon tube holding IRPMI.
- Add 500 µl Pen Strep antibiotic and agitate the Mixture.
- Store at 27° C for not more than 7 days.

Subculturing THP-1 Cell Lines:

- Take a cryovial holding the THP-1 cells from the -80° C freezer and immediately thaw the contents using a preheated water bath at 37° C.
- Transfer the contents of the vial to a falcon holding 10 ml IRPMI.
- Centrifuge at 1500 rpm for 10 Mins.
- Remove the supernatant add 5 ml IRPMI and Centrifuge again at 1500 rpm for 10 Mins.
- Remove the supernatant a second time and dissolve the pellet in 1 ml CRPMI.
- Fill 2 T25 flasks with 9.5 ml CRPMI and add 500 µl of CRPMI with the cells to each flask.
- Store the flasks at 37° C with 5% CO₂.

- Conduct sterility check after 3 days using BHI agar or check for colour change of CRPMI.



Fig 4.3: T25 Flask containing THP-1 Cell Suspension

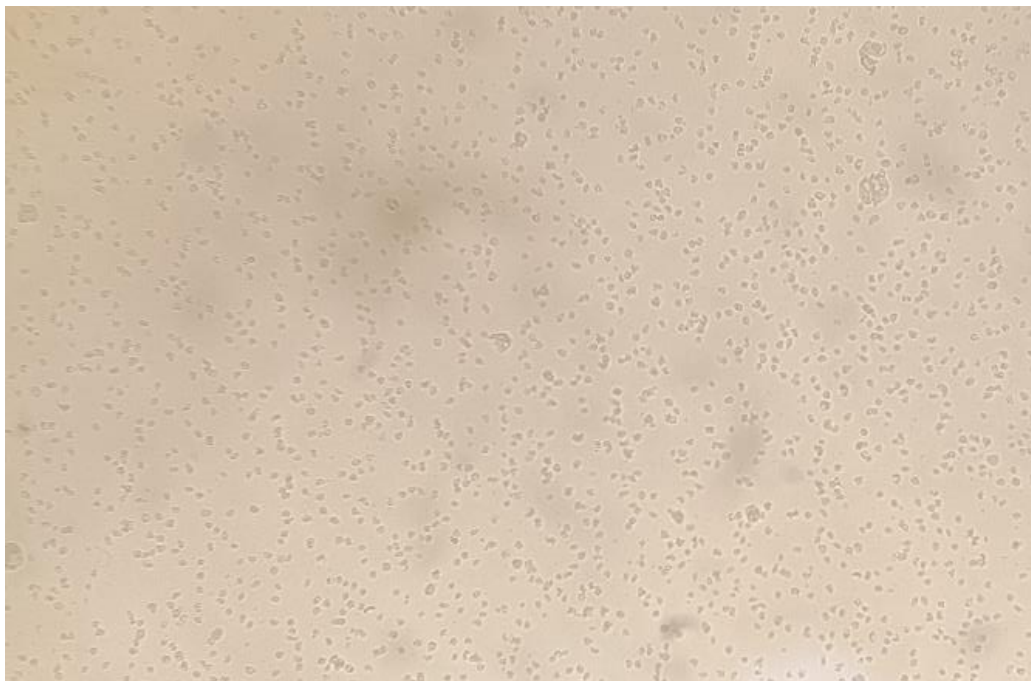


Fig 4.4: THP-1 cells as viewed under 10X microscope.

THP-1 Differentiation and Infection:

- Dissolve 1mg PMA (phorbol 12-myristate-13-acetate) in 0.81 ml DMSO.
- Aliquot 15 μ l to a 1.5ml falcon and add 1.95 ml sterile CRPMI to create PMA stock.
- Take a sterile 24 well plate and aliquot 1 ml of THP-1 cell suspension to all the wells.
- From the PMA stock add 10 μ l to all the wells except the undifferentiated control wells.
- Incubate for 48 hours at 37°C.

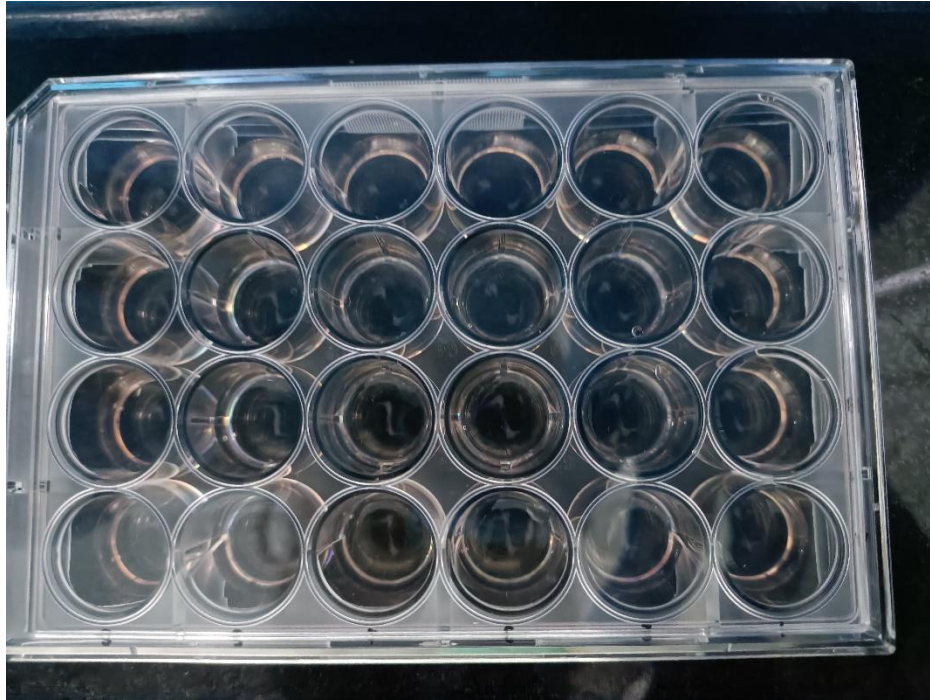


Fig 4.5: Differentiated and adherent THP-1 cells

Preparation of Infection Culture:

- Prepare 1 MF standard matched suspension of desired M. TB strain.
- Add 1 ml matched suspension to 2 ml 7H9 broth to create 3 ml 10^8 dilution suspension.
- Now from the 3 ml suspension take 1 ml and add to 9 ml 7H9 broth. This is to reduce the number of cells to 10^7 .
- For each ml of cells, add 10 μ l of 10^7 cell suspension.

Infection Assay:

- Take the differentiation plate after 48 hours and add 10 μ l of 10^7 cell suspension to all the wells.
- Incubate for 3 hours.
- Discard the media and add fresh, sterile CRPMI.
- Incubate for 1 hour.
- Add drug combinations as predestined in the assay template.
- Incubate for 72 hours.
- Discard media and immediate wash the wells with PBS.
- Add 100 μ l dist. H₂O and incubate for 15 Mins.
- Add 900 μ l 7H9 with 0.05% Tween 80
- Scrap cells and plate 1 ml of each well in individual 7H11 + PANTA agar plates.
- Incubate for a minimum of 21 days.

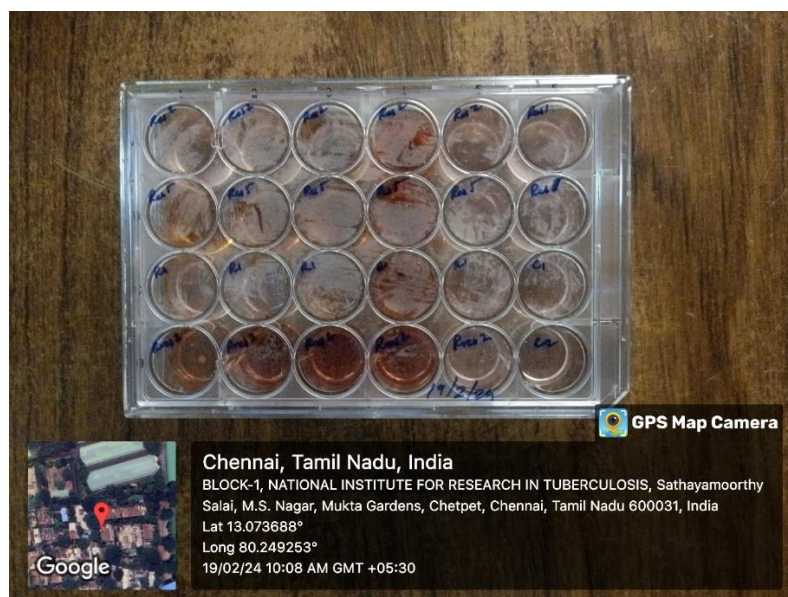


Fig 4.6: Infection assay plate for Res 2, Res 5, R1 and Rres 2 to test Mi+Rif Combinations

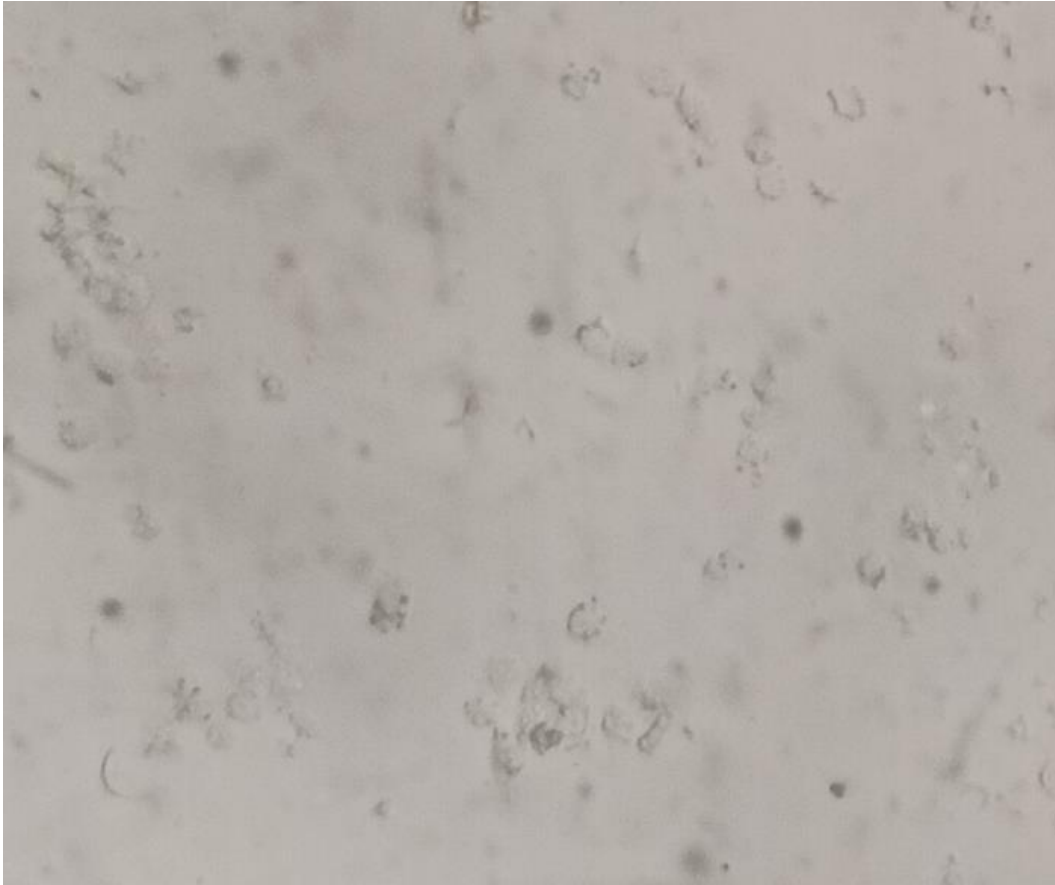


Fig 4.7: THP-1 cells infected with M. TB under 10X microscope.

