

# ***In silico* AND *In vitro* VALIDATION: ASSESSING ALLICIN'S ANTIMYCOBACTERIAL ACTIVITY**

Submitted in partial fulfillment of the requirement for the award of  
Bachelor of Technology Degree in Biotechnology.

By

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**DEPARTMENT OF BIOTECHNOLOGY  
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

**SATHYABAMA**  
INSTITUTE OF SCIENCE AND TECHNOLOGY  
(DEEMED TO BE UNIVERSITY)  
Accredited with Grade "A++" by NAAC  
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**APRIL – 2024**



# SATHYABAMA

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## DEPARTMENT OF BIOTECHNOLOGY

### BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **Ilhan Shamir Ali. M (40230051)** who carried out the project entitled "***In Silico* AND *In Vitro* VALIDATION: ASSESSING ALLICIN'S ANTIMYCOBACTERIAL ACTIVITY**" under our supervision from **November 2023** to **March 2024**.

  
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## DECLARATION

I, **Ilhan Shamir Ali M (40230051)**, hereby declare that the Project Report entitled "***In Silico AND In Vitro* VALIDATION: ASSESSING ALLICIN'S ANTIMYCOBACTERIAL ACTIVITY**" done by me under the guidance of **Dr. Jayshree Nellore Ph.D., Department of Biotechnology (Internal)** and **Dr. R. Sam Ebenezer Ph.D., Centre for Ocean Research, Sathyabama Research Park (External) at Sathyabama Institute of Science and Technology, Chennai, TamilNadu, India** is submitted in partial fulfillment of the requirements for the award of **Bachelor of Technology Degree in Biotechnology**.

**DATE:** 03/04/2024

**PLACE:** Chennai

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**SIGNATURE OF THE CANDIDATE**

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## ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (M.Tb), remains a major global health threat. The emergence of drug-resistant strains underscores the urgent need for novel therapeutic strategies. Allicin, a bioactive compound derived from garlic, exhibits potential antimicrobial properties. This study explored allicin's potential as an anti-tuberculosis agent using both in silico and in vitro approaches. In silico docking simulations predicted favorable interactions between allicin and the Mycobacterial ATP synthase rotor ring, a crucial metabolic enzyme. In vitro experiments demonstrated significant partial inhibition of *M.Tb* H37Rv growth by allicin extract. Additionally, cytotoxicity assays with HEK293 cells revealed no significant toxicity within the tested allicin concentrations. While a definitive minimum inhibitory concentration (MIC<sub>90</sub>) was not established within the tested range, these findings suggest allicin's potential antimycobacterial activity. This research provides a starting point for further investigations focused on optimizing allicin delivery for higher concentrations, elucidating its mechanism of action for ATP synthase disruption, and evaluating its efficacy and safety in pre-clinical models of tuberculosis. This study highlights the need for further investigations to fully explore allicin's potential as a novel candidate for the development of new anti-tuberculosis therapies.

**Keywords:** Antibacterial, *Mycobacterium tuberculosis*, allicin, natural products, ATP synthase, drug development, in silico modeling

## TABLE OF CONTENTS

| CHAPTER<br>NO. | TITLE                                                             | PAGE<br>NO. |
|----------------|-------------------------------------------------------------------|-------------|
|                | LIST OF FIGURES                                                   | vi          |
|                | LIST OF ABBREVIATIONS                                             | viii        |
|                | LIST OF TABLES                                                    | ix          |
| 1.             | <b>INTRODUCTION</b>                                               | 1           |
| 2.             | <b>REVIEW OF LITERATURE</b>                                       | 3           |
|                | 2.1 BACKGROUND ON TUBERCULOSIS                                    | 3           |
|                | 2.2 CONVENTIONAL TUBERCULOSIS TREATMENT                           | 3           |
|                | 2.2.1 First-Line TB Treatment                                     | 4           |
|                | 2.2.2 Second-Line TB Treatment: Addressing Drug<br>Resistance     | 5           |
|                | 2.2.3 Third-Line TB Treatment: A Last Resort for<br>Complex Cases | 6           |
|                | 2.3 DRUG DELIVERY CHALLENGES                                      | 7           |
|                | 2.3.1 Solubility and Bioavailability Challenges<br>Forms          | 7           |
|                | 2.3.2 Sustained Drug Release Systems                              | 8           |
|                | 2.3.3 Targeted Drug Delivery                                      | 8           |
|                | 2.3.4. Patient Adherence and the Importance of Dosage<br>Forms    | 9           |

|        |                                                        |    |
|--------|--------------------------------------------------------|----|
| 2.4    | NOVEL DRUG DELIVERY STRATEGIES                         | 9  |
| 2.4.1  | Nanoparticle-based Drug Delivery Systems               | 10 |
| 2.4.2  | Inhalable Drug Delivery                                | 12 |
| 2.4.3  | Liposomal Drug Delivery                                | 13 |
| 2.4.4  | Implantable Drug Delivery Systems                      | 15 |
| 2.4.5  | Theranostic Approaches for TB                          | 16 |
| 2.5    | ALLICIN'S POTENTIAL WITHIN NOVEL DRUG DELIVERY SYSTEMS | 16 |
| 2.5.1  | Future Research Directions                             | 18 |
| 2.5.2  | Conclusion                                             | 18 |
| 3.     | <b>AIM AND SCOPE</b>                                   | 19 |
| 3.1    | AIM                                                    | 19 |
| 3.2    | SCOPE                                                  | 19 |
| 4.     | <b>MATERIALS AND METHODS</b>                           | 21 |
| 4.1    | <i>In Silico</i>                                       | 21 |
| 4.1.1  | Protein Preparation                                    | 21 |
| 4.1.2  | Ligand Preparation                                     | 21 |
| 4.1.3  | Molecular Docking Using PyRx                           | 22 |
| 4.1.4. | Validation of Molecular Docking Using iGEMDOCK         | 22 |
| 4.1.5. | Visualization Of Docking Using Biovia Discovery Studio | 22 |
| 4.2    | <i>In Vitro</i>                                        | 22 |
| 4.2.1  | Preparation of Crude Garlic Extract                    | 22 |
| 4.2.2  | Characterization of Crude Garlic Extracts              | 24 |

|           |                                                           |    |
|-----------|-----------------------------------------------------------|----|
| 4.2.3     | Separation of Allicin From Crude Garlic Extract           | 25 |
| 4.2.4     | Maintenance Of <i>M. Tb</i> In LJ Slants                  | 25 |
| 4.2.5     | MIC <sub>90</sub> Determination of Allicin Extract        | 26 |
| 4.2.6     | Cell Culture                                              | 27 |
| 4.2.7     | Assessment Of % Inhibition - MTT Assay On<br>HEK293 Cells | 28 |
| 4.3       | STATISTICAL ANALYSIS                                      | 28 |
| <b>5.</b> | <b>RESULTS AND DISCUSSION</b>                             | 29 |
| 5.1       | <i>In Silico</i> ANALYSIS                                 | 29 |
| 5.1.1     | Water Solubility                                          | 31 |
| 5.1.1.1   | Interpretation of Table 5.2                               | 32 |
| 5.1.2     | Lipophilicity                                             | 33 |
| 5.1.2.1   | Interpretation of Table 5.3                               | 34 |
| 5.1.3     | Pharmacokinetic Properties                                | 35 |
| 5.1.3.1   | Interpretation of Table 5.4                               | 36 |
| 5.1.4     | Toxicity Prediction – PRO TOX II                          | 37 |
| 5.1.4.1   | Interpretation of Table 5.5                               | 38 |
| 5.1.5     | Docking Results From PyRx                                 | 38 |
| 5.1.5.1   | Intepretation of Docking Results                          | 39 |
| 5.1.6     | Validation of Docking using iGEM DOCK:                    | 40 |
| 5.1.6.1   | Interpretation of iGEM Docking                            | 41 |

|          |                                                                    |    |
|----------|--------------------------------------------------------------------|----|
|          | 5.1.7 Binding Sites of Allicin                                     | 42 |
|          | 5.2 <i>In Vitro</i> STUDIES                                        | 43 |
|          | 5.2.1 HPLC-LCMS Analysis of Crude Garlic Extract                   | 43 |
|          | 5.2.2 HPLC-LCMS Separation of Allicin                              | 44 |
|          | 5.2.3 MIC 90 Determination of Allicin Extract by Cord<br>Formation | 45 |
|          | 5.2.4 Assessment of % Viability - MTT Assay on<br>HEK293 Cells     | 48 |
|          | 5.3 STATISTICAL ANALYSIS OF MIC 90 DETERMINATION                   | 49 |
|          | <b>DISCUSSION</b>                                                  | 51 |
| <b>6</b> | <b>SUMMARY AND CONCLUSION</b>                                      | 52 |
|          | <b>REFERENCES</b>                                                  |    |
|          | <b>ANNEXURE</b>                                                    |    |

## LIST OF FIGURES

| Figure no | Name of figure                                                                                                                                    | Page number |
|-----------|---------------------------------------------------------------------------------------------------------------------------------------------------|-------------|
| 4.1       | A. Centrifugation of Garlic Extract with Milli-Q Water and Ethyl Acetate<br>B. Separation and Storage of Aqueous Extract (Milli-Q Water Fraction) | 24          |
| 4.2       | A. Growth of Mycobacterium tuberculosis on LJ Slant Figure                                                                                        | 26          |
| 4.3       | Visual Representation of Cord Formation Assay for MIC 90 Determination                                                                            | 27          |
| 4.4       | A. HEK293 Cells Seeded in a 96-Well Plate for MTT Assay<br>B. Formation of Formazan Crystals in MTT Assay After 36-Hour Incubation                | 28          |
| 5.1       | 2D Chemical Structure of Allicin                                                                                                                  | 31          |
| 5.2       | Binding of Allicin to the Mycobacterial ATP synthase rotor ring                                                                                   | 40          |
| 5.3       | 2D Interaction Diagram of Allicin Binding to the Mycobacterial ATP Synthase Rotor Ring                                                            | 42          |

|      |                                                                                                                                                                                    |    |
|------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----|
| 5.4  | Top binding sites of Allicin on the Mycobacterial ATP synthase rotor ring—<br>Image from Biovia Discovery studio                                                                   | 43 |
| 5.5  | HPLC-LCMS Chromatogram of Garlic Extract (Extraction Method 1)                                                                                                                     | 44 |
| 5.6  | HPLC-LCMS Chromatogram of Garlic Extract (Extraction Method 2)                                                                                                                     | 44 |
| 5.7  | HPLC Chromatogram of allicin separation from garlic extract (2:1 water:ethyl acetate ratio) with UV detection at 254 nm                                                            | 45 |
| 5.8  | A. M.Tb H37Rv Growth Control (No Inhibition)<br>B. Partial Inhibition of M.Tb H37Rv by Allicin Extract (400 µg/mL)<br>C. Complete Inhibition of M.Tb H37Rv by Rifampicin (1 µg/mL) | 47 |
| 5.9  | Bar graph showing % of viable cells with each allicin concentration and rifampicin                                                                                                 | 48 |
| 5.10 | Confirmation of Mycobacterium tuberculosis Cord Formation by Acid-Fast Staining                                                                                                    | 48 |
| 5.11 | Effect of Allicin Concentration on HEK293 Cell Viability                                                                                                                           | 49 |
| 5.12 | Effect of Allicin Extract on M. Tuberculosis H37Rv Growth: Results of Student's t-Test Analysis                                                                                    | 50 |

## LIST OF ABBREVIATIONS

| ACRONYM                           | EXPANSION                                                    |
|-----------------------------------|--------------------------------------------------------------|
| <b>ATP synthase</b>               | Adenosine Triphosphate synthase                              |
| <b>CYP</b>                        | Cytochrome P450                                              |
| <b>DMSO</b>                       | Dimethyl sulfoxide                                           |
| <b>ESOL</b>                       | Estimation Programs for Organic Liquids                      |
| <b>GI</b>                         | Gastrointestinal                                             |
| <b>HEK</b>                        | Human Embryonic Kidney cells                                 |
| <b>HIV</b>                        | Human Immunodeficiency Virus                                 |
|                                   | Minimum Inhibitory Concentration required to inhibit 90% of  |
| <b>MIC<sub>90</sub></b>           | bacterial growth                                             |
| <b><i>M.Tb</i></b>                | Mycobacterium tuberculosis                                   |
| <b>MTT</b>                        | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| <b>MDR-TB</b>                     | Multidrug Resistant Tuberculosis                             |
| <b>XDR-TB</b>                     | Extensively drug-resistant                                   |
| <b>INH</b>                        | Isoniazid                                                    |
| <b>BBB</b>                        | Blood-Brain Barrier                                          |
| <b>LogP or LogP<sub>o/w</sub></b> | Octanol/Water Partition Coefficient                          |
| <b>PDB</b>                        | Protein Data Bank                                            |
|                                   | High-performance liquid chromatography coupled with mass     |
| <b>HPLC-LCMS</b>                  | spectrometry                                                 |
| <b>RMSD</b>                       | Root Mean Square Deviation                                   |
| <b>LJ Slant</b>                   | Lowenstein-Jensen Slant                                      |