CHAPTER 5

RESULTS AND DISCUSSIONS

5.1 Insilico Analysis of Potential Efflux Pump Inhibitors:

Through literature survey, four efflux pump inhibitors that can act on rifampicin-specific MDR-MTB strains were identified. The authors had identified these compounds using molecular docking and thus the actual drug ability of the compounds was unknown. Hence, the four compounds were analyzed insilico to identify their physicochemical and ADMET properties using ADMETLab software.

ADMET Parameters and their Meaning:

Table 5.1: ADMET Properties and their Meaning

ADMET Classification	Property	Meaning
Physicochemical Properties	Log S	The logarithm of aqueous solubility value. The first step in the drug absorption process is the disintegration of the tablet or capsule, followed by the dissolution of the active drug. Low solubility is detrimental to good and complete oral absorption, and early measurement of this property is of great importance in drug discovery
	log D7.4	An eligible drug usually needs to keep a balance between lipophilicity and hydrophilicity to dissolve in the body fluid and penetrate the bio membrane effectively. Therefore, it is important to estimate the n-octanol/water distribution coefficients at physiological pH (logD7.4) values for candidate compounds

	Log P	The logarithm of the n-octanol/water distribution coefficient. log P possess a leading position with considerable impact on both membrane permeability and hydrophobic binding to macromolecules, including the target receptor as well as other proteins like plasma proteins, transporters, or metabolizing enzymes
Adsorption	Caco2 Permeability	The human colon adenocarcinoma cell lines (Caco-2), as an alternative approach for the human intestinal epithelium, has been commonly used to estimate in vivo drug permeability due to their morphological and functional Similarities. Thus, Caco-2 cell permeability has also been an important index for an eligible candidate drug compound.
	HIA	The human intestinal absorption of an oral drug is the essential prerequisite for its apparent efficacy. The close relationship between oral bioavailability and intestinal absorption has also been proven and HIA can be seen an alternative indicator for oral bioavailability to some extent.
Distribution	PPB (Plasma Protein Binding)	PPB can directly influence the oral bioavailability because the free concentration of the drug is at stake when a drug binds to serum proteins in this process.
Metabolism	P450 CYP1A2 inhibitor	Based on the chemical nature of biotransformation, the process of drug metabolism reactions can be divided into two broad categories: phase I (oxidative reactions) and phase II (conjugative reactions). The
	P450 CYP1A2 Substrate	
	P450 CYP3A4	

	inhibitor	human cytochrome P450 Family (phase I enzymes) contains 57 isozymes, and these isozymes metabolize approximately two-thirds of known drugs in human with 80% of this attribute to five isozymes—1A2, 3A4, 2C9, 2C19 and 2D6. Most of these CYPs responsible for phase I reactions are concentrated in the liver.
	P450 CYP3A4 substrate	
	P450 CYP2C9 inhibitor	
	P450 CYP2C9 substrate	
	P450 CYP2C19 inhibitor	
	P450 CYP2C19 substrate	
	P450 CYP2D6 inhibitor	
	P450 CYP2D6 substrate	
Elimination	T 1/2 (Half Lifetime)	The half-life of a drug is a hybrid concept that involves clearance and volume of distribution, and it is arguably more appropriate to have reliable estimates of these two properties instead.
	CL (Clearance Rate)	Clearance is an important pharmacokinetic parameter that defines, together with the volume of distribution, the half-life, and thus the frequency of dosing of a drug
Toxicity	LD50 (LD50 of acute toxicity)	Median lethal dose (LD50) usually represents the acute toxicity of Chemicals. It is the dose amount of a tested molecule to kill 50 % of the treated animals within a given period.

5.2 Compound Specific Insilico Results and Interpretation

- **Myoinositol**

Graph 5.1: ADMET Properties of Myoinositol

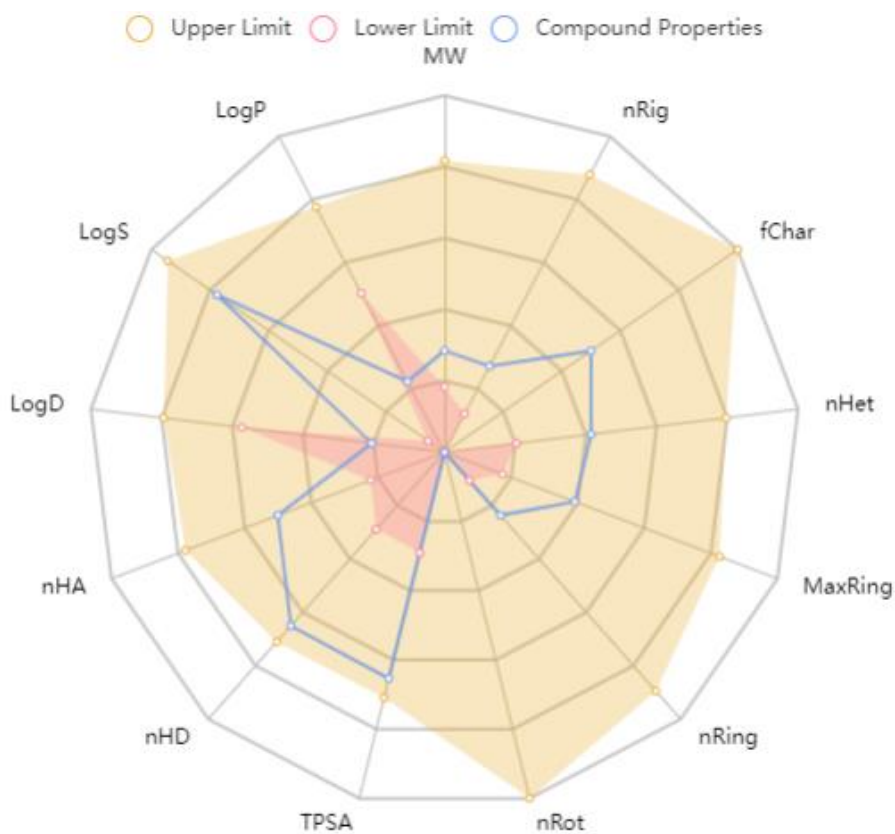


Table 5.2: ADMET Properties of Myoinositol

ADMET Classification	Property	Suggested Values	Myoinositol	
			Predicted Values	Interpretation
Physicochemical Properties	Log S	Optimal: higher than -4 log mol/L $\mu\text{g/mL}$: Low solubility; 10–60 $\mu\text{g/mL}$: Moderate solubility; >60 $\mu\text{g/mL}$: High	97869.807 $\mu\text{g/mL}$	Very high solubility

		solubility		
	log D7.4	< 1: Solubility high; Permeability low by passive transcellular diffusion; Permeability possible via paracellular if MW < 200; Metabolism low; 1 to 3: Solubility moderate; Permeability moderate; Metabolism low. 3 to 5: Solubility low; Permeability high; Metabolism moderate to high. > 5: Solubility low; Permeability high; Metabolism high.	0.778	High solubility, low permeability, and low metabolism
	Log P	Optimal: 0< Log <3: poor aqueous solubility; LogP <3: poor lipid bilayer permeability;	-3.835	Poor lipid bilayer permeability

		LogP>3: poor aqueous solubility		
Adsorption	Caco2 Permeability	Optimal: higher than -5.15 cm/s	-5.836 cm/s	Moderately optimal Caco2 permeability
	HIA	A molecule with an absorbance of less than 30% is poorly absorbed. Accordingly, molecules with a HIA >30% were classified as HIA- (Category 0), while molecules with a HIA < 30% were classified as HIA+ (Category 1). The output value is the probability of being HIA+, within the range of 0 to 1.	0.4	Low probability of being poorly absorbed
Distribution	PPB (Plasma Protein Binding)	A compound is considered to have a proper PPB if it has predicted value < 90%, and drugs with high protein-bound may have a low therapeutic index	18.58%	Has proper plasma protein binding

Metabolism	P450 CYP1A2 inhibitor	Category 0: Non-substrate / Non-inhibitor; Category 1: substrate / inhibitor. The output value is the probability of being substrate / inhibitor, within the range of 0 to 1.	0.002	Low probability of being inhibitor
	P450 CYP1A2 Substrate		0.222	Low probability of being substrate
	P450 CYP3A4 inhibitor		0	Low probability of being inhibitor
	P450 CYP3A4 substrate		0.03	Low probability of being substrate
	P450 CYP2C9 inhibitor		0.002	Low probability of being inhibitor
	P450 CYP2C9 substrate		0.257	Low probability of being substrate
	P450 CYP2C19 inhibitor		0.057	Low probability of being inhibitor
	P450 CYP2C19 substrate		0.144	Low probability of being substrate
Elimination	T_{1/2} (Half Lifetime)	Range: >8h: high; 3h< Cl < 8h: moderate; <3h low	1.546 h	Low T _{1/2}
	CL (Clearance Rate)	Range: >15 mL/Min/kg: high; 5mL/Min/kg< Cl < 15mL/Min/kg:	1.183 mL/Min/kg	Low Clearance Rate

		moderate; <5ml/Min/kg: low		
Toxicity	LD50 (LD50 of acute toxicity)	High-toxicity: 1~50 mg/kg; Toxicity: 51~500 mg/kg; low-toxicity: 501~5000 mg/kg.	2622.097 mg/kg	Low Toxicity