## LIST OF TABLES

| <b>Table no</b> | <b>Table name</b>                                                                                             | <b>Page no</b> |
|-----------------|---------------------------------------------------------------------------------------------------------------|----------------|
| 2.1             | WHO recommended doses of the Second line Anti Tb drugs                                                        | 6              |
| 2.2             | WHO recommended doses of the Third line Anti Tb drugs                                                         | 7              |
| 2.3             | Nanocarriers and Their Impact on Drug Bioavailability                                                         | 11             |
| 4.1             | Mycobacterium tuberculosis Protein Target and PDB ID                                                          | 21             |
| 4.2             | Comparison of Extraction Solvent Ratios for Method 1 and Method 2                                             | 23             |
| 5.1             | Properties of Allicin                                                                                         | 29             |
| 5.2             | Water solubility of Allicin                                                                                   | 32             |
| 5.3             | Lipophilicity of Allicin                                                                                      | 34             |
| 5.4             | Pharmacokinetic properties of Allicin                                                                         | 36             |
| 5.5             | Toxicity prediction - PRO TOX II                                                                              | 37             |
| 5.6             | Docking results from PyRx                                                                                     | 39             |
| 5.7             | Docking validation using iGEM                                                                                 | 41             |
| 5.8             | XYZ Coordinates of the top binding site of Allicin on the Mycobacterial ATP synthase rotor ring               | 42             |
| 5.9             | Results of the Cord Formation Assay for MIC <sub>90</sub> Determination of Allicin Extract against M.Tb H37Rv | 46             |



# CHAPTER 1

## INTRODUCTION

Despite decades of effort, tuberculosis (TB) remains one of the deadliest infectious diseases worldwide, killing over a million people each year. This chronic and highly contagious disease is primarily caused by *Mycobacterium*, with *M. tuberculosis* and *M. bovis* being the notable strains responsible. While TB typically targets the lungs, it can also manifest as extrapulmonary TB, affecting various bodily organs, including the eyes, bones, neurons, and blood vessels. Alarmingly, approximately one-third of the world's population remains susceptible to TB infection, with untreated active TB cases potentially leading to the infection of 10 to 15 individuals annually (Banyal, S., *et al.*, 2013).

The World Health Organization (WHO) underscored the gravity of this issue in 2008 when it reported an estimated 9.4 million TB cases worldwide, with a significant portion of these cases originating in India, where TB prevalence is notably high, accounting for one-fifth of the global TB burden (Patil, K., *et al.*, 2018).

However, combating TB presents a daunting challenge, particularly due to the complexity of TB treatment, often requiring long-term medication regimens and the emergence of drug-resistant (MDR) tuberculosis bacterial strains (MDR-TB), has necessitated innovative approaches to enhance the effectiveness and patient compliance of therapy (De Maio, F., *et al.*, 2019).

In this context, exploring natural compounds like allicin, derived from garlic, offers a promising alternative approach. Allicin is a potent organosulfur compound responsible for the pungent aroma and flavor of garlic. Numerous studies have demonstrated the antibacterial, antifungal, and antiviral properties of allicin, making it an attractive candidate for exploring its potential as an anti-TB agent. Allicin has exhibited broad-spectrum antimicrobial activity against various bacterial species, including *Mycobacterium tuberculosis* (Ye, C.L., *et al.*, 2013).

Its ability to inhibit the growth and survival of *M. tuberculosis* suggests its potential as an anti-TB agent. Several studies report its ability to disrupt essential bacterial processes, hindering cell wall synthesis and interfering with vital metabolic pathways. Moreover, allicin may enhance the host's immune response, aiding in the

fight against TB and studies have reported synergistic effects when allicin is combined with conventional anti-TB drugs, such as isoniazid and rifampicin (Gupta, S., *et al.*, 2020).

These synergistic interactions may enhance the efficacy of existing treatments and potentially reduce the development of drug resistance. Allicin has been shown to be effective against drug-resistant strains of *M. tuberculosis*, including multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains (Borlinghaus, J., *et al.*, 2014, Pyun, M.S. & Shin, S., 2020).

This property makes allicin a promising candidate for combating the growing challenge of drug resistance in TB treatment. In addition to its direct antimicrobial activity, allicin has demonstrated immunomodulatory effects, enhancing the host's immune response against pathogens (Arreghán, V.A., *et al.*, 2015). This property could potentially aid in the clearance of TB infection and improve treatment outcomes.

The instability of allicin in aqueous solutions presents a challenge. However, various strategies, including encapsulation in nanoparticles, liposomal formulations, and chemical modifications, are being explored to improve its stability and bioavailability (Miron, T., *et al.*, 2002, Zheng, X., *et al.*, 2013).

## CHAPTER 2

### REVIEW OF LITERATURE

#### 2.1 BACKGROUND ON TUBERCULOSIS

Tuberculosis (TB) remains a persistent global health threat, characterized by its chronic and highly contagious nature. It is primarily caused by *Mycobacterium*, with *M. tuberculosis* and *M. bovis* being the notable strains responsible. While TB typically targets the lungs, it can also manifest as extrapulmonary TB, affecting various bodily organs. Alarmingly, approximately one-third of the world's population remains susceptible to TB infection, with untreated active TB cases potentially leading to the infection of 10 to 15 individuals annually (Banyal *et al.*, 2013).

The World Health Organization (WHO) underscored the gravity of this issue in 2008 when it reported an estimated 9.4 million TB cases worldwide, with India facing a notably high TB prevalence, accounting for one-fifth of the global TB burden (Patil *et al.*, 2018). However, combating TB presents a daunting challenge, particularly due to the complexity of TB treatment, often requiring long-term medication regimens and the emergence of drug-resistant (MDR) tuberculosis bacterial strains (MDR-TB), has necessitated innovative approaches to enhance the effectiveness and patient compliance of therapy (De Maio *et al.*, 2019). One such compound with potential is allicin, derived from garlic. Allicin exhibits antimicrobial properties, and our research aims to investigate its effectiveness against tuberculosis and explore the potential for optimizing its delivery through novel systems. In recent years, novel drug delivery systems have emerged as a promising frontier in the battle against this infectious disease. This search for innovation has led to the exploration of novel drug delivery systems, which offer the potential to improve the precision, safety, and efficacy of TB treatment.

#### 2.2 CONVENTIONAL TB TREATMENT: STANDARD ANTIBIOTIC REGIMENS

Tuberculosis (TB) treatment is categorized into first-line, second-line, and third-line treatments, depending on the drugs used and their respective roles in managing TB. Here's an overview of these treatment categories:

### **2.2.1 First-Line TB Treatment**

The cornerstone of tuberculosis (TB) management is first-line treatment, the standard regimen used to combat drug-susceptible TB. This regimen combines antibiotics highly effective against most *Mycobacterium tuberculosis* strains. The primary objective of first-line treatment is twofold: to achieve a cure for the TB infection and to prevent the emergence of drug-resistant strains. The standard first-line TB regimen comprises four key drugs:

1. Isoniazid (INH): Isoniazid is a key component of first-line TB treatment. It inhibits the synthesis of mycolic acids, an essential component of the TB cell wall, ultimately leading to bacterial death (Vilchèze and Jacobs, 2019).
2. Rifampicin (RMP): Rifampicin targets the RNA polymerase of *M. tuberculosis*, disrupting RNA synthesis and protein production, leading to bacterial death (Piccaro *et al.*, 2014).
3. Pyrazinamide (PZA): Pyrazinamide's mechanism is not entirely understood, but it is believed to work best in an acidic environment, disrupting bacterial metabolism (Zhang *et al.*, 2014).
4. Ethambutol (EMB): Ethambutol interferes with the synthesis of arabinogalactan, another component of the TB cell wall, inhibiting bacterial growth (Palomino and Martin, 2014).

The standard treatment for drug-susceptible TB involves administering these four drugs—isoniazid, rifampicin, ethambutol, and pyrazinamide—for six months (Dua *et al.*, 2018). Approximately four decades ago, a shorter therapeutic regimen was introduced, comprising isoniazid, rifampicin, pyrazinamide, and ethambutol for two months, followed by a subsequent four-month regimen of isoniazid and rifampicin. This treatment approach has remained largely unchanged since its inception (Dartois and Rubin, 2022). In clinical studies, cure rates for patients receiving first-line anti-TB medications, including rifampicin, isoniazid, pyrazinamide, and ethambutol, have reached impressive levels of up to 95%. However, success rates can vary, dropping as low as 65% in certain regions (Dartois and Rubin, 2022). Despite the efficacy of these drugs, an important challenge arises due to the rapid metabolism and excretion of the drugs once administered, limiting their efficacy in therapy. A key factor in successful antibiotic therapy is achieving and

maintaining a critical drug concentration within the body for an adequate duration. Free antibiotics, while effective, have a rapid onset of action and a brief duration of activity. Consequently, maintaining therapeutic levels of these antibiotics at specific sites in the body necessitates frequent dosing throughout the day. Moreover, the need to increase drug doses to achieve therapeutic benefits, often because of limited drug absorption, can result in a higher incidence of adverse effects (Xiong *et al.*, 2014). This risk stems from primary resistance, where individuals are initially infected with drug-resistant *M. tuberculosis* strains (Sharma *et al.*, 2020). It is compounded by issues like poor patient compliance and inconsistent adherence to drug treatment. Patients infected with strains resistant to isoniazid and rifampicin, collectively referred to as multidrug-resistant (MDR) TB, pose a significant clinical challenge, as they are almost untreatable with standard first-line therapies. Treating MDR-TB involves administering pyrazinamide concurrently with second-line drugs (Seung *et al.*, 2015).

### **2.2.2 Second-Line TB Treatment: Addressing Drug Resistance**

When first-line therapies fail due to drug resistance, the treatment of TB becomes more complex. Second-line treatment regimens are employed for cases such as multidrug-resistant (MDR) or extensively drug-resistant (XDR) TB.

Second-line treatment is used when TB strains are resistant to one or more of the first-line drugs, leading to MDR-TB or XDR-TB. Second-line drugs are less effective, more toxic, and often more costly than first-line agents (Sosnik *et al.*, 2010). The choice of second-line drugs and the duration of treatment can vary depending on the resistance profile of the TB strain. Successful completion of second-line treatment is critical in managing drug-resistant TB. Recommended dosages for various second-line anti-TB drugs are provided in Table 2.1

**Table 2.1 : WHO recommended doses of the Second line Anti Tb drugs**

| <b>Second Line anti-Tb Drugs</b> | <b>Dosing</b>                                                              |
|----------------------------------|----------------------------------------------------------------------------|
| Cycloserine                      | Initial : 250 mg per day<br>Maintenance : 500mg to 1g per Day per os       |
| Ethionamide                      | 15 to 20 mg/kg/Day per os                                                  |
| Kanamycin                        | 5 to 7.5mg/kg/dose divided 8 to 12 h IM or IV                              |
| Capreomycin                      | Initial : 1g/day ( 60 -120 days )<br>Maintenance: 1g – 2 to 3 times a week |
| P-aminosalicylic(PAS)            | 4g/Day per os                                                              |

Second-line TB treatment is usually more challenging and requires a more extended duration of treatment, ranging from 9 to 24 months, to achieve a cure (Sosnik *et al.*, 2010). Successful completion of second-line treatment is critical in managing drug-resistant TB.

### ***2.2.3 Third-Line TB Treatment: A Last Resort for Complex Cases***

Third-line TB treatment is reserved for cases where MDR-TB or XDR-TB does not respond to second-line drugs or when there are severe adverse effects from second-line medications. These drugs have limited efficacy, and their use is often considered a last resort. Third-line drugs may include (Table 2.2):

- Linezolid
- Imipenem-cilastatin
- Clofazimine
- Amoxicillin-clavulanate
- Meropenem
- Thioacetazone

**Table 2.2 : WHO recommended doses of the Third line Anti Tb drugs**

| Third- line anti-TB drugs    | Dosing                                                          |
|------------------------------|-----------------------------------------------------------------|
| Clarithromycin               | 500mg twice daily                                               |
| Linezolid                    | 600mg once daily                                                |
| Thioacetazone                | 150mg/day                                                       |
| Imipenem                     | 1000mg IV twice daily                                           |
| Amoxicillin and clauvulanate | Amoxicillin 2000mg/day and Clauvalnate 125mg/day twice daily    |
| Bedaquiline                  | 400mg /day for 14 days<br>200mg/day 3 times a week for 22 weeks |
| Delamanid                    | 100mg twice daily for 24 weeks                                  |

Third-line TB medications have limited efficacy or have not demonstrated consistent effectiveness (Future Microbiology, 2023). The World Health Organization (WHO) does not include these drugs in its primary recommendations (World Health Organization, 2011).