- **Palmitic Acid**

Graph 5.2: ADMET Properties of Palmitic Acid

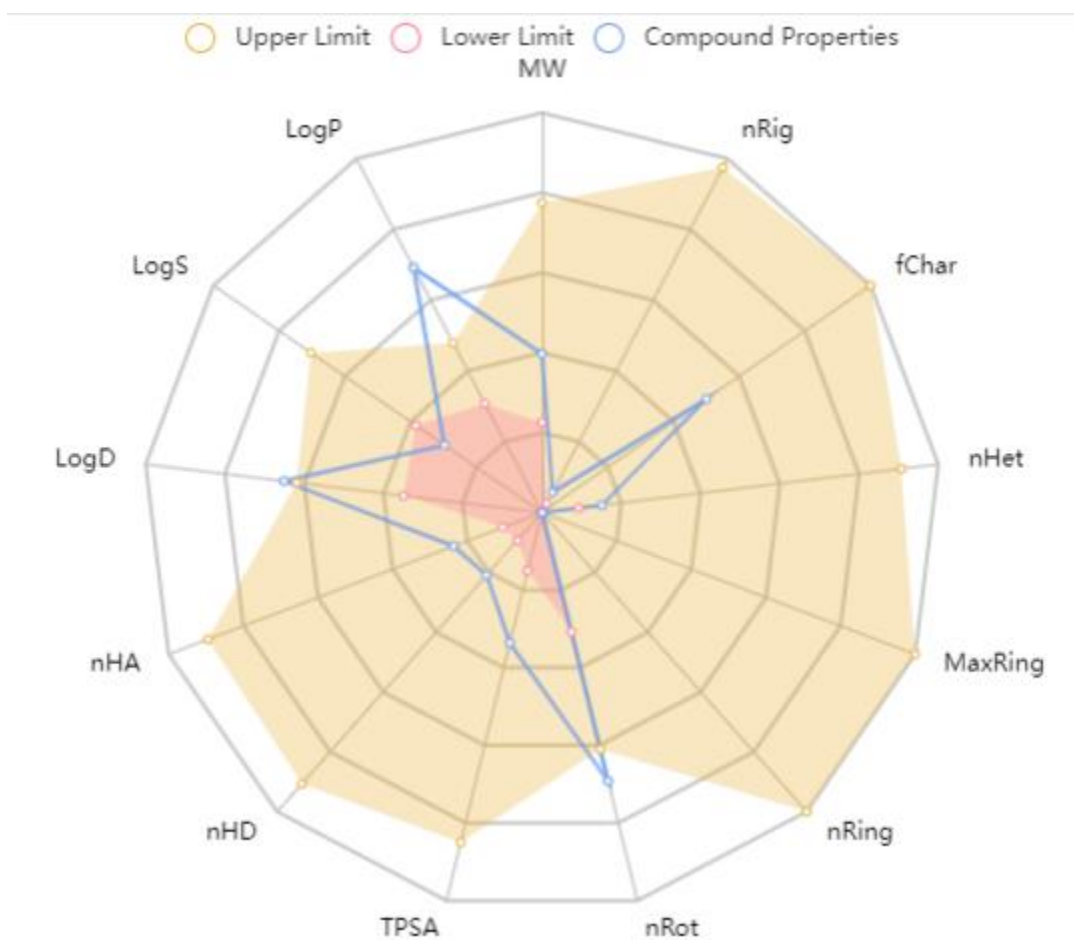


Table 5.3: ADMET Properties of Palmitic Acid

ADMET Classification	Property	Suggested Values	Palmitic Acid	
			Predicted Values	Interpretation
Physicochemical Properties	Log S	Optimal: higher than $-4 \log \text{ mol/L}$ $<10 \mu\text{g/mL}$: Low solubility; $10\text{--}60 \mu\text{g/mL}$: Moderate solubility; $>60 \mu\text{g/mL}$: High solubility	$0.822 \mu\text{g/mL}$	Low solubility
	log D7.4	< 1 : Solubility high; Permeability low by passive transcellular diffusion; Permeability possible via paracellular if MW < 200 ; Metabolism low; 1 to 3 : Solubility moderate; Permeability moderate; Metabolism low. 3 to 5 : Solubility low; Permeability high; Metabolism moderate to high. > 5 : Solubility low;	1.467	Moderate solubility, moderate permeability, and low metabolism

		Permeability high; Metabolism high.		
	Log P	Optimal: $0 < \text{LogP} < 3$: poor aqueous solubility; $\text{LogP} < 3$: poor lipid bilayer permeability; $\text{LogP} > 3$: poor aqueous solubility	5.552	Poor aqueous solubility
Adsorption	Caco2 Permeability	Optimal: higher than -5.15 cm/s	-4.658 cm/s	Optimal Caco2 permeability
	HIA	A molecule with an absorbance of less than 30% is poorly absorbed. Accordingly, molecules with a HIA $> 30\%$ were classified as HIA- (Category 0), while molecules with a HIA $< 30\%$ were	0.8	High probability of being poorly absorbed

		classified as HIA+(Category 1). The output value is the probability of being HIA+, within the range of 0 to 1.		
Distribution	PPB (Plasma Protein Binding)	A compound is considered to have a proper PPB if it has predicted value < 90%, and drugs with high protein-bound may have a low therapeutic index	75.60%	Has moderately good plasma protein binding
Metabolism	P450 CYP1A2 inhibitor	Category 0: Non-substrate / Non-inhibitor; Category 1: substrate / inhibitor. The output value is the probability of being substrate / inhibitor, within the range of 0 to 1.	0.331	Low probability of being inhibitor
	P450 CYP1A2 Substrate		0.142	Low probability of being substrate
	P450 CYP3A4 inhibitor		0.001	Low probability of being inhibitor
	P450 CYP3A4 substrate		0.008	Low probability of being substrate
	P450 CYP2C9 inhibitor		0.008	Low probability of being inhibitor
	P450 CYP2C9		0.925	High

	substrate			probability of being substrate
	P450 CYP2C19 inhibitor		0.013	Low probability of being inhibitor
	P450 CYP2C19 substrate		0.2	Low probability of being substrate
Elimination	T_{1/2} (Half Lifetime)	Range: >8h: high; 3h< Cl < 8h: moderate; <3h low	1.608 h	Low T _{1/2}
	CL (Clearance Rate)	Range: >15 mL/Min/kg: high; 5mL/Min/kg< Cl < 15mL/Min/kg: moderate; <5ml/Min/kg: low	1.231 mL/Min/kg	Low Clearance Rate
Toxicity	LD50 (LD50 of acute toxicity)	High-toxicity: 1~50 mg/kg; Toxicity: 51~500 mg/kg; low-toxicity: 501~5000 mg/kg.	14287.914 mg/kg	Low Toxicity

- **Dodecanoic Acid**

Graph 5.3: ADMET Properties of Dodecanoic Acid

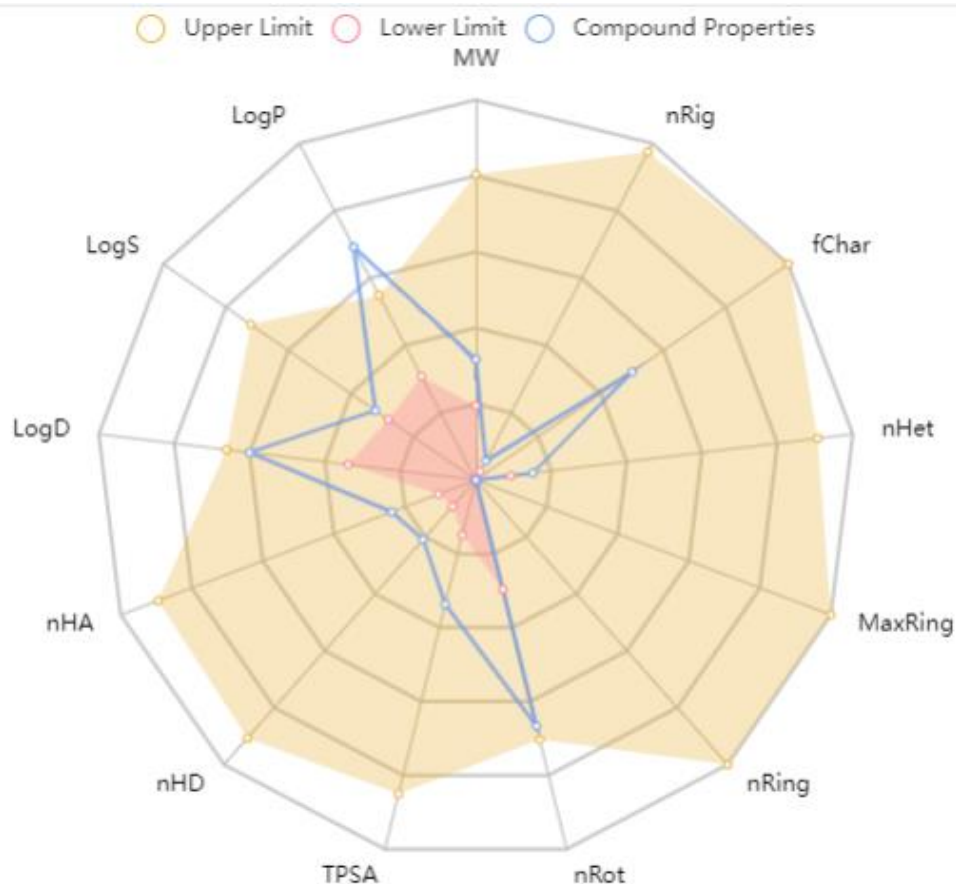


Table 5.4: ADMET Properties of Dodecanoic Acid

ADMET Classification	Property	Suggested Values	Dodecanoic Acid	
			Predicted Values	Interpretation
Physicochemical Properties	Log S	Optimal: higher than -4 log mol/L	11.878 µg/mL	Moderate solubility
		<10 µg/mL: Low solubility;		
		10–60 µg/mL: Moderate solubility;		
		>60 µg/mL: High		

		solubility		
	log D7.4	< 1: Solubility high; Permeability low by passive transcellular diffusion; Permeability possible via paracellular if MW < 200; Metabolism low; 1 to 3: Solubility moderate; Permeability moderate; Metabolism low. 3 to 5: Solubility low; Permeability high; Metabolism moderate to high. > 5: Solubility low; Permeability high; Metabolism high.	1.099	Moderate solubility, moderate permeability, and low metabolism
	Log P	Optimal: 0< LogP <3: poor aqueous solubility; LogP <3: poor lipid bilayer permeability; LogP>3: poor	3.992	Slightly poor aqueous solubility

		aqueous solubility		
Adsorption	Caco2 Permeability	Optimal: higher than - 5.15 cm/s	-4.569 cm/s	Optimal Caco2 permeability
	HIA	A molecule with an absorbance of less than 30% is poorly absorbed. Accordingly, molecules with a HIA >30% were classified as HIA- (Category 0), while molecules with a HIA < 30% were classified as HIA+(Category 1). The output value is the probability of being HIA+, within the range of 0 to 1.	0.8	High probability of being poorly absorbed
Distribution	PPB (Plasma Protein Binding)	A compound is considered to have a proper PPB if it has predicted value < 90%, and drugs with high protein-bound may have a low therapeutic index	73.23%	Has moderately good plasma protein binding
Metabolism	P450 CYP1A2 inhibitor	Category 0: Non-substrate / Non-	0.331	Low probability of

		inhibitor; Category 1: substrate / inhibitor.		being inhibitor
	P450 CYP1A2 Substrate	The output value is the probability of being substrate / inhibitor, within the range of 0 to 1.	0.142	Low probability of being substrate
	P450 CYP3A4 inhibitor		0.001	Low probability of being inhibitor
	P450 CYP3A4 substrate		0.008	Low probability of being substrate
	P450 CYP2C9 inhibitor		0.008	Low probability of being inhibitor
	P450 CYP2C9 substrate		0.925	High probability of being substrate
	P450 CYP2C19 inhibitor		0.013	Low probability of being inhibitor
	P450 CYP2C19 substrate		0.2	Low probability of being substrate
Elimination	T 1/2 (Half Lifetime)	Range: >8h: high; 3h< Cl < 8h: moderate; <3h low	1.538 h	Low T 1/2
	CL (Clearance Rate)	Range: >15 mL/Min/kg: high; 5mL/Min/kg< Cl < 15mL/Min/kg: moderate;	1.162 mL/Min/kg	Low Clearance Rate

		<5ml/Min/kg: low		
Toxicity	LD50 (LD50 of acute toxicity)	High-toxicity: 1~50 mg/kg; Toxicity: 51~500 mg/kg; low-toxicity: 501~5000 mg/kg.	6834.845 mg/kg	Low Toxicity

- **Methyl Stearate**

Graph 5.4: ADMET Properties of Methyl Stearate

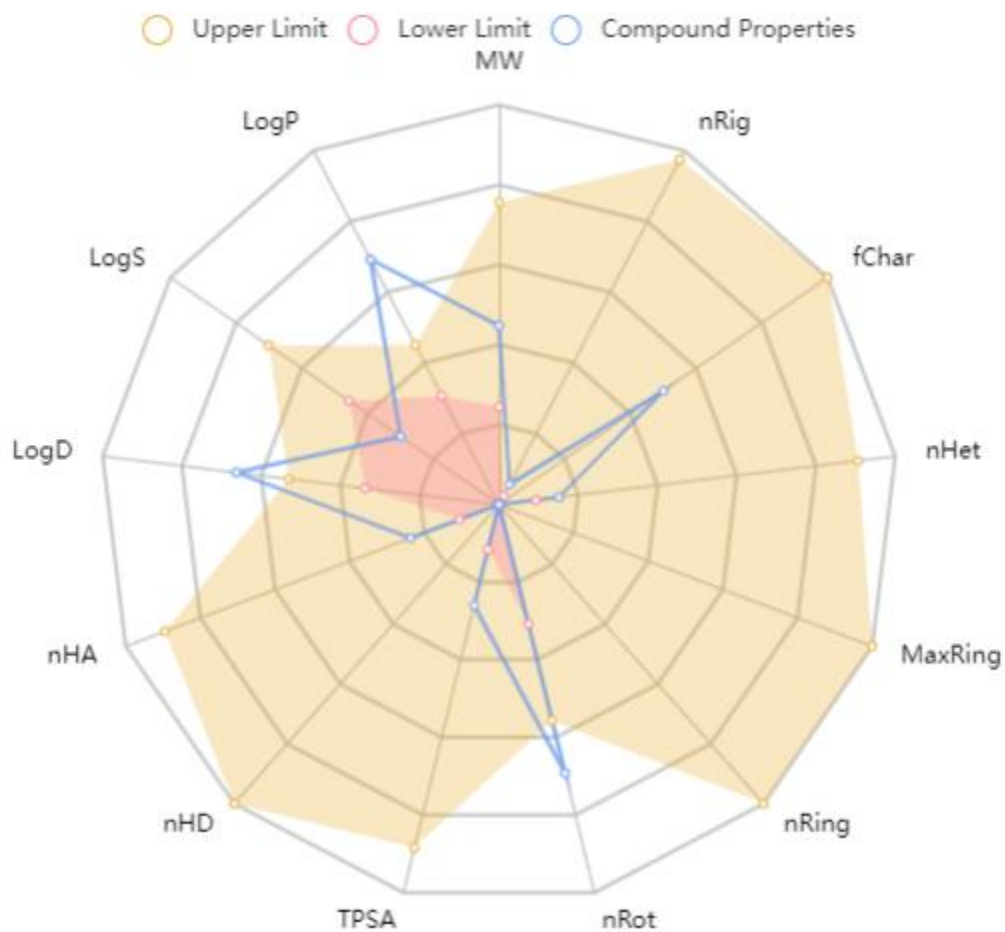


Table 5.5: ADMET Properties of Methyl Stearate

ADMET Classification	Property	Suggested Values	Methyl Stearate	
			Predicted Values	Interpretation
Physicochemical Properties	Log S	Optimal: higher than -4 log mol/L <10 µg/mL: Low solubility; 10–60 µg/mL: Moderate solubility; >60 µg/mL: High solubility	3.899 µg/mL	Low solubility
	log D7.4	< 1: Solubility high; Permeability low by passive transcellular diffusion; Permeability possible via paracellular if MW < 200; Metabolism low; 1 to 3: Solubility moderate; Permeability moderate; Metabolism low. 3 to 5: Solubility low; Permeability high; Metabolism moderate to high. > 5: Solubility low;	2.752	Moderate solubility, moderate permeability, and low metabolism

		Permeability high; Metabolism high.		
	Log P	Optimal: $0 < \text{LogP} < 3$: poor aqueous solubility; $\text{LogP} < 3$: poor lipid bilayer permeability; $\text{LogP} > 3$: poor aqueous solubility	6.421	Poor aqueous solubility
Adsorption	Caco2 Permeability	Optimal: higher than - 5.15 cm/s	-4.555 cm/s	Optimal Caco2 permeability
	HIA	A molecule with an absorbance of less than 30% is poorly absorbed. Accordingly, molecules with a HIA >30% were classified as HIA- (Category 0), while molecules with a HIA < 30% were classified as	0.745	Moderately high probability of being poorly absorbed

		HIA+(Category 1). The output value is the probability of being HIA+, within the range of 0 to 1.		
Distribution	PPB (Plasma Protein Binding)	A compound is considered to have a proper PPB if it has predicted value < 90%, and drugs with high protein-bound may have a low therapeutic index	72.94%	Has moderately good plasma protein binding
Metabolism	P450 CYP1A2 inhibitor	Category 0: Non-substrate / Non-inhibitor; Category 1: substrate / inhibitor. The output value is the probability of being substrate / inhibitor, within the range of 0 to 1.	0.846	High probability of being inhibitor
	P450 CYP1A2 Substrate		0.341	Low probability of being substrate
	P450 CYP3A4 inhibitor		0.014	Low probability of being inhibitor
	P450 CYP3A4 substrate		0.084	Low probability of being substrate
	P450 CYP2C9 inhibitor		0.051	Low probability of being inhibitor
	P450 CYP2C9		0.718	High

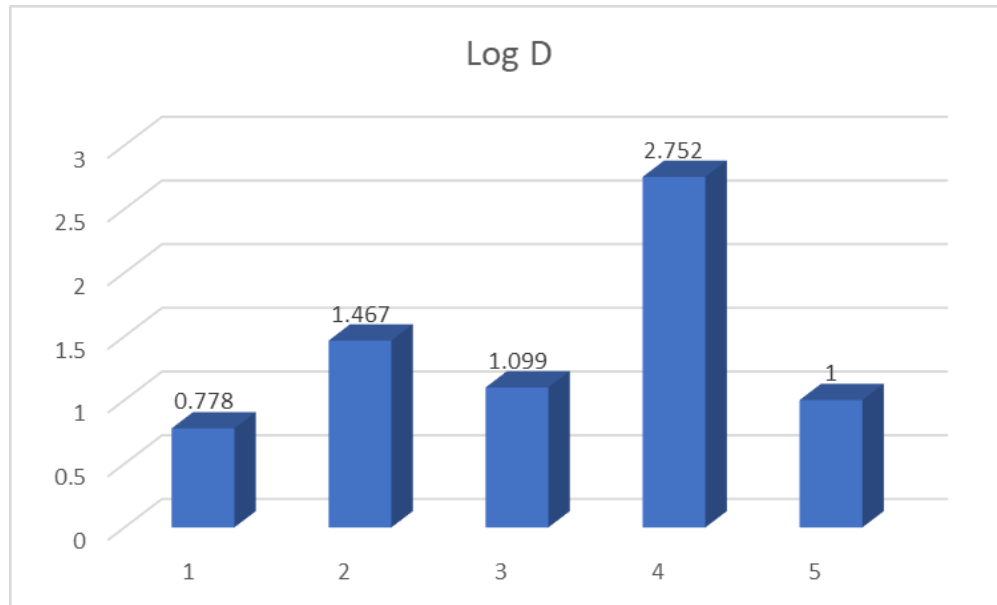
	substrate			probability of being substrate
	P450 CYP2C19 inhibitor		0.26	Low probability of being inhibitor
	P450 CYP2C19 substrate		0.256	Low probability of being substrate
Elimination	T 1/2 (Half Lifetime)	Range: >8h: high; 3h< Cl < 8h: moderate; <3h low	1.74 h	Low T 1/2
	CL (Clearance Rate)	Range: >15 mL/Min/kg: high; 5mL/Min/kg< Cl < 15mL/Min/kg: moderate; <5ml/Min/kg: low	1.193 mL/Min/kg	Low Clearance Rate
Toxicity	LD50 (LD50 of acute toxicity)	High-toxicity: 1~50 mg/kg; Toxicity: 51~500 mg/kg; low-toxicity: 501~5000 mg/kg.	12273.271 mg/kg	Low Toxicity