In summary, the choice of TB treatment depends on the susceptibility profile of the TB strain. First-line treatment is reserved for drug-susceptible TB, second-line treatment for drug-resistant TB, and third-line treatment remains a last resort in challenging cases. Proper management of TB, including drug resistance, requires careful consideration of the specific drugs to be used and close medical supervision

### 2.3 DRUG DELIVERY CHALLENGES IN TB THERAPY

Despite the progress made with conventional tuberculosis (TB) treatments, their effectiveness is often hindered by several drug delivery challenges.

#### **2.3.1 Solubility and Bioavailability Challenges**

Drug solubility and bioavailability are crucial for effective tuberculosis (TB) therapy. The poor aqueous solubility of many anti-TB drugs is a significant pharmaceutical challenge, necessitating the development of appropriate formulation strategies (Amrutha *et al.*, 2020). Limited solubility can compromise drug absorption and bioavailability, resulting in suboptimal drug levels at target sites. This, in turn, can allow TB bacteria to persist and potentially develop resistance (Di *et al.*, 2012).

Furthermore, compounds with insufficient solubility face a higher risk of attrition and increased costs in drug development (Di *et al.*, 2012). For example, isoniazid (INH) absorption can be reduced when taken with food due to the presence of fatty substances (Lin *et al.*, 2010).

Drug metabolism is another critical factor. It involves transforming the drug into a more hydrophilic compound for elimination through the kidneys (Gaedigk *et al.*, 2017). This process primarily occurs within the liver. For highly permeable drugs like INH, transporters have a minimal impact on its absorption into hepatocytes (Shugarts and Benet, 2009). The blood-brain barrier (BBB) presents an additional challenge in treating extrapulmonary TB. This highly selective barrier protects the brain but also restricts the passage of many drugs. Consequently, achieving therapeutic drug concentrations within the brain to combat TB infection remains a significant hurdle in treatment. Innovative approaches, such as nanotechnology-based drug delivery systems and lipid-based formulations, hold promise in enhancing the solubility and bioavailability of anti-TB drugs.

### **2.3.2 Sustained Drug Release Systems**

The absence of sustained drug release systems is another limitation in conventional TB therapy. Such systems enhance drug bioavailability, improve targeting precision, reduce drug toxicity, and crucially, ensure patient adherence (Ahmad and Khuller, 2008).

Researchers are exploring various strategies, including polymer-based drug delivery systems, microparticles, and implants, to achieve sustained drug release. These innovations aim to optimize TB treatment by maintaining therapeutic drug levels over extended periods, improving overall outcomes.

### **2.3.3 Targeted Drug Delivery**

Precision in drug delivery is paramount in TB therapy. To address limitations of conventional treatments that may not effectively reach infected tissues, researchers are developing targeted drug delivery systems capable of delivering drugs specifically to TB-infected areas. Drug targeting generally refers to the ability of a drug to accumulate selectively in the target organ or tissue (Torchilin, 2010).

Targeted drug delivery falls into two main categories: passive and active. Passive targeting leverages the enhanced permeability and retention (EPR) effect, where nanoparticles tend to accumulate in areas like tumors due to leaky vasculature (Schafer-Korting, 2010; Yhee *et al.*, 2013). Active targeting in tuberculosis treatment involves attaching specific ligands to drugs that recognize and bind to receptors expressed by cells at the infected site. This approach lowers the effective drug dose needed, reducing unnecessary distribution and potential side effects (Torchilin, 2010 ; Pinheiro *et al.*, 2011).

Nanoparticles functionalized with ligands or antibodies that recognize TB-specific receptors hold potential for precise drug targeting. Such approaches minimize collateral damage to healthy tissues, enhance drug efficacy, and reduce the risk of resistance development.

#### **2.3.4. Patient Adherence and the Importance of Dosage Forms**

Successful drug delivery also relies heavily on patient adherence to treatment regimens. Innovative dosage forms and strategies play a crucial role in improving adherence. Ensuring patient adherence to protracted TB treatment regimens remains a formidable challenge. Non-adherence can lead to treatment failure and the emergence of drug-resistant strains. Innovations in dosage forms, including pediatric-friendly formulations and fixed-dose combinations, are critical for simplifying treatment and improving patient compliance.

Drug delivery challenges in TB therapy underscore the need for continued research. The potential of natural compounds like allicin, when coupled with innovative delivery systems, warrants further investigation. By exploring potential synergies between natural products and cutting-edge drug delivery technologies, we may unlock novel therapeutic avenues. This research contributes to the ongoing effort to develop more effective and patient-friendly TB treatments, contributing to the global fight against these persistent diseases.

## **2.4 NOVEL DRUG DELIVERY STRATEGIES**

Tuberculosis (TB) remains a formidable global health challenge, necessitating continuous exploration of innovative drug delivery approaches to enhance treatment effectiveness and patient friendliness. Novel drug delivery

strategies are at the forefront of efforts to advance TB therapy, with their primary objectives being the improvement of drug efficacy, minimization of adverse effects, and enhancement of patient adherence. This section offers a comprehensive examination of cutting-edge drug delivery methods that hold immense potential to transform TB treatment, addressing the multifaceted challenges of TB therapy and ultimately contributing to the global mission to combat this infectious disease (Verma *et al.*, 2022). The fusion of pharmaceutical science and technology has opened doors for the development of ideal drug delivery systems, offering the potential to treat diseases more effectively with superior medication delivery technologies.

#### **2.4.1 Nanoparticle-based Drug Delivery Systems**

Nanoparticles, with at least one dimension between 1-100 nanometres, offer a promising avenue for revolutionizing drug delivery (Griffiths *et al.*, 2010). Researchers are actively investigating the use of nanoparticle-encapsulated pharmaceuticals as an alternative to administering antibiotics and other medications in their free form (Sosnik *et al.*, 2010; Kreuter, 2004). Advantages of nanoparticle-based drug delivery systems for TB treatment include:

- I. **Enhanced Stability and Prolonged Action:** Nanoparticles protect drugs, maintaining high stability and exerting therapeutic effects over an extended period.
- II. **High Drug Loading Capacity:** These versatile carriers can encapsulate multiple medications within their matrix.
- III. **Reduced Adverse Effects:** Nanoparticle-based drug delivery systems tend to exhibit fewer adverse effects compared to traditional pharmaceuticals, improving patient safety and comfort.
- IV. **Improved Bioavailability:** Nanoparticles facilitate controlled, delayed, and sustained drug release, optimizing the drug's availability to the body. Specific examples of enhanced bioavailability with various nanocarriers are outlined in Table 2.3
- V. **Versatile Administration Methods:** These systems can be adapted for various administration routes, including oral delivery and inhalation, enhancing treatment flexibility.

- VI. Minimized Side Effects and Enhanced Patient Compliance: By reducing side effects and offering convenient dosing regimens, nanoparticle-based drug delivery systems promote patient adherence to treatment, a crucial factor in TB therapy.

**Table 2.3 : Nanocarriers and Their Impact on Drug Bioavailability**

| VII. Drug | Nanocarrier                          | Bioavailability (%) | Reference                      |
|-----------|--------------------------------------|---------------------|--------------------------------|
| Isoniazid | Solid lipid nanoparticles (SLNs)     | 69-95               | (Reddy <i>et al.</i> , 2012)   |
|           | Nanostructured lipid carriers (NLCs) | 70-95               | (Jain <i>et al.</i> , 2009)    |
|           | Liposomes                            | 80-95               | (Kumar <i>et al.</i> , 2009)   |
|           | Chitosan nanoparticles               | 75-90               | (Bajpai <i>et al.</i> , 2005)  |
|           | Dendrimers                           | 70-90               | (Moghimi <i>et al.</i> , 2001) |
|           | Micelles                             | 75-90               | (Kataoka <i>et al.</i> , 2001) |
|           | Polymer nanoparticles                | 70-90               | (Langer, 1990)                 |
|           | Carbon nanotubes                     | 75-90               | (Prato, 2005)                  |
|           | Mesoporous silica nanoparticles      | 70-90               | (Slowing <i>et al.</i> , 2008) |
|           | Quantum dots                         | 75-90               | (Slowing <i>et al.</i> , 2008) |

#### **2.4.1.1 Targeted Drug Delivery**

Targeted drug delivery aims to increase the precision of drug delivery through the use of ligands that bind to specific receptors expressed by cell populations at the diseased site (Kingsley *et al.*, 2006). Ligands such as sugars, monoclonal

antibodies, RNA/DNA aptamers, or peptides are often added to nanoparticle surfaces to achieve this targeted approach (Kingsley *et al.*, 2006). For instance, mannose has been used as a targeting ligand for nanoparticles due to its interactions with mannose receptors on the surface of macrophages (Chono *et al.*, 2007). By increasing the targeted delivery of TB medications to infected cells, these techniques increase treatment efficacy while reducing adverse effects[45].

#### **2.4.1.2 Potentials of Nanoparticles**

Nanoparticles can improve the absorption of the active ingredients, lower the therapeutic dosage, and make elements more soluble. Additionally, they are stable, non-toxic, non-thrombogenic, non-immunogenic, non-inflammatory (they do not activate neutrophils), and they avoid the reticuloendothelial system, making them favorable when employed in blood. Polymeric nanoparticles are occasionally employed to access certain tissues or do activities on the cell surface (Buzea *et al.*, 2007). Moxifloxacin is a fluoroquinolone antibiotic which is used against *M. tuberculosis* (Gosling *et al.*, 2003). This resulted in effective inhibition of intracellular *M. tuberculosis* growth at a much lower concentration, potentially reducing side effects (Kisich *et al.*, 2007). In another study, encapsulating frontline drugs rifampicin, isoniazid, and pyrazinamide in poly(DLlactide-co-glycolide (PLG) nanoparticles (PLG-NP) significantly extended their mean residence time and elimination half-life compared to the free forms when administered orally (Pandey *et al.*, 2003; Booyesen *et al.*, 2013).

#### **2.4.2 Inhalable Drug Delivery**

Antimicrobial medications for respiratory infections are often administered orally or parenterally. However, systemic delivery can result in low drug concentrations at the infection site due to poor pulmonary distribution. Direct drug delivery to the lungs via inhalation offers the potential for significantly higher drug concentrations, enhancing therapeutic efficacy (Wood and Swanson, 2007). Inhaled therapies, including medications delivered via aerosols, have been widely used for conditions such as cystic fibrosis (Borsje *et al.*, 2000). Researchers are actively investigating the use of inhalable drug delivery systems for tuberculosis treatment.

Several studies have focused on the physico-chemical characteristics of formulations and how different aerosol delivery systems can deliver anti-TB drugs

effectively to the lungs. Studies in animal models have demonstrated the potential of these systems, but further research is needed to evaluate their efficacy in realistic infection scenarios (Muttill *et al.*, 2009). Currently, there are no commercially available inhalable anti-TB medications for human use (Muttill *et al.*, 2009).

To effectively target *Mycobacterium tuberculosis* (*M.tb*), particularly within alveolar macrophages (AMΦs), inhaled anti-TB drugs require specialized delivery systems. These systems must have aerodynamic properties that allow for deep lung penetration (ideally 1-3 μm particle size) and facilitate endocytosis by AMΦs (Edwards *et al.*, 1998). Encapsulating drugs within carriers like microparticles, nanoparticles, liposomes, micelles, or dendrimers can address these challenges (Muttill *et al.*, 2007; Madhav and Kala, 2011; Gradon and Sosnowski, 2014). Such systems protect drugs from degradation, prolong their release, improve macrophage uptake, and potentially reduce drug toxicity.

Dry powder inhalers (DPIs) offer advantages as a delivery device for inhalable TB therapies. They are propellant-free, portable, user-friendly, and cost-effective. DPIs are breath-actuated, facilitating rapid, high-dose administration and efficient drug deposition within the lungs (Pilcer and Amighi, 2010).

Inhaled therapy for TB offers numerous potential benefits, including:

- Dosage Reduction: Significantly lower doses may be needed compared to oral therapy.
- Reduced Side Effects: Potential for fewer adverse effects, including liver, kidney, and peripheral nervous system toxicity.
- Targeted Delivery: Directly targets infected macrophages.
- Shorter Treatment Duration: Potential to shorten treatment, improving cost-effectiveness and adherence.
- Repurposing Potential: Systemically toxic drugs could be re-evaluated for inhalable delivery

#### **2.4.3 Liposomal Drug Delivery**

Liposomes are versatile nanoscale vesicles composed of lipid materials, often phospholipids and cholesterol, that mimic cell membranes. They encapsulate drugs and come in various sizes, charges, and compositions, influencing their

behavior within the body (Kaur *et al.*, 2014). Liposomes efficiently interact with macrophages, offering opportunities for targeted drug delivery or sustained release over time. This makes them valuable delivery systems for diseases like cancer, and they continue to make substantial therapeutic contributions, with numerous formulations already on the market or in clinical trials (Chang, 2012).

Liposomes can encapsulate both hydrophilic and hydrophobic drugs, making them suitable carriers for diverse molecules (Schwendener and Schott, 2009). For TB treatment, a liposome-in-hydrogel system for localized delivery to treat bone TB has shown promising results (Alexander *et al.*, 2013; Liu *et al.*, 2019). This system incorporated DINH-loaded liposomes into a thermosensitive PLGA-PEG-PLGA hydrogel matrix, demonstrating self-healing benefits and sustained drug release.