5.3 Comparison of Solubility Values of EPI:

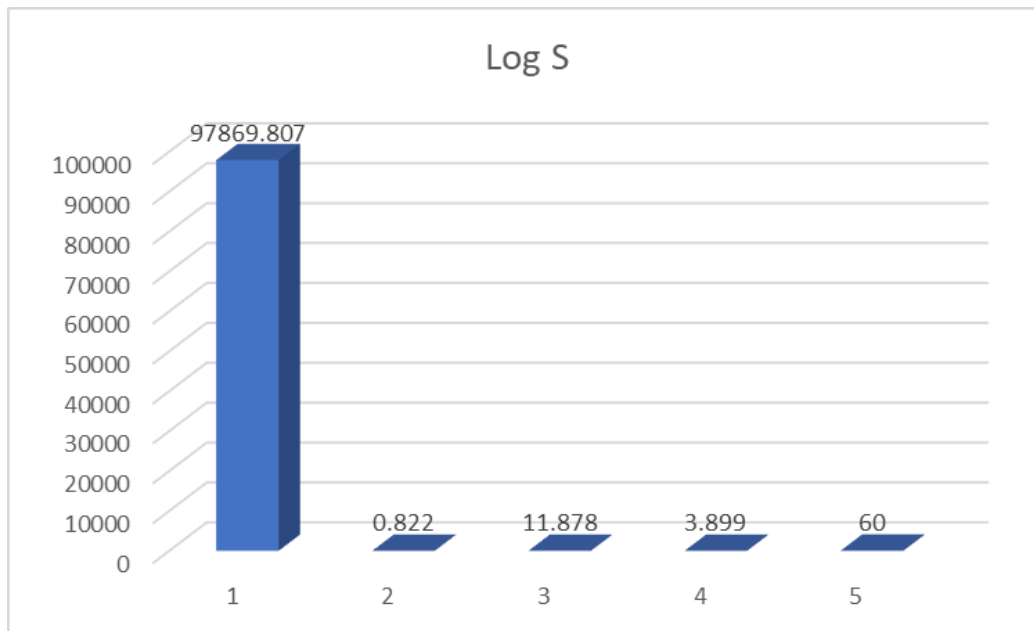
Table 5.6: Solubility values of EPI

EPI	Log D	Log S	Log P
MI	0.778	97869.81	-3.835
PA	1.467	0.822	5.552
DA	1.099	11.878	3.992
MS	2.752	3.899	6.421

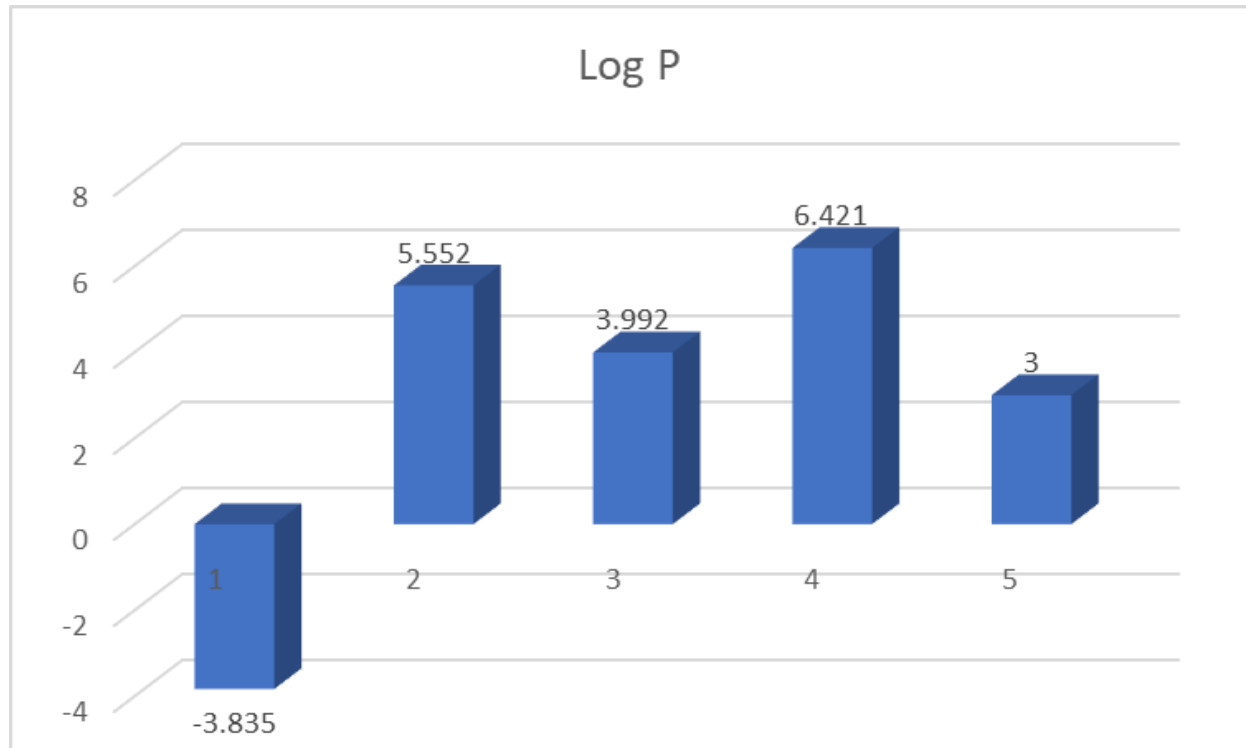
Graph 5.5: Log D values of EPI and Upper Limit Value



Graph 5.6: Log S values of EPI and Lower Limit Value



Graph 5.7: Log P values of EPI and Upper Limit Value



- Data No. 5 in all the graphs is the upper limit/lower limit. It is evident here from the Log S, Log D and Log P graphs that except myoinositol, the other 3 EPI exhibit solubility characteristics that do not fit the desired range.

5.4 Myoinositol MIC Results: (All concentrations are given in µg/mL)

Table 5.7: Myoinositol MIC Assay Results

Microdilution Broth Assay												
Strain Name		Myoinositol MIC						Remarks		Mi has no inhibitory effect on M. TB strains		
	1	2	3	4	5	6	7	8	9	10	11	12
	1600	800	400	200	100	50	25	12.5	6.25	3.125	1.5625	0
Res 1	P	P	P	P	P	P	P	P	P	P	P	P
Res 2	P	P	P	P	P	P	P	P	P	P	P	P
XDR1	P	P	P	P	P	P	P	P	P	P	P	P
XDR2	P	P	P	P	P	P	P	P	P	P	P	P
Rres 5	P	P	P	P	P	P	P	P	P	P	P	P
Rres 9	P	P	P	P	P	P	P	P	P	P	P	P

Observation: It is evident after testing in a variety of different MDR strains that myoinositol has no standalone inhibitory effect

5.5 Rifampicin MIC and Combination Assay Results: (All concentrations are in µg/mL)

I. Res 1 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 01 Rifampicin MIC						Remarks		MIC = 256 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	P	P	P	P	P	P	P	P	P
B	N	N	N	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 01 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
←Rif Mi↓	1 0	2 0.25	3 0.5	4 1	5 2	6 4	7 8	8 16	9 32	10 64	11 128	12 256
A 400	P	N	N	N	N	N	N	N	N	N	N	N
B 200	P	N	N	N	N	N	N	N	N	N	N	N
C 100	P	P	P	P	N	N	N	N	N	N	N	N
D 50	P	P	P	P	P	P	P	N	N	N	N	N
E 25	P	P	P	P	P	P	P	N	N	N	N	N
F 12.5	P	P	P	P	P	P	P	N	N	N	N	N
G 6.25	P	P	P	P	P	P	P	P	P	N	N	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 256 µg/mL and Mi+Rif MIC was 100+8 µg/mL

II. Res 2 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 02 Rifampicin MIC							Remarks		MIC = 128 µg/mL	
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	N	P	P	P	P	P	P	P	P
B	N	N	N	N	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 02 Mi+Rif Combination Assay							Remarks		Reduction in MIC noted	
← Rif	1 0	2 0.25	3 0.5	4 1	5 2	6 4	7 8	8 16	9 32	10 64	11 128	12 256
Mi↓												
A 400	P	P	P	P	P	P	N	N	N	N	N	N
B 200	P	P	P	P	P	P	N	N	N	N	N	N
C 100	P	P	P	P	P	P	P	P	N	N	N	N
D 50	P	P	P	P	P	P	P	P	N	N	N	N
E 25	P	P	P	P	P	P	P	P	N	N	N	N
F 12.5	P	P	P	P	P	P	P	P	N	N	N	N
G 6.25	P	P	P	P	P	P	P	P	P	N	N	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 128µg/mL and Mi+Rif MIC was 200+32µg/mL

III. Res 3 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 03 Rifampicin MIC						Remarks		MIC = 32 µg/mL		
	1	2	3	4	5	6	7	8	9	10	11	12
	1024	512	256	128	64	32	16	8	4	2	1	0.5
A	N	N	N	N	N	N	P	P	P	P	P	P
B	N	N	N	N	N	N	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 03 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
←Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	0.0312	0.0625	0.125	0.25	0.5	1	2	4	8	16	32
A 400	P	P	P	P	P	P	P	P	P	P	P	N
B 200	P	P	P	P	P	P	P	P	P	P	P	N
C 100	P	P	P	P	P	P	P	P	P	P	P	N
D 50	P	P	P	P	P	P	P	P	P	P	P	N
E 25	P	P	P	P	P	P	P	P	P	P	P	N
F 12.5	P	P	P	P	P	P	P	P	P	P	P	N
G 6.25	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 32 µg/mL and Mi+Rif MIC did not change.

IV. Res 4 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 04 Rifampicin MIC						Remarks		MIC = 128 µg/mL		
	1	2	3	4	5	6	7	8	9	10	11	12
	1024	512	256	128	64	32	16	8	4	2	1	0.5
A	N	N	N	N	P	P	P	P	P	P	P	P
B	N	N	N	N	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 04 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
← Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	0.125	0.25	0.5	1	2	4	8	16	32	64	128
A 400	P	P	P	P	P	P	P	P	P	P	P	N
B 200	P	P	P	P	P	P	P	P	P	P	P	N
C 100	P	P	P	P	P	P	P	P	P	P	P	N
D 50	P	P	P	P	P	P	P	P	P	P	P	N
E 25	P	P	P	P	P	P	P	P	P	P	P	N
F 12.5	P	P	P	P	P	P	P	P	P	P	P	N
G 6.25	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 128µg/mL and Mi+Rif MIC did not change

V. Res 5 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 05 Rifampicin MIC						Remarks		MIC = 256 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8	9	10	11	12
A	N	N	N	P	P	P	P	P	P	P	P	P
B	N	N	N	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 05 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
←Rif	1 0	2 0.25	3 0.5	4 1	5 2	6 4	7 8	8 16	9 32	10 64	11 128	12 256
A 400	P	P	P	P	P	P	P	P	P	N	N	N
B 200	P	P	P	P	P	P	P	P	P	N	N	N
C 100	P	P	P	P	P	P	P	P	P	N	N	N
D 50	P	P	P	P	P	P	P	P	P	P	N	N
E 25	P	P	P	P	P	P	P	P	P	P	P	N
F 12.5	P	P	P	P	P	P	P	P	P	P	P	N
G 6.25	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 256 µg/mL and Mi+Rif MIC was 100+64µg/mL

VI. Res 7 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 07 Rifampicin MIC						Remarks		MIC = 64 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	N	N	P	P	P	P	P	P	P
B	N	N	N	N	N	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 07 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
← Rif	1 0	2 0.0625	3 0.125	4 0.25	5 0.5	6 1	7 2	8 4	9 8	10 16	11 32	12 64
Mi↓												
A 800	P	P	P	P	P	P	P	P	P	P	P	N
B 400	P	P	P	P	P	P	P	P	P	P	P	N
C 200	P	P	P	P	P	P	P	P	P	P	P	N
D 100	P	P	P	P	P	P	P	P	P	P	P	N
E 50	P	P	P	P	P	P	P	P	P	P	P	N
F 25	P	P	P	P	P	P	P	P	P	P	P	N
G 12.5	P	P	P	P	P	P	P	P	P	P	P	N
H 0.00	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 64 µg/mL and Mi+Rif MIC did not change.

VII. Res 8 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 08 Rifampicin MIC							Remarks		MIC = 512 µg/mL	
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	P	P	P	P	P	P	P	P	P	P
B	N	N	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 08 Mi+Rif Combination Assay							Remarks		No reduction in MIC	
← Rif	1 0	2 0.5	3 1	4 2	5 4	6 8	7 16	8 32	9 64	10 128	11 256	12 512
Mi↓												
A 800	P	P	P	P	P	P	P	P	P	P	P	N
B 400	P	P	P	P	P	P	P	P	P	P	P	N
C 200	P	P	P	P	P	P	P	P	P	P	P	N
D 100	P	P	P	P	P	P	P	P	P	P	P	N
E 50	P	P	P	P	P	P	P	P	P	P	P	N
F 25	P	P	P	P	P	P	P	P	P	P	P	N
G 12.5	P	P	P	P	P	P	P	P	P	P	P	N
H 0.00	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 512 µg/mL and Mi+Rif MIC did not change

VIII. Res 9 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 09 Rifampicin MIC						Remarks		MIC = 256 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	P	P	P	P	P	P	P	P	P
B	N	N	N	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 09 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
←Rif Mi↓	1 0	2 0.25	3 0.5	4 1	5 2	6 4	7 8	8 16	9 32	10 64	11 128	12 256
A 800	P	P	P	P	P	P	P	P	P	P	N	N
B 400	P	P	P	P	P	P	P	P	P	P	N	N
C 200	P	P	P	P	P	P	P	P	P	P	N	N
D 100	P	P	P	P	P	P	P	P	P	P	N	N
E 50	P	P	P	P	P	P	P	P	P	P	N	N
F 25	P	P	P	P	P	P	P	P	P	P	N	N
G 12.5	P	P	P	P	P	P	P	P	P	P	N	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 256µg/mL and Mi+Rif MIC was 800+128µg/mL

IX. Res 10 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 10 Rifampicin MIC						Remarks		MIC = 256 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	P	P	P	P	P	P	P	P	P
B	N	N	N	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 10 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
← Rif	1 0	2 0.25	3 0.5	4 1	5 2	6 4	7 8	8 16	9 32	10 64	11 128	12 256
A 800	P	P	P	P	P	P	P	P	P	P	P	N
B 400	P	P	P	P	P	P	P	P	P	P	P	N
C 200	P	P	P	P	P	P	P	P	P	P	P	N
D 100	P	P	P	P	P	P	P	P	P	P	P	N
E 50	P	P	P	P	P	P	P	P	P	P	P	N
F 25	P	P	P	P	P	P	P	P	P	P	P	N
G 12.5	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 256 µg/mL and Mi+Rif MIC did not change.

X. Res 11 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 11 Rifampicin MIC						Remarks		MIC = 128 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	N	P	P	P	P	P	P	P	P
B	N	N	N	N	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 11 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
← Rif	1 0	2 0.125	3 0.25	4 0.5	5 1	6 2	7 4	8 8	9 16	10 32	11 64	12 128
A 800	P	P	P	P	P	P	P	P	P	P	P	N
B 400	P	P	P	P	P	P	P	P	P	P	P	N
C 200	P	P	P	P	P	P	P	P	P	P	P	N
D 100	P	P	P	P	P	P	P	P	P	P	P	N
E 50	P	P	P	P	P	P	P	P	P	P	P	N
F 25	P	P	P	P	P	P	P	P	P	P	P	N
G 12.5	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: The Rif MIC was found to be 128 µg/mL and Mi+Rif MIC did not change.

XI. Res 12 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 12 Rifampicin MIC						Remarks		MIC = 512 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	P	P	P	P	P	P	P	P	P	P	P
B	N	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 12 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
← Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
Mi↓												
A 800	P	P	P	P	P	P	P	P	P	N	N	N
B 400	P	P	P	P	P	P	P	P	P	P	N	N
C 200	P	P	P	P	P	P	P	P	P	P	N	N
D 100	P	P	P	P	P	P	P	P	P	P	N	N
E 50	P	P	P	P	P	P	P	P	P	P	N	N
F 25	P	P	P	P	P	P	P	P	P	P	N	N
G 12.5	P	P	P	P	P	P	P	P	P	P	N	N
H 0	P	P	P	P	P	P	P	P	P	P	N	N

Observation: The Rif MIC was found to be 512 µg/mL and Mi+Rif MIC was 800+256 µg/mL

XII. Res 13 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Res 13 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Res 13 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
←Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
Mi↓												
A 800	P	P	P	P	P	P	P	P	N	N	N	N
B 400	P	P	P	P	P	P	P	P	N	N	N	N
C 200	P	P	P	P	P	P	P	P	N	N	N	N
D 100	P	P	P	P	P	P	P	P	P	N	N	N
E 50	P	P	P	P	P	P	P	P	P	N	N	N
F 25	P	P	P	P	P	P	P	P	P	N	N	N
G 12.5	P	P	P	P	P	P	P	P	P	N	N	N
H 0	P	P	P	P	P	P	P	P	P	N	N	N

Observation: The Rif MIC was found to be >1024µg/mL and Mi+Rif MIC was 200+128 µg/mL

XIII. Rres 2 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Rres 2 Rifampicin MIC							Remarks		MIC >1024 µg/mL	
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Rres 2 Mi+Rif Combination Assay							Remarks		Reduction in MIC noted	
← Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
A 800	P	P	P	P	P	P	P	P	N	N	N	N
B 400	P	P	P	P	P	P	P	P	N	N	N	N
C 200	P	P	P	P	P	P	P	P	N	N	N	N
D 100	P	P	P	P	P	P	P	P	N	N	N	N
E 50	P	P	P	P	P	P	P	P	N	N	N	N
F 25	P	P	P	P	P	P	P	P	N	N	N	N
G 12.5	P	P	P	P	P	P	P	P	N	N	N	N
H 0	P	P	P	P	P	P	P	P	P	N	N	N

Observation: The Rif MIC was found to be 512 µg/mL and Mi+Rif MIC was 100+8 µg/mL

XIV. Rres 5 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Rres 5 Rifampicin MIC							Remarks		MIC >1024 µg/mL	
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Rres 5 Mi+Rif Combination Assay							Remarks		Reduction in MIC noted	
← Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
A 800	P	P	P	P	P	P	P	P	P	N	N	N
B 400	P	P	P	P	P	P	P	P	P	N	N	N
C 200	P	P	P	P	P	P	P	P	P	N	N	N
D 100	P	P	P	P	P	P	P	P	P	P	N	N
E 50	P	P	P	P	P	P	P	P	P	P	N	N
F 25	P	P	P	P	P	P	P	P	P	P	N	N
G 12.5	P	P	P	P	P	P	P	P	P	P	N	N
H 0	P	P	P	P	P	P	P	P	P	P	N	N

Observation: The Rif MIC was found to be >1024µg/mL and Mi+Rif MIC was 200+256 µg/mL

XV. Rres 9 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		Rres 9 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		Rres 9 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
←Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
Mi↓												
A 800	P	P	P	P	P	P	P	P	P	P	P	P
B 400	P	P	P	P	P	P	P	P	P	P	P	P
C 200	P	P	P	P	P	P	P	P	P	P	P	P
D 100	P	P	P	P	P	P	P	P	P	P	P	P
E 50	P	P	P	P	P	P	P	P	P	P	P	P
F 25	P	P	P	P	P	P	P	P	P	P	P	P
G 12.5	P	P	P	P	P	P	P	P	P	P	P	P
H 0	P	P	P	P	P	P	P	P	P	P	P	P

Observation: The Rif MIC was found to be >1024µg/mL and Mi+Rif MIC did not change.

XVI. XDR1 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR1 Rifampicin MIC							Remarks		MIC = 512 µg/mL	
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	P	P	P	P	P	P	P	P	P	P
B	N	N	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR1 Mi+Rif Combination Assay							Remarks		Reduction in MIC noted	
←Rif	1 0	2 0.5	3 1	4 2	5 4	6 8	7 16	8 32	9 64	10 128	11 256	12 512
A 800	P	P	P	N	N	N	N	N	N	N	N	N
B 400	P	P	P	N	N	N	N	N	N	N	N	N
C 200	P	P	P	N	N	N	N	N	N	N	N	N
D 100	P	P	P	N	N	N	N	N	N	N	N	N
E 50	P	P	P	N	N	N	N	N	N	N	N	N
F 25	P	P	P	N	N	N	N	N	N	N	N	N
G 12.5	P	P	P	N	N	N	N	N	N	N	N	N
H 0	P	P	P	P	N	N	N	N	N	N	N	N

Observation: The Rif MIC was found to be 512 µg/mL and Mi+Rif MIC was 12.5+2 µg/mL

XVII. XDR2 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR2 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR2 Mi+Rif Combination Assay						Remarks		No reduction in MIC		
←Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	1	2	4	8	16	32	64	128	256	512	1024
A 800	P	P	P	P	P	P	P	P	P	P	P	P
B 400	P	P	P	P	P	P	P	P	P	P	P	P
C 200	P	P	P	P	P	P	P	P	P	P	P	P
D 100	P	P	P	P	P	P	P	P	P	P	P	P
E 50	P	P	P	P	P	P	P	P	P	P	P	P
F 25	P	P	P	P	P	P	P	P	P	P	P	P
G 12.5	P	P	P	P	P	P	P	P	P	P	P	P
H 0	P	P	P	P	P	P	P	P	P	P	P	P

Observation: The Rif MIC was found to be >1024µg/mL and Mi+Rif MIC did not change.