Due to their composition and size, liposomes are often cleared from circulation by the mononuclear phagocyte system (MPS) (Immordino *et al.*, 2006). This characteristic, while sometimes viewed as a limitation, can be advantageous for targeted delivery of antimicrobial drugs to treat infections like TB that reside within MPS cells (Deol *et al.*, 1997).

For TB treatment, liposomes can be customized to enhance specificity through surface modifications like the attachment of ligands. This active targeting approach promotes accumulation in infected tissues, potentially increasing therapeutic efficacy while reducing toxicity to healthy tissues.

Additional advantages of liposomal drug delivery for TB treatment include:

- Versatility: Suitable for various administration routes, including inhalation and intravenous injection.
- Encapsulation: Accommodates drugs with different solubilities.
- Biocompatibility: Promotes safety for use in the body.
- Targeted Delivery: Can be designed to accumulate in specific target tissues.

Liposomes must be carefully tailored in size for optimal delivery within the respiratory tract (Gill *et al.*, 2007). In the context of tuberculosis, liposomal drug delivery utilizes two key targeting strategies:

**Passive Targeting:** This approach relies on the inherent distribution of liposomes composed of particular lipids. Liposomes of suitable size (<5  $\mu\text{m}$ ) and composition

are readily taken up by alveolar macrophages (AMs) via phagocytosis, making this strategy beneficial for pulmonary TB treatment, especially when using inhalation delivery (El-Ridy *et al.*, 2007). In extrapulmonary TB, intravenous administration can also benefit from passive targeting by the phagocytic cells of the MPS.

**Active Targeting:** This involves modifications to the liposome surface, such as attaching ligands (proteins, peptides, etc.) to target specific cells. Other strategies include coating with PEG to extend circulation time or incorporating negatively charged lipids to increase AM uptake. Ligand-anchored liposomes, macrophage activators like tuftsin, and strategies targeting mannose receptors all hold potential in TB treatment (Olsson *et al.*, 2011; Vyas *et al.*, 2004; El-Ridy *et al.*, 2007).

#### **2.4.4 Implantable Drug Delivery Systems**

Implantable drug delivery systems (IDDSs) offer a promising solution to challenges faced with oral drug administration, including issues of bioavailability, stability, toxicity, and dosing frequency. Developed to reduce dosing frequency, prolong drug release, improve patient adherence, and minimize systemic side effects, IDDSs hold interest for delivering a wide range of medications (Martín del Valle *et al.*, 2009). Antibiotics, especially those with limited oral bioavailability or needing site-specific targeting, could particularly benefit from this delivery approach (Schmidmaier *et al.*, 2006).

For tuberculosis treatment, IDDSs present a potential avenue for more effective and patient-centric drug delivery (Dong *et al.*, 2014). Implants typically release drugs over extended periods, from days to years (Kumar and Pillai, 2018). They can be designed for systemic effects (often via intravenous, intramuscular, or subcutaneous routes) or localized effects by placing implants directly at target sites (Laser *et al.*, 2015).

Preclinical studies have demonstrated the potential of implants for TB treatment. A single implant containing isoniazid (INH) in a PLGA copolymer maintained therapeutic INH levels for up to 8 weeks in rabbits (Kailasam *et al.*, 1994). Research in mice using isoniazid and pyrazinamide implants has also shown promise. By bypassing first-pass metabolism and potential drug degradation in the GI tract, implantable systems can enhance the bioavailability of some anti-TB drugs (Dash and Cudworth, 1998; Liao *et al.*, 2020).

Additional advantages of IDDSs include:

- Improved Patient Compliance: Implants can significantly reduce dosing frequency, potentially simplifying treatment regimens.
- Reversible: Implants can be removed early if needed, such as in the case of adverse effects (Dash and Cudworth, 1998).

#### **2.4.5 Theranostic Approaches for TB**

Theranostics, a rapidly evolving field in medicine, offers a promising approach to integrate diagnosis and treatment of TB. Theranostic agents enable targeted drug delivery and real-time imaging of drug distribution and therapeutic response. This allows for treatment monitoring, individualized adjustments, and ultimately, more efficient therapies (Rabin *et al.*, 2008). A promising class of theranostic agents for TB utilizes fluorophores with aggregation-induced emission (AIE) properties. These molecules exhibit enhanced fluorescence brightness when aggregated and can be designed to include photosensitizing properties. In TB theranostics, AIE fluorophores offer the dual benefits of diagnosis and treatment (Rabin *et al.*, 2008). For example, the RIF-loaded AIE carrier (TTD+RIF NPs) accumulated in TB granulomas via the EPR effect, providing a strong fluorescent signal for diagnosis and demonstrating potent antibacterial efficacy against drug-resistant TB strains (Wang *et al.*, 2018). Another approach involves combining biocompatible hyaluronic acid with the drug rifampicin and the dye Cy5.5 to create GSH-activatable RIF@Cy5.5-HA-NG. This compound targeted Mycobacterium TB with high selectivity and biocompatibility and demonstrated its potential as an anti-tuberculosis therapeutic with focused imaging and treatment capabilities (Liao *et al.*, 2020; Zheng *et al.*, 2022).

### **2.5 ALLICIN'S POTENTIAL WITHIN NOVEL DRUG DELIVERY SYSTEMS**

While allicin exhibits promising antimicrobial activity against Mycobacterium tuberculosis, its direct use presents limitations in stability, bioavailability, and potential for rapid degradation in biological environments (Wang *et al.*, 2014). The novel drug delivery strategies explored in this review offer exciting avenues for overcoming these challenges, potentially harnessing the full therapeutic potential of allicin within optimized systems.

**Nanoparticles for Allicin Delivery:** Nanoparticle-based drug delivery systems hold immense potential for enhancing allicin's therapeutic profile. Their small size and customizable surface properties make them ideal carriers for both targeted delivery and protection of encapsulated molecules (Patra *et al.*, 2018). Allicin-loaded nanoparticles could be functionalized with ligands for targeted delivery to macrophages harboring *Mycobacterium tuberculosis*, potentially increasing efficacy (Ahmad *et al.*, 2006). Various biocompatible materials, including lipids, polymers, and even natural biomolecules, could be used to create these nanoscale carriers, enhancing allicin stability and controlling its release profile.

**Liposomes for Targeted Delivery:** Liposomal formulations, with their biocompatibility and ability to encapsulate molecules of varying solubilities, present another promising carrier system for allicin (Bulbake *et al.*, 2017). Liposomes can be designed for passive targeting or functionalized with ligands for active targeting to infected macrophages. The lipid bilayer of liposomes could shield allicin from degradation, while their internal aqueous compartment can maintain its soluble form for controlled release. This versatile system has the potential to optimize allicin's pharmacokinetics and reduce adverse effects.

**Inhalable Delivery for Pulmonary TB:** Direct delivery of allicin to the lungs via inhalation offers potential advantages for treating pulmonary TB (Sosnik *et al.*, 2010). Aerosolized allicin formulations within optimized carrier systems, such as microparticles or dry powder inhalers, could achieve high local drug concentrations and more rapid therapeutic action at the infection site. Careful design of these systems is crucial for ensuring that allicin reaches its intended site of action within the complex airway architecture.

**Implantable Delivery for Sustained Release:** While more research is needed, implantable allicin delivery systems could be valuable for chronic TB cases or localized therapy (García-González *et al.*, 2016). Biodegradable materials could be designed to release allicin in a controlled manner over extended periods, potentially improving patient adherence and reducing the need for frequent dosing. Minimally invasive surgical techniques could be employed for implant placement and subsequent removal after the course of treatment.

### **2.5.1 Future Research Directions**

Realizing the potential of allicin-loaded delivery systems for TB treatment requires rigorous research across multiple stages. In vitro studies are needed to investigate allicin's stability within different carrier materials, its release kinetics, and potential interactions with carrier components. Additionally, cellular studies could evaluate the uptake of allicin-loaded delivery systems by infected macrophages and their anti-mycobacterial efficacy. In vivo models are essential to evaluate the safety and efficacy of these systems in realistic TB infection scenarios, providing insights into their pharmacokinetics, biodistribution, and therapeutic potential. Finally, translational research efforts will be crucial for bridging the gap between promising laboratory findings and the development of allicin-based therapies with potential for clinical application.

### **2.5.2 Conclusion**

The integration of allicin into the novel drug delivery strategies described in this review offers a promising avenue for developing more effective and patient-centric TB therapies. By addressing the limitations associated with allicin's direct use, these innovative approaches could unlock its full potential as a valuable anti-tuberculous agent. This research direction has the potential to make a significant contribution to the global fight against this persistent and devastating infectious disease.

## CHAPTER 3

### AIM AND SCOPE OF PRESENT INVESTIGATION

#### 3.1 AIM

To computationally identify potential protein targets of allicin in *M. tuberculosis* through molecular docking studies and subsequently validate the in vitro anti-tubercular activity of garlic-derived allicin extract using culture-based assays.

#### 3.2 SCOPE

This project aims to explore the potential of allicin, a bioactive compound derived from garlic, as a novel anti-tuberculosis therapeutic. It employs a combined computational and experimental approach to investigate allicin's potential mode of action and its effectiveness against *Mycobacterium tuberculosis*. Specifically, the scope of this project encompasses:

- **Computational Target Identification:** Molecular docking studies will be used to computationally predict potential protein targets of allicin within *Mycobacterium tuberculosis*. These predictions will guide subsequent experimental validation.
- **Allicin Extraction and Characterization:** Crude garlic extract will be prepared, and analytical techniques, such as HPLC-LCMS, will be employed to confirm the presence and isolate allicin.
- **In Vitro Anti-tubercular Activity:** The anti-tubercular activity of the garlic-derived allicin extract will be evaluated using culture-based assays, such as the cord formation assay. This will assess allicin's ability to inhibit the growth of *Mycobacterium tuberculosis*.
- **Cytotoxicity Assessment:** The potential cytotoxicity of allicin will be evaluated using the MTT assay to determine any adverse effects on mammalian cells. This information is crucial for assessing the safety profile of allicin.

**Significance and Outcomes :**

- Tuberculosis remains a major global health challenge, and the rise of drug-resistant strains necessitates the discovery of new treatment options. This project seeks to contribute to this effort by exploring the potential of allicin, a widely available natural compound, as an anti-tubercular agent. The computational and experimental approach allows for the identification of potential mechanisms of action of allicin in combating TB and provides insights into its safety profile. If successful, this work could provide valuable leads for the future development of allicin-based or allicin-inspired therapeutics against tuberculosis.

## CHAPTER 4

### MATERIALS AND METHODS:

#### 4.1 IN SILICO SURVIVAL STUDIES

##### 4.1.1 PROTEIN PREPARATION

The *Mycobacterium tuberculosis* membrane protein, Mycobacterial ATP synthase rotor ring, was selected for docking studies (Table 4.1):

**Table 4.1 : *Mycobacterium tuberculosis* Protein Target and PDB ID**

| Name                                  | PDB ID |
|---------------------------------------|--------|
| Mycobacterial ATP synthase rotor ring | 4V1G   |

The Mycobacterial ATP synthase rotor ring was selected as the target for docking studies with allicin. ATP synthase is a crucial enzyme for energy production in *Mycobacterium tuberculosis*. Its disruption offers a potential therapeutic strategy for combating tuberculosis infections. Additionally, previous research has suggested that natural compounds (Ahmad, Z., *et al.*, 2013) may have inhibitory effects on ATP synthase function.

Crystal structures for these target were retrieved from the RCSB Protein Data Bank (PDB) <https://www.rcsb.org/>. Structure were pre-processed using Molegro Molecular Viewer software by removing water molecules and non-standard residues. Binding sites for the target was identified using Biovia Discovery Studio and PyMOL software. The top binding site for the protein, identified as the most favourable for docking studies, was selected for further analysis.

##### 4.1.2 LIGAND PREPARATION

The structure of allicin was retrieved from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>) (PubChem ID: 65036) in SDF format Relevant physicochemical properties, including canonical SMILES, molecular formula, and molecular weight, were also obtained from PubChem. Drug-likeness properties were evaluated using the Molsoft webserver (<https://molsoft.com/>), and pharmacokinetic properties and medicinal chemistry considerations were analyzed

using the SwissADME website (<http://www.swissadme.ch/>). Toxicity risk assessment was conducted using the Protox-III website (<https://comptox.charite.de/protox3/>). The allicin compound was prepared for docking by loading it into PyRx software and converting it into a PDBQT file.

#### **4.1.3 MOLECULAR DOCKING USING PyRx**

Molecular docking simulations were performed to investigate potential interactions and binding affinities between allicin and the selected *Mycobacterium tuberculosis* protein targets. PyRx software, which provides a graphical user interface (GUI) for the AutoDock Vina molecular docking engine, was utilized for these simulations.

#### **4.1.4. VALIDATION OF MOLECULAR DOCKING USING iGEMDOCK**

To enhance the reliability of the molecular docking studies, the results obtained using PyRx were validated using iGEMDOCK, an integrated graphical environment for molecular docking. This additional validation step strengthens confidence in the predicted binding interactions between allicin and the *Mycobacterium tuberculosis* protein targets.

#### **4.1.5. VISUALIZATION OF DOCKING USING BIOVIA DISCOVERY STUDIO**

BIOVIA Discovery Studio was used to visualize the docking results between allicin and the selected targets. This visualization software allowed for detailed examination of protein-ligand interactions, aiding in understanding the potential mechanisms by which allicin may disrupt target protein function. High-quality images were generated using BIOVIA Discovery Studio to facilitate analysis and presentation of the results.

### **4.2 IN VITRO STUDIES :**

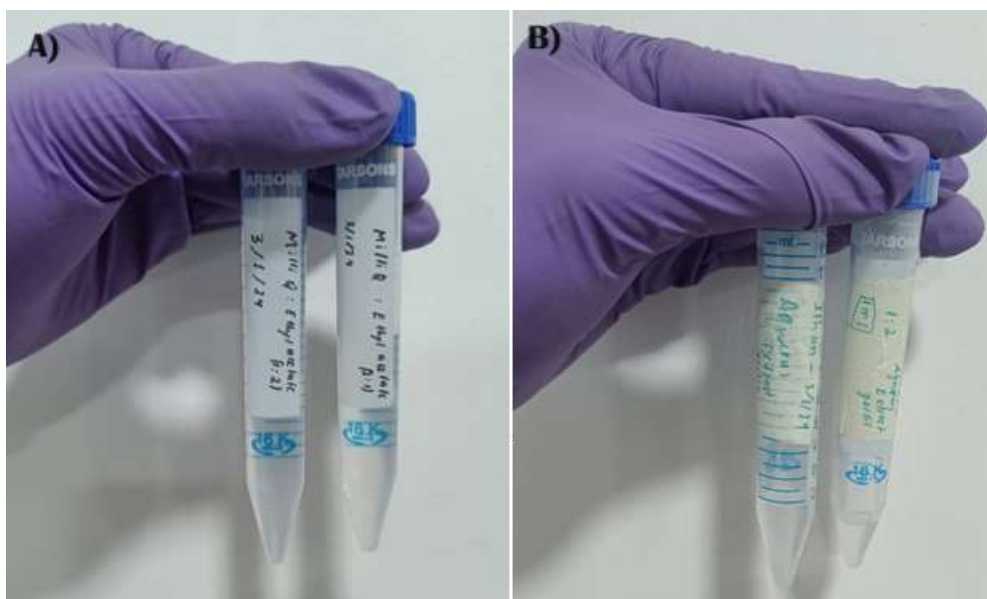
#### **4.2.1 PREPARATION OF CRUDE GARLIC EXTRACTS**

This study employed two modified extraction methods based on the protocol established by Sasidharan S. et al. (2010) to prepare crude garlic extracts from fresh Indian garlic cloves. The variation between the methods lies in the differing ratios of Milli-Q water to ethyl acetate (Table 4.2).

**TABLE 4.2: Comparison of Extraction Solvent Ratios for Method 1 and Method 2**

| Method              | Milli-Q Water (mL) | Ethyl Acetate (mL) | Ratio |
|---------------------|--------------------|--------------------|-------|
| Extraction Method 1 | 4                  | 8                  | 1:2   |
| Extraction Method 2 | 4                  | 2                  | 2:1   |

Fresh garlic cloves (0.1 g) were first homogenized with 1 mL of Milli-Q water to facilitate the release of desired compounds by breaking down cell walls. An additional 3 mL of Milli-Q water was then added to the homogenate to increase the overall volume and create a more suitable aqueous environment for water-soluble extractions. The mixture was then combined with ethyl acetate in the specific ratios outlined in Table 4.2 (1:2 for Method 1 and 2:1 for Method 2). Ethyl acetate, a suitable solvent for extracting hydrophobic compounds, was chosen to target allicin present in the garlic cloves. Following adequate interaction between the phases, centrifugation was performed to achieve separation of the aqueous layer (containing water-soluble components) from the organic layer (containing the ethyl acetate and extracted hydrophobic compounds). Finally, the aqueous extracts from both methods were collected and stored at 4°C for approximately one week before HPLC-LCMS analysis to detect the presence of allicin.



**Fig 4.1 (A) Centrifugation of Garlic Extract with Milli-Q Water and Ethyl Acetate (B) Separation and Storage of Aqueous Extract (Milli-Q Water Fraction)**

#### **4.2.2 Characterization of Crude Garlic Extracts**

High-performance liquid chromatography coupled with mass spectrometry (HPLC-LCMS) is a powerful analytical technique for characterizing complex natural products like crude garlic extracts. It allows for separation of compounds based on their physicochemical properties, as well as identification through molecular mass and fragmentation patterns. This study employed HPLC-LCMS under the following conditions to achieve several goals:

- HPLC Conditions: C18 column, gradient elution 20-60% acetonitrile over 17 min.

Firstly, it aimed to confirm the presence of allicin, the primary bioactive compound in garlic. HPLC-LCMS has proven effective for allicin detection and characterization in garlic extracts [Lee, S., *et al.* (2003)]. Secondly, this method enabled the investigation of other potentially active compounds within the garlic extract, providing insight into its overall chemical composition

#### **4.2.3 SEPERATION OF ALLICIN FROM CRUDE GARLIC EXTRACT**

To isolate allicin from the crude garlic extracts (prepared with both 1:2 and 2:1 milliql:ethyl acetate ratios), HPLC-LCMS separation was employed. The extracts were diluted, filtered, and injected onto a reverse-phase C18 column. A gradient elution program was used to separate the compounds, starting with water: methanol (90:10, v/v) and gradually increasing the methanol percentage over 30 minutes (example: 0-5 min: 90:10; 5-25 min: gradient to 50:50; 25-30 min: hold at 50:50). Separation was monitored with a UV detector set at 254 nm. Fractions were collected based on the UV signal and analysed by LCMS in positive ionization mode to confirm the presence of allicin. LCMS parameters included a capillary voltage of 3.5 kV, cone voltage of 30 V, source temperature of 120°C, and an m/z range of 50-500.

#### **4.2.4 MAINTENANCE OF *M.Tb* IN LJ SLANTS**

To maintain a viable culture of *Mycobacterium tuberculosis* (*M.Tb*) H37Rv strain for experimentation, commercially prepared Lowenstein-Jensen (LJ) slants (HiMedia) were used. All procedures involving *M.Tb* were conducted within a Biosafety Level-Tuberculosis (BSL-TB) laboratory with strict adherence to safety protocols, including the use of appropriate personal protective equipment (PPE). A sterile inoculating loop was used to aseptically transfer a loopful of *M.Tb* H37Rv from a previously established culture with confluent growth onto the surface of a fresh LJ slant, employing a four-quadrant streaking technique for optimal distribution. The inoculated slant was labelled with the strain name, date, and relevant identifiers, then incubated at 37°C. Slants were periodically monitored for characteristic *M.Tb* growth (dry, rough, buff-coloured colonies), typically appearing within 1-3 weeks but potentially extending up to 6 weeks. Upon successful growth confirmation, LJ slants were either used immediately for subculture or sealed with Parafilm and stored at 4°C for longer-term maintenance of culture viability. Figure 4.2 visually depicts the growth of *Mycobacterium tuberculosis* on an LJ slant.



**Figure 4.2: Growth of *Mycobacterium tuberculosis* on LJ Slant**

#### **4.2.5 MIC<sub>90</sub> Determination of Allicin Extract**

Determining the minimum inhibitory concentration (MIC<sub>90</sub>) of a potential antimicrobial agent is crucial for assessing its efficacy and establishing appropriate dosages. The MIC<sub>90</sub> represents the lowest concentration of a compound that inhibits the growth of 90% of a bacterial population. In this study, the cord formation assay was performed to determine the MIC<sub>90</sub> of a concentrated allicin extract against *Mycobacterium tuberculosis* (*M.Tb*) H37Rv.

A 0.75 ml aliquot of purified allicin extract was concentrated at 30°C for 4 hours in an Eppendorf Concentrator Plus to reduce solvent volume [Janská et al., 2021]. The concentrated extract was weighed, the concentration adjusted to 28000 µg/ml (28.8 mg/ml) with DMSO, and then serially diluted in 7H9 broth within a 96-well plate to achieve final concentrations of 400, 200, 100, 50, 25, 12.5, and 6.5 µg/ml. Each allicin concentration was inoculated in triplicate with 100 µl of *M.Tb* H37Rv liquid culture. To confirm the strain's drug sensitivity, two wells were inoculated with *M.Tb* H37Rv and 10 µl of diluted rifampicin control (1 µg/ml final concentration), along with a media contamination control (7H9 broth only) and two growth controls (7H9 broth and *M.Tb* H37Rv). The sealed plate was incubated at 37°C for one week, then visually examined for cord formation (a characteristic clumping of *M.Tb* under inhibition). The MIC<sub>90</sub> was determined as the lowest allicin concentration preventing visible cord formation in at least 90% of the replicate wells.



**Figure 4.3: Visual Representation of Cord Formation Assay for MIC<sub>90</sub> Determination**

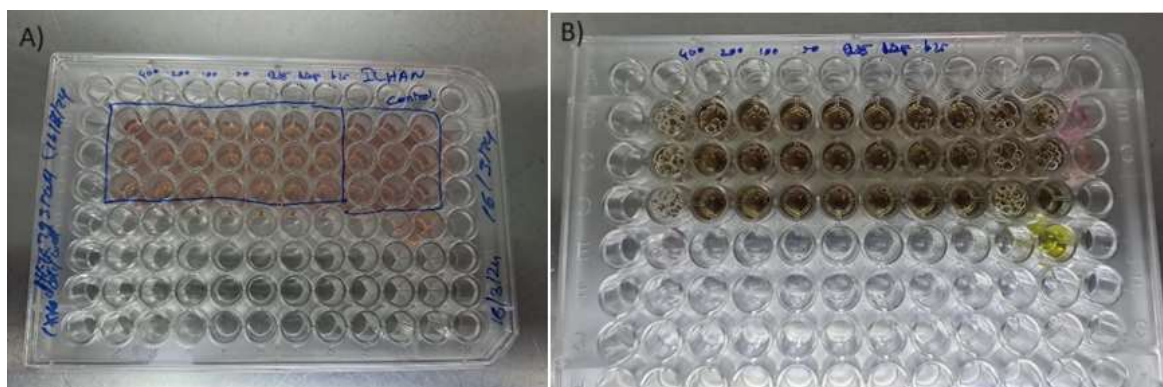
#### **4.2.6 CELL CULTURE**

Human Embryonic Kidney (HEK) cells, specifically the HEK293 cell line, are a widely used model system for various research applications, including drug toxicity testing and studying cellular pathways. In this study, HEK293 cells (purchased from TANUVAS, Chennai) were selected for their reliability, rapid growth rate, and susceptibility to transfection. These qualities make them suitable for preliminary investigations of a compound's potential cytotoxicity, providing insights into its safety profile before testing in more complex disease models.

The HEK293 cells were maintained in DMEM medium containing 100 mg/L penicillin and 100 mg/L streptomycin, supplemented with 10% foetal bovine serum at 37°C in a 5% CO<sub>2</sub> atmosphere. Subculturing was performed after the cells reached 80% - 90% confluency. Prior to passaging, the media was discarded, and the cells were washed using 2 ml of PBS. The cells were then trypsinized with 2 ml of trypsin and incubated in a CO<sub>2</sub> incubator for 2 minutes to encourage cell detachment. 2 ml of serum-containing media was added to the flask to inactivate the trypsin. The cells were centrifuged at 900 rpm for 3 minutes. The supernatant was discarded, and the pellet was collected by gentle aspiration. The pellet was suspended in growth media supplemented with 10% Foetal bovine serum. The cells were then seeded into 6-well and 96-well plates and incubated for 24 hours.

#### 4.2.7 ASSESSMENT OF % INHIBITION – MTT ASSAY ON HEK293 CELLS

HEK293 cells were plated in 96-well microplates, with DMEM culture medium supplemented with 10% foetal bovine serum, and incubated at 37°C and 5% CO<sub>2</sub> overnight. The next day, the cells were subject to treatment with allicin for a period of 36 hours in the following concentrations (in triplicates): 400 µg/ml, 200 µg/ml, 100 µg/ml, 50 µg/ml, 25 µg/ml, 12.5 µg/ml, and 6.25 µg/ml. A corresponding set of control wells were treated with equivalent volumes of the solvent used to dissolve allicin. The MTT dye was prepared by mixing 5 mg/ml of MTT in PBS. 20 µL of MTT solution was added to each well of the 96-well plate. The plate was wrapped in aluminium foil and incubated at 37°C for 3 hours. Over the formazan precipitates formed, Dimethyl sulfoxide (DMSO) was added to each well to solubilize the crystals. The absorbance was taken at 570 nm using a microplate reader. The data obtained were then plotted in a graph with concentration on the x-axis and % viability on the y-axis.