XVIII. XDR5 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR5 Rifampicin MIC						Remarks		MIC = 1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	P	P	P	P	P	P	P	P	P	P	P
B	N	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR5 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
← Rif Mi↓	1	2	3	4	5	6	7	8	9	10	11	12
	0	1	2	4	8	16	32	64	128	256	512	1024
A 800	P	P	P	P	P	P	P	P	P	P	N	N
B 400	P	P	P	P	P	P	P	P	P	P	N	N
C 200	P	P	P	P	P	P	P	P	P	P	N	N
D 100	P	P	P	P	P	P	P	P	P	P	N	N
E 50	P	P	P	P	P	P	P	P	P	P	P	N
F 25	P	P	P	P	P	P	P	P	P	P	P	N
G 12.5	P	P	P	P	P	P	P	P	P	P	P	N
H 0	P	P	P	P	P	P	P	P	P	P	P	N

Observation: Rif MIC was found to be 1024 µg/mL and Mi+Rif MIC was 100+512 µg/mL

XIX. XDR6 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR6 Rifampicin MIC						Remarks		MIC = 256 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	N	N	N	P	P	P	P	P	P	P	P	P
B	N	N	N	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR6 Mi+Rif Combination Assay						Remarks		No growth		
←Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	0.25	0.5	1	2	4	8	16	32	64	128	256
A 800	N	N	N	N	N	N	N	N	N	N	N	N
B 400	N	N	N	N	N	N	N	N	N	N	N	N
C 200	N	N	N	N	N	N	N	N	N	N	N	N
D 100	N	N	N	N	N	N	N	N	N	N	N	N
E 50	N	N	N	N	N	N	N	N	N	N	N	N
F 25	N	N	N	N	N	N	N	N	N	N	N	N
G 12.5	N	N	N	N	N	N	N	N	N	N	N	N
H 0	N	N	N	N	N	N	N	N	N	N	N	N

Observation: Rif MIC was found to be 1024µg/mL, but no growth in combination assay

XX. XDR7 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR7 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1	2	3	4	5	6	7	8	9	10	11	12
	1024	512	256	128	64	32	16	8	4	2	1	0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR7 Mi+Rif Combination Assay						Remarks		No growth		
← Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	1	2	4	8	16	32	64	128	256	512	1024
A 800	N	N	N	N	N	N	N	N	N	N	N	N
B 400	N	N	N	N	N	N	N	N	N	N	N	N
C 200	N	N	N	N	N	N	N	N	N	N	N	N
D 100	N	N	N	N	N	N	N	N	N	N	N	N
E 50	N	N	N	N	N	N	N	N	N	N	N	N
F 25	N	N	N	N	N	N	N	N	N	N	N	N
G 12.5	N	N	N	N	N	N	N	N	N	N	N	N
H 0	N	N	N	N	N	N	N	N	N	N	N	N

Observation: Rif MIC was found to be 1024µg/mL, but no growth in combination assay

XXI. XDR8 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR8 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR8 Mi+Rif Combination Assay						Remarks		No growth		
← Rif	1	2	3	4	5	6	7	8	9	10	11	12
Mi↓	0	1	2	4	8	16	32	64	128	256	512	1024
A 800	N	N	N	N	N	N	N	N	N	N	N	N
B 400	N	N	N	N	N	N	N	N	N	N	N	N
C 200	N	N	N	N	N	N	N	N	N	N	N	N
D 100	N	N	N	N	N	N	N	N	N	N	N	N
E 50	N	N	N	N	N	N	N	N	N	N	N	N
F 25	N	N	N	N	N	N	N	N	N	N	N	N
G 12.5	N	N	N	N	N	N	N	N	N	N	N	N
H 0	N	N	N	N	N	N	N	N	N	N	N	N

Observation: Rif MIC was found to be 1024µg/mL, but no growth was observed in combination assay

XXII. XDR9 MIC and Combination Assay Results:

Microdilution Broth Assay												
Strain Name		XDR9 Rifampicin MIC						Remarks		MIC >1024 µg/mL		
	1 1024	2 512	3 256	4 128	5 64	6 32	7 16	8 8	9 4	10 2	11 1	12 0.5
A	P	P	P	P	P	P	P	P	P	P	P	P
B	P	P	P	P	P	P	P	P	P	P	P	P

Checkerboard Assay												
Assay Name		XDR9 Mi+Rif Combination Assay						Remarks		Reduction in MIC noted		
←Rif	1 0	2 1	3 2	4 4	5 8	6 16	7 32	8 64	9 128	10 256	11 512	12 1024
Mi↓												
A 800	P	P	P	P	P	P	P	P	P	N	N	N
B 400	P	P	P	P	P	P	P	P	P	N	N	N
C 200	P	P	P	P	P	P	P	P	P	P	N	N
D 100	P	P	P	P	P	P	P	P	P	P	N	N
E 50	P	P	P	P	P	P	P	P	P	P	N	N
F 25	P	P	P	P	P	P	P	P	P	P	N	N
G 12.5	P	P	P	P	P	P	P	P	P	P	N	N
H 0	P	P	P	P	P	P	P	P	P	P	N	N

Observation: Rif MIC was found to be >1024µg/mL and Mi+Rif MIC was 400+256 µg/mL

5.6 Strains Showing Significant Reduction in MIC In the Presence of Myoinositol:

Graph 5.8: RIF MIC and Mi+Rif MIC Scatter Plots

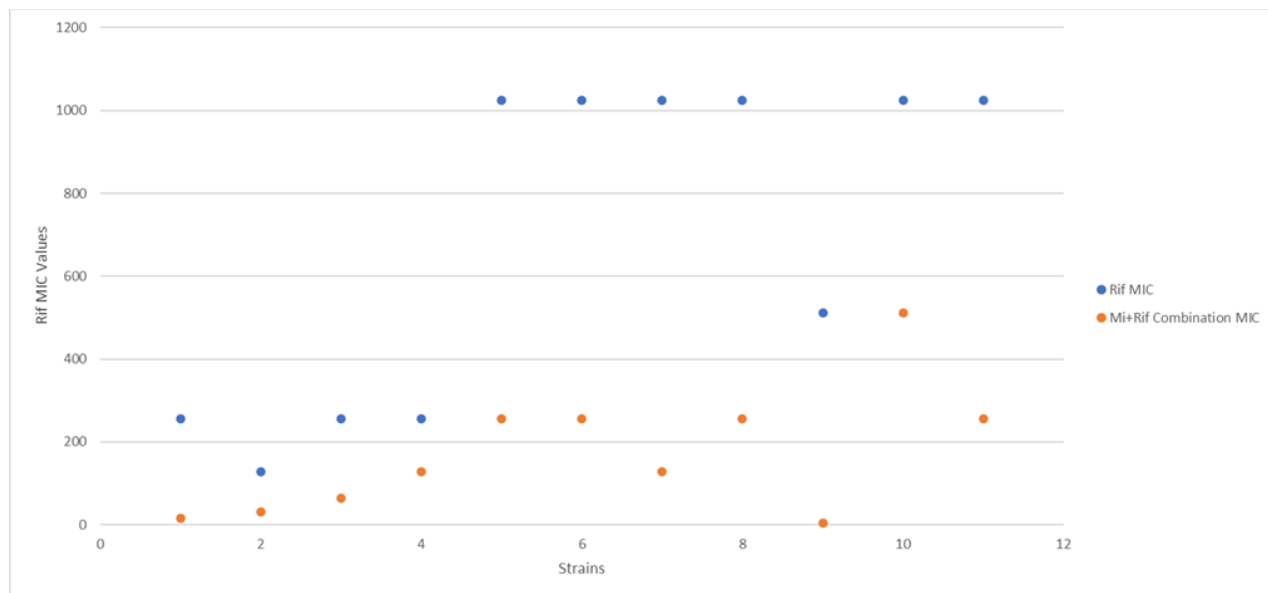


Table 5.8: Rif MIC and Reduced Mi+Rif MIC Values

Strain Name	Rif MIC	Mi+Rif Combination MIC
Res 1	256	16
Res 2	128	32
Res 5	256	64
Res 9	256	128
Res 12	1024	256
Res 13	1024	256
Rres 2	1024	128
Rres 5	1024	256
XDR1	512	2
XDR5	1024	512
XDR9	1024	256

5.7 Infection Assay Results

- Infection assay template and results for Res 1 MDR Strain for testing Mi+Rif combination and 2 MS (EL, C1) encapsulated formulations.

Infection Assay Template						
	1	2	3	4	5	6
A	Mi 100 + Rif 4	Mi 100 + Rif 8	Mi 100 + Rif 16	Mi 100 + Rif 32	Mi 100 + Rif 64	EL 4
B	EL 256	EL 128	EL 64	EL 32	EL 16	EL 8
C	C1 256	C1 128	C1 64	C1 32	C1 16	C1 8
D	C1 4	Mi Control	Rif 512 Control	Rif 8 Control	Infected Control	Infected Control

Infection Assay Results						
	1	2	3	4	5	6
A	0	0	0	0	1	2
B	0	C	39	0	1	C
C	0	0	342	321/C	C	306
D	C/63	276	C	1	C	290

Observation: All readings are given in CFU/ml. Due to contamination and insufficient growth; this infection assay result is not considered and needs to be repeated.

- Infection assay template and results for Res 2, Res 5 MDR Strains, R1 XDR strain and Rres 2 Mono-resistant strain for testing Mi+Rif Combinations

Infection Assay Template						
	1	2	3	4	5	6
A	Res 2 4000 + 4	Res 2 4000 + 8	Res 2 4000 + 16	Res 2 512	Res 2 Control	Res 1 Control
B	Res 5 200 + 64	Res 5 200 + 128	Res 5 200 + 256	Res 5 512	Res 5 Control	Res 1 Control
C	R1 800 + 2	R1 800 + 0.4	R1 800 + 8	R1 512	R1 Control	Uninfected Control
D	Rres 2 400 + 128	Rres 2 400 + 256	Rres 2 400 + 512	Rres 2 512	Rres 2 Control	Uninfected Control

Infection Assay Results						
	1	2	3	4	5	6
A	342	164	146	N	389	375
B	N	N	N	N	345	367
C	306	314	326	N	324	311
D	78/C	69/C	C	N/C	179	359/C

Observation: All readings are given in CFU/ml. Due to insufficient growth and inconsistency in the readings, this infection assay result is not considered and needs to be repeated.

5.8 Paired Samples t Test using IBM-SPSS:

GET DATA

/TYPE=TXT

/FILE="C:\Users\User\Desktop\Combination Data.csv"

/DELCASE=LINE

/DELIMITERS=","

/ARRANGEMENT=DELIMITED

/FIRSTCASE=1

/IMPORTCASE=ALL

/VARIABLES=

V1 F4.0

V2 F4.0.

CACHE.

EXECUTE.

DATASET NAME DataSet1 WINDOW=FRONT.

T-TEST PAIRS=V1 WITH V2 (PAIRED)

/CRITERIA=CI (.9500)

/MISSING=ANALYSIS.

T-Test

Table 5.9: IBM-SPSS Parameters Notes for t Test

Output Created	09-MAR-2024 17:02:14	
Comments		
	Data	C:\Users\User\Desktop\Combination Data.csv
	Active Dataset	DataSet1
Input	Filter	<none>
	Weight	<none>
	Split File	<none>

N of Rows in Working Data File	22
Definition of Missing Value Handling	User defined Missing values are treated as Missing.
Cases Used	Statistics for each analysis are based on the cases with no Missing or out-of-range data for any variable in the analysis.
Syntax	T-TEST PAIRS=V1 WITH V2 (PAIRED) /CRITERIA=CI (.9500) /MISSING=ANALYSIS.
Resources	Processor Time Elapsed Time
	00:00:00.00 00:00:00.00

[DataSet1]

Table 5.10: Paired Samples Statistics

	Mean	N	Std. Deviation	Std. Error Mean
Pair 1 V1	568.73	22	407.644	86.910
V2	335.45	22	359.378	76.620

Table 5.11: Paired Samples Correlations

	N	Correlation	Sig.
Pair 1 V1 & V2	22	.670	.001

Table 5.12: Paired Samples Test

	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
Pair 1 V1 - V2	233.273	314.789	67.113	93.703	372.843	3.476	21	.002

Observation: Here V1 is a data set containing standalone rifampicin MIC and V2 is a data set containing the rifampicin MIC after addition of myoinositol.

5.9 Discussion of Results

By interpreting the insilico analysis done for the four potential EPI namely, Myoinositol, Dodecanoic Acid (DA), Palmitic Acid (PA) and Methyl Stearate (MS), it is found that the solubility parameters required for drugability of DA, PA and MS are found to not adhere to the suggested range. The Log P value, which indicates the permeability and hydrophobic binding to macromolecules including target protein pumps and the Log D value which indicates the target's lipophilicity and hydrophilicity to dissolve in body fluids and penetrate the biomembrane are solubility at oral absorption for PA and MS are found to be lesser than the lower limit. The only compound for which all three of these parameters are found to be good is Myoinositol. Although they are not drugable, we suggest that DA, PA, and MS be encapsulated using suitable nanoparticles to increase their drugability as they are really potent EPI.

The practical aim of this project is to find whether the EPI and the drug have one of these three different drug interactions: synergism, antagonism, and indifference. According to the Loewe Additivity Model, synergism refers to a situation where the combined effect of two or more drugs is greater than the sum of their individual effects. In other words, synergy occurs

when drugs work together to produce a stronger therapeutic effect than they would individually. Antagonism, on the other hand, occurs when the combined effect of two or more drugs is less than the sum of their individual effects, meaning that the drugs interfere with each other's action. Indifference occurs when the combined effect of two drugs is equal to the sum of their individual effects, resulting in no significant enhancement or reduction in therapeutic activity.

MS was encapsulated successfully using an undisclosed nanoparticle and the efficacy of the four encapsulated formulations was tested in M. TB infected THP1 cells. But the tests did not lead to any positive results for any of the formulations. This leads us to investigate other formulations and thus no decipherable results were obtained for MS.

The evaluation of myoinositol as an efflux pump inhibitor (EPI) and its potential synergism with rifampicin presents intriguing insights into combating multidrug-resistant strains of *Mycobacterium tuberculosis* (M. TB). Myoinositol, a naturally occurring sugar alcohol, has exhibited promising properties against M. TB due to its ability to disrupt efflux pump activity. As observed in the MDB assay for Mi, in higher concentrations of Mi (1600µg/ml), there is significant shrinkage and deformation of M. TB cells which can be attributed to the presence of Mi. This phenomenon was found to not only be effective against MDR strains of M. TB but also against XDR and Mono-resistant strains.

The efficacy of myoinositol as an EPI can be attributed to several factors. Firstly, its structural similarity to glucose allows for efficient uptake by M. TB, facilitating its intracellular action in both THP1 cells and M. TB cells. Additionally, myoinositol has been found to interfere with efflux pump function through direct interaction with pump components thereby impeding drug extrusion. On analysis of the checkerboard assay results of the tested 22 strains, 11 strains have shown reduction in MIC of rifampicin with almost 5 strains showing a significant reduction in MIC (>3 step reduction in MIC). The fact that Myoinositol is effective was also statistically proven through a paired samples t test done for all before and after Mi MIC values of the twenty-two strains. The calculated t at (-3.476) is far less than the table t value (1.7) at a significance of 5% which can be interpreted to mean that Mi has a significant effect in the reduction of MIC of strains.

But when tested in combination with isoniazid, no reduction in MIC was observed in any strains. Further investigation through literature survey found that Myoinositol can only inhibit the efflux pumps that are specific to rifampicin. It can also be stated that the failure of Myoinositol in the other strains can be attributed to the fact that they possess some other means of exhibiting multi-drug resistance.

Comparative analysis with existing efflux pump inhibitors, such as piperine and verapamil, sheds light on the diverse mechanisms by which these compounds potentiate the activity of antimicrobial agents. Piperine, a bioactive compound found in black pepper, has been shown to inhibit efflux pump activity in *M. TB* by modulating gene expression or interfering with pump assembly. The present study also provides additional novel findings, in which synergism between PIP and SM was observed in the *M. tuberculosis* reference strain, being observed effect was similar in susceptible and MDR clinical isolates. The aminoglycoside SM was first used to treat TB in 1946. However, resistance to SM was observed as soon as 1948. *M. tuberculosis* was found to develop resistance to SM because of its sole usage for TB treatment. The synergistic effect between PIP and SM that was observed in this study raised the possibility that SM added to PIP could be, in some cases, useful as treatment for resistant TB. This draws attention to further studies of combinations of available drugs, including those that are no longer used for the treatment of TB (e.g., SM), which may be a quick and economical strategy for helping patients who are infected with drug-resistant TB. [57][39]

Similarly, Verapamil, a calcium channel blocker, exerts its EPI effects by binding to specific pump proteins and preventing drug expulsion. Verapamil, characterized by a pKa of 8.92 and a cLogP of 5.23, is considered a lipophilic weak base or a cationic amphiphile due to its tertiary amine, which becomes protonated at physiological pH. These properties suggest its potential to dissolve in lipid bilayers. Indeed, previous biophysical studies have demonstrated that verapamil can be inserted into artificial membranes, altering their electrical properties. This aligns with indirect observations indicating its association with mycobacterial membranes. It was also found to increase the efficacy of bedaquiline in mice to an extent that is the same as that to which it increases systemic bedaquiline exposure in plasma. They concluded that verapamil's adjunctive activity in vivo is likely due to enhanced systemic exposure to companion drugs via effects on mammalian transporters rather than to inhibition of bacterial pumps.[47][48]

Of note, verapamil demonstrates its effects across a broad range of concentrations, consistent with observed potentiation both in vivo and in vitro. At low Micromolar concentrations, which are typically achieved in patients and mirrored in mice, a pharmacokinetic interaction with rifampin is observed. This interaction results in increased oral exposure over 8 hours, likely due to the inhibition of P-glycoprotein (P-gp) and other eukaryotic transporters known to be targeted by verapamil. Similar effects are noted when rifampin is administered to steady state levels.

The results obtained from testing the synergism of piperine and verapamil with rifampicin underscore the potential of combination therapy in overcoming drug resistance in M.TB. Other findings demonstrate that the co-administration of piperine or verapamil with rifampicin significantly enhances the bactericidal activity of rifampicin against drug-resistant strains of M.TB. This synergistic effect likely arises from the dual inhibition of efflux pumps by the EPIs, leading to increased intracellular concentrations of rifampicin and improved bacterial killing.

Hence the difference between the mode of action of myoinositol and verapamil is that although verapamil is a known efflux pump inhibitor, its effects are more targeted towards eukaryotic transporters. But with myoinositol, it is guaranteed that it only affects the efflux pump inhibitors.

CHAPTER 6

SUMMARY AND CONCLUSION

The various techniques and drugs innovated for curing tuberculosis are vast and numerous. Amidst this landscape, the present study sheds light on the therapeutic potential of myoinositol as an efflux pump inhibitor (EPI) and its synergistic interaction with rifampicin in combating drug-resistant *M. tuberculosis*. Through rigorous experimentation and analysis, our study has unveiled promising prospects for this novel combination therapy.

Notably, the statistical significance of our findings has been underscored by conducting a paired t-test on the before and after minimum inhibitory concentration (MIC) values obtained through the combination assays. This statistical validation reinforces the efficacy of the myoinositol-rifampicin combination in addressing drug-resistant strains of *M. tuberculosis*.

However, the journey towards combating tuberculosis is far from over. Further study is warranted to delve into the underlying mechanisms of action governing the synergy between myoinositol and rifampicin. Understanding these mechanisms will not only deepen our comprehension of tuberculosis pathogenesis but also pave the way for optimizing treatment regimens. By refining and optimizing these regimens, we can strive towards achieving enhanced clinical efficacy in the management of drug-resistant tuberculosis cases.

In conclusion, the integration of myoinositol as an efflux pump inhibitor with rifampicin presents a promising avenue in the battle against drug-resistant *M. tuberculosis*. As we continue to unravel the intricacies of this therapeutic synergy, we move closer towards the goal of effectively combating tuberculosis and alleviating the burden it imposes on global health.

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