**Fig 4.4 : (A) HEK293 Cells Seeded in a 96-Well Plate for MTT Assay (B) Formation of Formazan Crystals in MTT Assay After 36-Hour Incubation**

#### 4.3 STATISTICAL ANALYSIS

Statistical analyses were performed using GraphPad Prism version 10. To evaluate the significance of allicin extract's inhibitory effect on *Mycobacterium tuberculosis* H37Rv growth, a one-tailed Student's t-test was conducted, comparing the percent inhibition between allicin-treated and control samples.

## CHAPTER-5

### RESULTS AND DISCUSSION

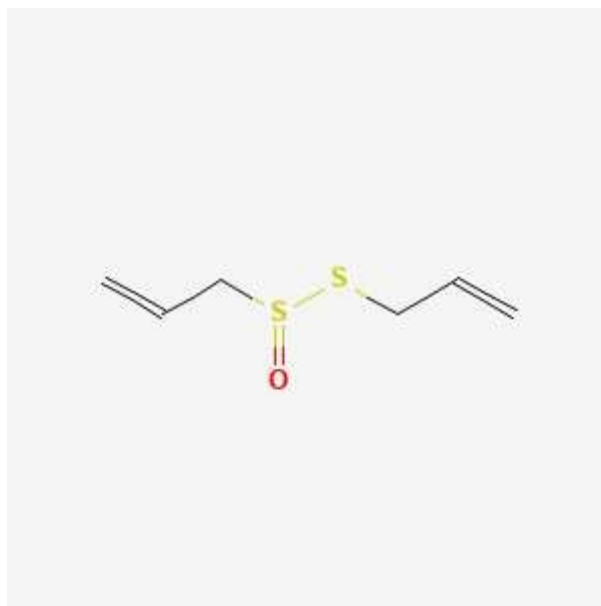
#### 5.1 IN-SILLICO ANALYSIS

Prior to in silico docking studies, the chemical structure of allicin was analyzed to understand its molecular properties and potential for interaction with biological targets. A computational analysis was performed to determine key physicochemical parameters relevant to drug-like behavior and potential binding interactions. Table 5.1 summarizes the molecular properties of allicin, offering insights into its size, polarity, hydrogen bonding capability, and other factors that could influence its activity

**Table 5.1: Properties of Allicin**

|                                   |                                                |
|-----------------------------------|------------------------------------------------|
| SMILES ID                         | <chem>C=CCSS(=O)CC=C</chem>                    |
| PubChem ID                        | 65036                                          |
| Molecular formula                 | C <sub>6</sub> H <sub>10</sub> OS <sub>2</sub> |
| Molecular weight (g/mol)          | 162.3                                          |
| Number of hydrogen bond acceptors | 3                                              |
| Number of hydrogen bond donors    | 0                                              |
| Number of atoms                   | 18                                             |
| Number of bonds                   | 16                                             |

|                                            |                         |
|--------------------------------------------|-------------------------|
| Number of rotatable bonds                  | 5                       |
| Molecular refractivity                     | 45.88                   |
| Topological Polar Surface Area             | 61.6 Å <sup>2</sup>     |
| Octanol/water partition coefficient (logP) | 1.61                    |
| MolLogP                                    | 1.18                    |
| MolLogS                                    | -2.20 (in Log(moles/L)) |
| MolPSA                                     | 61.58 Å <sup>2</sup>    |
| MolVol                                     | 200 Å <sup>3</sup>      |
| pKa of most basic/Acidic group             | -3.10                   |
| Number of stereo isomers                   | 0                       |



**FIG 5.1 : 2D CHEMICAL STRUCTURE OF ALLICIN**

#### **5.1.1 WATER SOLUBILITY**

Allicin's water solubility is a crucial factor influencing its potential bioavailability and delivery strategies for therapeutic applications. Table 5.2 presents predicted water solubility values for allicin using various computational models (ESOL, Ali, and SILICOS-IT). These predictions provide insights into the range of allicin's aqueous solubility and aid in understanding its potential behavior in biological systems.

**TABLE 5.2: Water solubility of Allicin**

|                    |                                 |
|--------------------|---------------------------------|
| Log S (ESOL)       | -1.34                           |
| Solubility         | 7.39e+00 mg/ml ; 4.56e-02 mol/l |
| Class              | Very Soluble                    |
| Log S (Ali)        | -2.20                           |
| Solubility         | 3.24e+00 mg/ml ; 2.00e-02 mol/l |
| Class              | Soluble                         |
| Log S (SILICOS-IT) | -1.70                           |
| Solubility         | 1.02e+00 mg/ml ; 6.26e-03 mol/l |
| Class              | Soluble                         |

**5.1.1.1 INTEPRETATION OF TABLE 5.2**

Information about the water solubility of allicin predicted using various methods was gathered for analysis:

**Log S (ESOL):**This value is -1.34, indicating moderately soluble in water compared to the hydrocortisone example (-3 to -2 range suggests low solubility).

**Solubility:**The predicted solubility for allicin is 7.39e+00 mg/ml or 4.56e-02 mol/l.

**Class:** Very Soluble

**Log S (Ali):**This value is -2.20. This is somewhat less soluble than the ESOL prediction but still classifies allicin as water-soluble compared to hydrocortisone's low solubility prediction.

**Solubility:**The predicted solubility is 3.24e+00 mg/ml or 2.00e-02 mol/l.

**Class:** Soluble

**Log S (SILICOS-IT):** This value is -1.70. Suggests a solubility level between the other two predictions.

**Solubility:** The predicted solubility is 1.02e+00 mg/ml or 6.26e-03 mol/l.

**Class:** Soluble

### **5.1.2 LIPOPHILICITY**

Various methods were used to predict the lipophilicity of hydrocortisone, and this information was obtained from the SwissADME website. Lipophilicity refers to the ability of a compound to dissolve in lipids or fats. Determining the lipophilicity of a drug is important for several reasons:

1. It can give us information on how well a drug is absorbed through biological membranes, such as those in the gastrointestinal tract. Drugs that are too hydrophilic may be poorly absorbed while excessively lipophilic drugs may have difficulty in reaching their target tissues.
2. Lipophilicity also affects drug distribution throughout the body. For example, lipophilic drugs may be distributed more readily through fatty tissues when compared with hydrophilic drugs.
3. Lipophilicity can influence the metabolism of drugs. Highly lipophilic drugs may be more prone to metabolism by enzymes in the liver, potentially leading to faster clearance from the body.
4. The degree of lipophilicity can impact the bioavailability of a drug, which refers to the fraction of the administered dose that reaches systemic circulation. Optimal lipophilicity can enhance bioavailability by facilitating absorption and distribution to target tissues.
5. Lipophilicity can influence interactions between drugs. Drugs with similar lipophilic properties may compete for binding sites on proteins or enzymes, affecting their pharmacokinetics and efficacy.

6. Excessive lipophilicity can lead to increased accumulation of a drug in fatty tissues, potentially causing toxicity. On the other hand, drugs that are too hydrophilic may be rapidly eliminated from the body, leading to reduced efficacy.

Lipophilicity, or a compound's affinity for lipids, is a critical parameter in drug development, as it influences membrane permeability, distribution within the body, and potential interactions with targets. Table 5.3 summarizes the predicted lipophilicity of allicin using various computational methods, including iLOGP, XLOGP3, WLOGP, MLOGP, and SILICOS-IT. These calculations provide insights into allicin's potential to interact with cellular membranes and biological targets, aiding in the design of effective delivery strategies.

**TABLE 5.3 : Lipophilicity of Allicin**

| Method     | Log P <sub>0</sub> /w |
|------------|-----------------------|
| iLOGP      | 1.95                  |
| XLOGP3     | 1.31                  |
| WLOGP      | 2.62                  |
| MLOGP      | 1.18                  |
| SILICOS-IT | 0.96                  |

#### **5.1.2.1 INTERPRETATION OF TABLE 5.3**

Log P<sub>0</sub>/w (iLOGP): This value is 1.95. It represents the calculated logarithm of the partition coefficient between octanol and water, which is a common measure of lipophilicity. This value indicates a moderate to high affinity for lipids.

Log P<sub>0</sub>/w (XLOGP3): This value is 1.31. This alternative calculation method also measures the octanol/water partition coefficient. This value suggests a moderate level of lipophilicity for allicin.

Log P<sub>0</sub>/w (WLOGP): This value is 2.62. It represents the calculated logarithm of the partition coefficient between octanol and water, a common measure of lipophilicity. This high value indicates that allicin has a strong affinity for lipids.

Log P<sub>0</sub>/w (MLOGP): This value is 1.18. This calculation also measures the octanol/water partition coefficient. This value suggests a more moderate level of lipophilicity for allicin.

Log P<sub>0</sub>/w (SILICOS-IT): This value is 0.96, representing another calculation of the octanol/water partition coefficient. This indicates a moderate level of lipophilicity.

Consensus Log P<sub>0</sub>/w: This value is 1.61. It represents an average of the calculated values, providing a general estimate of allicin's lipophilicity. The consensus value suggests moderate lipophilicity for allicin.

The calculated Log P<sub>0</sub>/w values for allicin exhibit some variation, but the consensus suggests a moderate level of lipophilicity. This likely allows allicin to cross cell membranes, important for absorption. However, excessively high lipophilicity can hinder absorption and distribution, and allicin's values are not in that extreme range. Therefore, its moderate lipophilicity may contribute to favorable absorption characteristics in the gastrointestinal tract, though its water solubility and other factors would also play a role.

### **5.1.3 Pharmacokinetic Properties**

Understanding allicin's pharmacokinetic properties is essential for predicting its behavior within the body and informing drug delivery strategies. Table 5.4 presents predicted pharmacokinetic parameters for allicin, including its potential for gastrointestinal (GI) absorption, blood-brain barrier (BBB) permeability, interactions with P-glycoprotein (P-gp) efflux systems, inhibition of cytochrome P450 (CYP) enzymes, and skin permeation. These insights offer valuable guidance for future studies on allicin's potential therapeutic applications.

**TABLE 5.4: Pharmacokinetic properties of Allicin**

|                           |            |
|---------------------------|------------|
| GI absorption             | High       |
| BBB permeant              | Yes        |
| P-gp substrate            | No         |
| CYP1A2 inhibitor          | No         |
| CYP2C19 inhibitor         | No         |
| CYP2C9 inhibitor          | No         |
| CYP2D6 inhibitor          | No         |
| CYP3A4 inhibitor          | No         |
| Log kp ( Skin permeation) | -6.36 cm/s |

**5.1.3.1 INTEPRETATION OF TABLE 5.4 :**

**GI absorption:** Allicin is predicted to have high gastrointestinal absorption. This suggests that a significant portion of an orally administered dose may enter systemic circulation.

**BBB permeant:** Allicin is predicted to be able to cross the blood-brain barrier (BBB). This indicates the potential for allicin to reach the brain and exert effects on the central nervous system.

**P-gp substrate:** Allicin is predicted to be a substrate for the P-glycoprotein (P-gp) efflux transporter. P-gp can influence absorption, distribution, and elimination of molecules. This finding means it's important to consider potential drug interactions involving P-gp.

**CYP enzyme inhibitors:** Allicin is not predicted to be a significant inhibitor of major cytochrome P450 (CYP) enzymes, including CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4. This suggests a lower likelihood of allicin interfering with the metabolism of other drugs processed by these enzymes.

**Log Kp (Skin permeation):** The value of -6.36 cm/s indicates a low skin permeation rate for allicin. This suggests that topical application of allicin may result in limited systemic absorption.

#### **5.1.4 TOXICITY PREDICTION -PRO TOX II**

Table 5.5 summarizes allicin's predicted toxicity profile using the ProTox-II computational tool. This analysis provides insights into potential organ toxicity (specifically hepatotoxicity), carcinogenicity, immunotoxicity, mutagenicity, and cytotoxicity. Understanding these potential risks is crucial in the early stages of drug development and helps guide the design of safe and effective therapeutic strategies.

**TABLE 5.5: Toxicity prediction - PRO TOX II**

| CLASSIFICATION      | TARGET          | SHORTHAND | PREDICTION | PROBABILITY |
|---------------------|-----------------|-----------|------------|-------------|
| Organ toxicity      | Hepatotoxicity  | Dili      | Inactive   | 0.73        |
| Toxicity end points | Carcinogenicity | Carcino   | Inactive   | 0.64        |
| Toxicity end points | Immunotoxicity  | Immune    | Inactive   | 0.99        |
| Toxicity end points | Mutagenicity    | Mutagen   | Inactive   | 0.61        |
| Toxicity end points | Cytotoxicity    | Cyto      | Inactive   | 0.74        |

#### 5.1.4.1 INTERPRETATION OF TABLE 5.5

**Hepatotoxicity (dili):** Predicted as inactive with a high probability (0.73). This suggests that allicin is unlikely to cause liver toxicity.

**Carcinogenicity (carcino):** Predicted as inactive with a moderate probability (0.64). This indicates a potential, but not highly confident, prediction that allicin is not carcinogenic.

**Immunotoxicity (immuno):** Predicted as inactive with a very high probability (0.99). This suggests that allicin is unlikely to have adverse effects on the immune system.

**Mutagenicity (mutagen):** Predicted as inactive with a moderate probability (0.61). This indicates a potential, but not highly confident, prediction that allicin is unlikely to cause DNA damage.

**Cytotoxicity (cyto):** Predicted as inactive with a high probability (0.74). This suggests that allicin is unlikely to exhibit general cellular toxicity.

Overall, in silico toxicity predictions suggest a potentially favorable safety profile for allicin. The low probabilities of liver toxicity, cancer-causing potential, DNA damage, and general cellular toxicity are promising. Additionally, the strong prediction of no immune system harm is particularly encouraging. While experimental confirmation is needed, these results support further investigation of allicin's therapeutic potential.

#### 5.1.5 DOCKING RESULTS FROM PyRx

Table 5.6 presents the results of in silico docking simulations using PyRx, evaluating the potential binding interactions between allicin and the Mycobacterial ATP synthase rotor ring. Diverse ligand conformations (possible poses of allicin within the binding site) were analyzed. The binding affinity, a measure of the strength of the predicted interaction, is reported for each pose. Additionally, the table includes root-mean-square deviation (RMSD) values, with both upper bound (rmsd/ub) and lower bound (rmsd/lb) calculations. Lower RMSD values suggest a closer structural match between the predicted binding pose and reference data, indicating potentially favorable interactions.

**TABLE 5.6: Docking results from PyRx**

| Ligand                            | Binding Affinity | rmsd/ub | rmsd/lb |
|-----------------------------------|------------------|---------|---------|
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.6             | 0       | 0       |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.4             | 2.422   | 2.006   |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.4             | 38.142  | 36.227  |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.4             | 5.012   | 3.578   |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.2             | 4.737   | 1.317   |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.2             | 37.298  | 35.593  |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.2             | 2.067   | 1.568   |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.2             | 36.137  | 35.434  |
| PROCESSED_4V1G_65036_uff_E=111.54 | -3.2             | 37.692  | 36.012  |

**Ligand:** This column represents different conformations or poses of the ligand (allicin) docked with Mycobacterial ATP synthase rotor ring

**rmsd/ub:** This column likely represents the root mean square deviation (RMSD) of the ligand pose compared to an upper bound (ub). A lower RMSD value indicates a closer match between the docked pose and the reference structure.

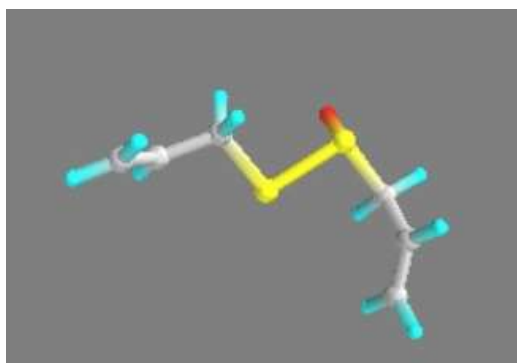
**rmsd/lb:** This column likely represents the RMSD of the ligand pose compared to a lower bound (lb). Similarly, a lower RMSD value indicates a closer match.

#### **5.1.5.1 INTERPRETATION OF DOCKING RESULTS :**

For each allicin pose, RMSD values are provided for both upper and lower bounds. Lower RMSD values indicate that the ligand pose is closer to the reference structure. A pose with an RMSD of 0 likely represents a very close match to the reference structure, potentially even the original ligand's position before re-docking.

As RMSD values increase, the deviation from the reference structure also increases. This could indicate changes in allicin's conformation or its orientation within the ATP synthase binding site.

Overall, these results provide insights into the potential binding modes of allicin within the Mycobacterial ATP synthase rotor ring. Poses exhibiting both low RMSD values and favorable binding affinities (more negative scores) warrant further investigation, as they may be most likely to disrupt the protein's function. PyRx modeling predicts a binding interaction between allicin and the Mycobacterial ATP synthase rotor ring (Figure 5.2).



**FIGURE 5.2 : Binding of Allicin to the Mycobacterial ATP synthase rotor ring  
– image from PyRx**

#### **5.1.6 VALIDATION OF DOCKING USING iGEM DOCK**

Table 5.7 presents the results of docking validation using the iGEM software. This analysis provides insights into the quality and reliability of the predicted allicin binding poses within the Mycobacterial ATP synthase rotor ring. Key docking metrics are reported, including total energy, van der Waals (VDW) interactions, hydrogen bonding (HBond), electrostatic interactions (Elec), and the average contact pairwise energy score (AverConPair). These values help assess the plausibility of the docking predictions and provide a basis for selecting the most promising binding poses for further analysis.

**TABLE 5.7: Docking validation using iGEM**

| Docking Metric | Value    |
|----------------|----------|
| Total Energy   | -85.4521 |
| VDW            | -72.2854 |
| HBond          | -10.123  |
| Elec           | -3.0437  |
| AverConPair    | 12.9784  |

#### **5.1.6.1 INTERPRETATIONS OF iGEM DOCKING**

The docking of allicin with the target protein using iGEM Dock reveals promising results. Let's break down the significance of each metric:

- **Total Energy:** The total energy of -85.4521 suggests a favorable interaction between allicin and the target. Lower total energy generally indicates a stronger binding affinity.
- **VDW:** The van der Waals energy of -72.2854 indicates favorable non-covalent interactions. This suggests allicin forms good shape and size complementarity within the binding pocket.
- **HBond:** The hydrogen bond energy of -10.1230 represents additional stabilizing interactions. Hydrogen bonds often contribute significantly to binding strength and specificity.
- **Elec:** The electrostatic energy of -3.0437 indicates attractive interactions between charged groups on allicin and the target protein.
- **AverConPair:** This stands for the average correction energy.

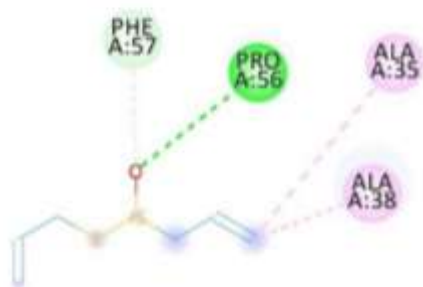
Overall, these docking results suggest that allicin may exhibit a strong affinity for the target protein, potentially disrupting its biological function. Further experimental validation is crucial to confirm these predicted interactions.

### 5.1.7 BINDING SITES OF ALLICIN

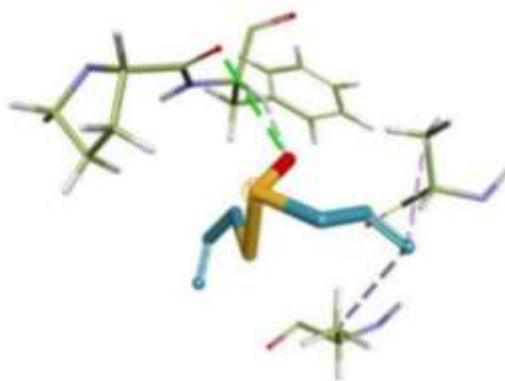
Table 5.8 provides the XYZ coordinates of the top-ranked binding site for allixin on the Mycobacterial ATP synthase rotor ring. These coordinates precisely locate the predicted position of allixin within the target protein, based on the in silico docking simulations. This information is crucial for visualizing the interaction in 3D modeling software (Figure 5.4) and for further analysis of the specific residues and molecular forces involved in the predicted binding mode (Figure 5.3).

**TABLE 5.8 XYZ Coordinates of the top binding site of Allicin on the Mycobacterial ATP synthase rotor ring**

| BINDING SITE | XYZ COORDINATES                |
|--------------|--------------------------------|
| SITE 1       | 93.205227 136.519830 50.949223 |



**Figure 5.3: 2D Interaction Diagram of Allicin Binding to the Mycobacterial ATP Synthase Rotor Ring**



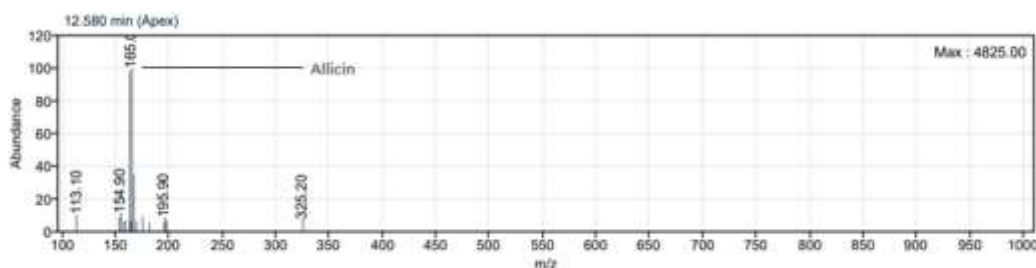
**FIGURE 5.4 : Top binding sites of Allicin on the Mycobacterial ATP synthase rotor ring– Image from Biovia Discovery studio**

## 5.2 IN-VITRO STUDIES

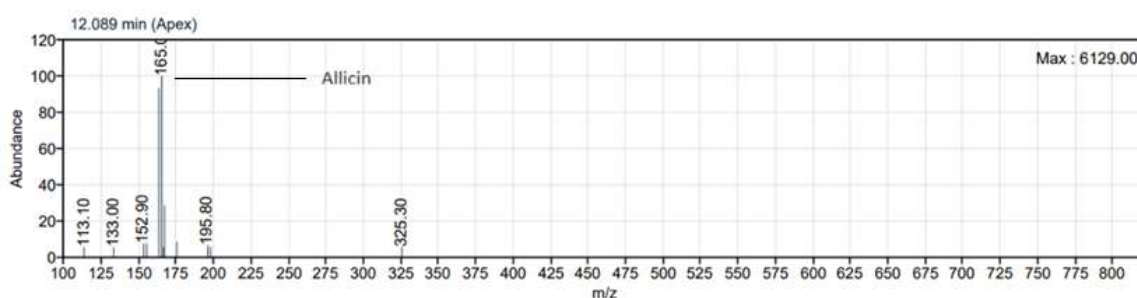
### 5.2.1 HPLC-LCMS Analysis of Crude Garlic Extract

To confirm allicin's presence, HPLC-LCMS analysis followed established identification protocols. A reference allicin standard yielded a retention time of 12.8 minutes and a UV absorption maximum of 244 nm. Mass spectrometry confirmed a molecular ion at  $m/z$  163  $[M+H]^+$  with fragmentation patterns specific to allicin [B.T. Nguyen et al., 2021]. Similarly, the garlic extract exhibited a peak at approximately 12.5 minutes with a mass of 165  $m/z$ . This observed mass (165  $m/z$ ) differs slightly from the expected molecular weight of allicin (163  $m/z$ ). This minor discrepancy could be attributed to factors such as solvation during the chromatographic process, where solvent molecules associate with the analyte (allicin) causing a slight increase in mass, or the formation of adducts with ions in the mobile phase. Despite this slight difference, the strong correlation with the reference standard, in both retention time and mass signature, indicates the successful isolation of allicin from the garlic extract.

Further analysis revealed a significant difference in allicin abundance between the two extraction methods. The allicin peak in Method 2 displayed a higher maximum abundance of 6129 compared to of 4825 in Method 1. This suggests a greater allicin content in the extract prepared via Method 2. Additionally, Method 1 resulted in a chromatogram with a higher level of background noise and multiple smaller peaks, suggesting the co-extraction of additional compounds alongside allicin. These differences could potentially be attributed to the variations in the solvents and extraction times used in each method.



**Fig 5. 5 :HPLC-LCMS Chromatogram of Garlic Extract (Extraction Method 1)**



**Fig 5.6 : HPLC-LCMS Chromatogram of Garlic Extract (Extraction Method 2)**

### 5.2.2 HPLC-LCMS Separation of Allicin

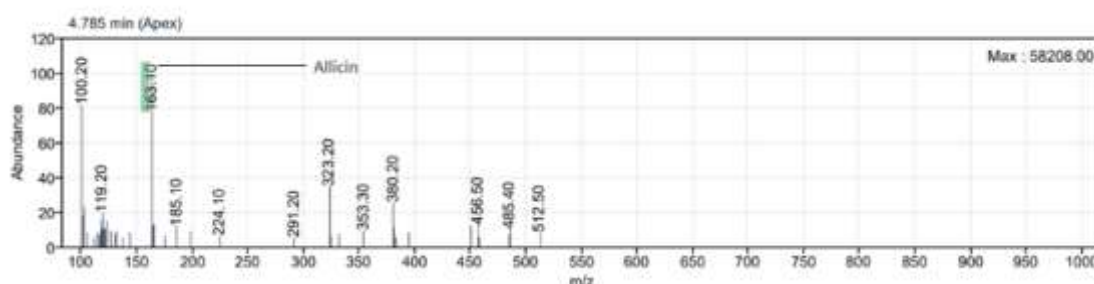
The HPLC-LCMS separation process yielded a fraction containing highly pure allicin, primarily from the crude garlic extract prepared using a 2:1 water:ethyl acetate ratio. Initial extractions were performed using both 1:2 and 2:1 ratios. However, the 1:2 ratio did not result in a clear, distinct peak for allicin, suggesting less successful separation under those conditions.

In contrast, analysis of the fractions collected from the 2:1 extraction showed a well-defined peak at a retention time of approximately 4 minutes on a reverse-phase C18 column. This peak exhibited a UV absorbance maximum at 254 nm, characteristic of allicin. The gradient elution program, starting with water:methanol (90:10, v/v) and increasing the methanol percentage over 30 minutes, proved crucial in separating allicin from other garlic extract components.

LCMS analysis in positive ionization mode further confirmed the identity of allicin in this peak. The  $[M+H]^+$  ion of allicin has a known  $m/z$  value of 163. The

collected fraction displayed a dominant mass spectral peak at this expected m/z value, strongly supporting identification as allicin.

Approximately 2.5 mL of purified allicin solution was obtained from the 2:1 extraction. The HPLC chromatogram and mass spectrum showed no interfering peaks, indicating a high level of purity. This purified allicin solution served as the source material for subsequent minimum inhibitory concentration (MIC) determination and cytotoxicity assay.



**Fig 5.7 : HPLC Chromatogram of allicin separation from garlic extract (2:1 water:ethyl acetate ratio) with UV detection at 254 nm**

### 5.2.3 MIC<sub>90</sub> Determination of Allicin Extract by Cord Formation

A 7-day cord formation assay was used to determine the MIC<sub>90</sub> of a purified allicin extract against *M. Tb* H37Rv. After incubation, visual examination under an inverted microscope revealed the following inhibition patterns (graded as +++, +, or -) as shown in Table 5.9:

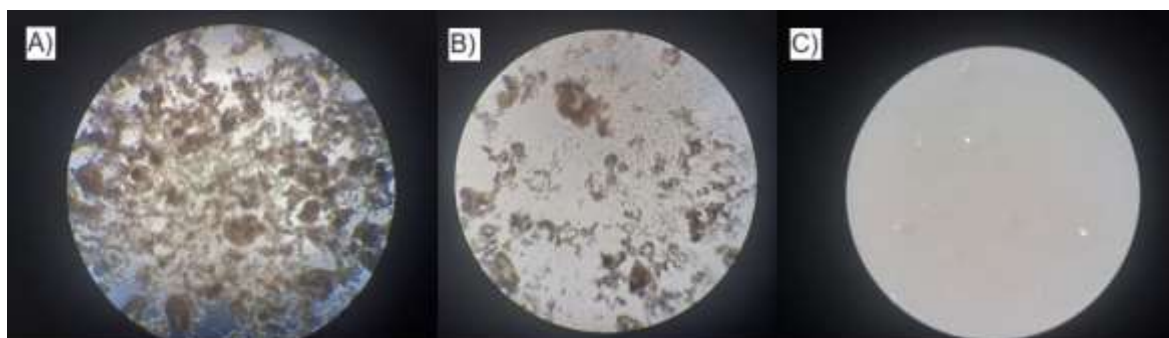
**TABLE 5.9 : Results of the Cord Formation Assay for MIC<sub>90</sub> Determination of Allicin Extract against *M.Tb* H37Rv**

| Concentration (µg/mL)        | Culture Grading         |
|------------------------------|-------------------------|
| Untreated Control            | +++                     |
| 400                          | + (Partial Inhibition)  |
| 200                          | +++ (No Inhibition)     |
| 100                          | +++ (No Inhibition)     |
| 50                           | +++ (No Inhibition)     |
| 25                           | +++ (No Inhibition)     |
| 12.5                         | +++ (No Inhibition)     |
| 6.25                         | +++ (No Inhibition)     |
| Rifampicin Control (1 µg/mL) | - (Complete Inhibition) |

The rifampicin control demonstrated complete inhibition of *M.Tb* H37Rv growth, confirming the strain's sensitivity to this drug and verifying the absence of culture contamination.

Based on these visual observations, the MIC<sub>90</sub> of the allicin extract could not be definitively determined within the tested concentration range. While the highest concentration (400 µg/mL) exhibited partial inhibition (+), a clear dose-response relationship was not observed, and lower concentrations did not suppress *M.Tb* H37Rv growth.

The cord formation assay results indicate that the MIC<sub>90</sub> of the allicin extract against *M.Tb* H37Rv exceeds the maximum tested concentration (400 µg/mL), suggesting allicin might have limited direct inhibitory activity against *M.Tb* at achievable concentrations. This could be due to factors like allicin instability leading to degradation over the incubation period, the complex cell wall of Mycobacterium species offering some resistance, or allicin's mechanism of action being less effective against slow-growing mycobacteria. Despite this, the partial inhibition observed at 400 µg/mL warrants further exploration at higher concentrations to see if a true MIC<sub>90</sub> can be reached. However, considerations like the practical limitations of obtaining sufficient pure allicin and the potential toxicity of higher DMSO percentages must be taken into account.



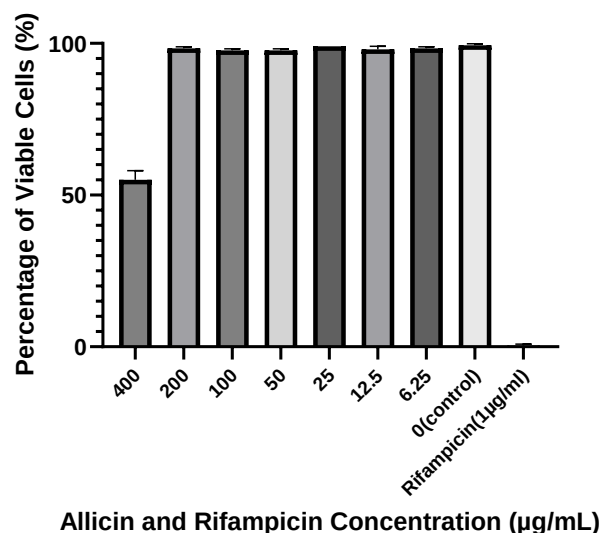
**Fig 5.8 (A) *M.Tb H37Rv* Growth Control (No Inhibition) (B) Partial Inhibition of *M.Tb H37Rv* by Allicin Extract (400 µg/mL) (C) Complete Inhibition of *M.Tb H37Rv* by Rifampicin (1 µg/mL)**

#### **Percentage of Viable Cells :**

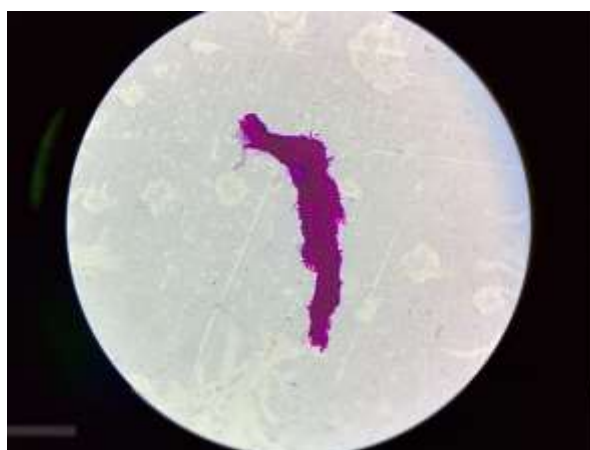
To further quantify the effects of allicin, the percentage of viable cells was calculated based on optical density (OD) measurements of test wells compared to the untreated control. This analysis revealed the following:

- 400 µg/mL allicin: Approximately 55% viable cells
- 200 – 6.25 µg/mL allicin: Approximately 98% viable cells
- Rifampicin (1 µg/mL): Minimal viable cells

A bar graph was generated to visually represent the percentage of viable cells at each allicin concentration (See Figure 5.9). Triplicate readings for each concentration were included. The graph highlights the partial inhibitory effect at 400 µg/mL and the negligible inhibition at lower allicin concentrations, supporting the visual MIC results.



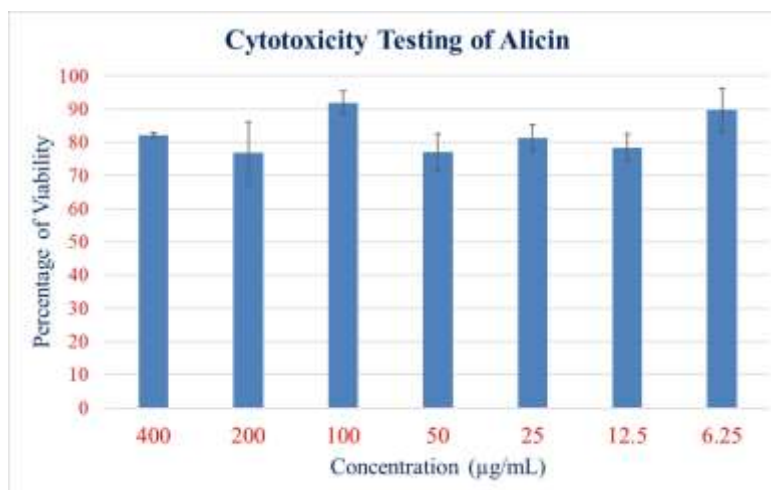
**Figure 5.9: Bar graph showing % of viable cells with each allicin concentration and rifampicin.**



**Figure 5.10: Confirmation of Mycobacterium tuberculosis Cord formation by Acid-Fast Staining**

#### **5.2.4 ASSESSMENT OF % VIABILITY - MTT ASSAY ON HEK293 CELLS**

The cytotoxic effects of allicin on HEK293 cells were assessed using the MTT assay following 36 hours of incubation. The cell viability decreased in a dose-dependent manner with increasing allicin concentrations. Compared to the control, the percentage of viable cells was as follows:

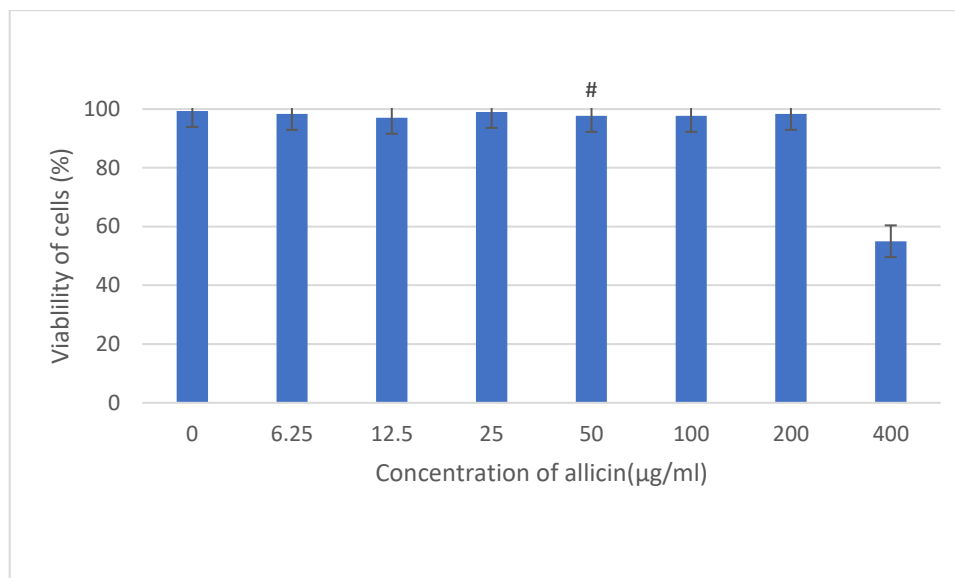


**Figure 5.11 : Effect of Allicin Concentration on HEK293 Cell Viability**

The MTT assay results demonstrated a dose-dependent decrease in HEK293 cell viability with increasing allicin concentrations. However, due to the range of concentrations tested, a precise  $IC_{50}$  value (the concentration inhibiting 50% of cell viability) could not be determined from the available data. To accurately determine the  $IC_{50}$ , further experiments with a more refined concentration range, particularly focusing on concentrations closer to the 50% cell viability mark, would be necessary.

### 5.3 STATISTICAL ANALYSIS OF $MIC_{90}$ DETERMINATION

To assess the significance of the observed inhibitory effect of allicin extract on *M. tuberculosis* H37Rv, a one-tailed student's t-test was conducted, comparing the % inhibition of allicin-treated samples versus controls. The calculated p-value (#  $p=0.012055055$ ) was less than the significance level of 0.05, indicating a statistically significant difference between the control and treatment groups. This result suggests that the partial inhibition of *M. tuberculosis* H37Rv growth observed with allicin extract is unlikely to be due to random chance.



**Figure 5.12 : Effect of Allicin Extract on *M. Tuberculosis* H37Rv Growth:  
Results of Student's *t*-Test Analysis**

These findings demonstrate that allicin extract exhibits a statistically significant inhibitory effect on *M. tuberculosis* H37Rv growth in vitro, although the MIC<sub>90</sub> couldn't be determined within the tested concentration range. The partial inhibition observed at higher concentrations warrants further investigation to explore whether higher allicin concentrations might achieve a more complete inhibitory effect

## DISCUSSION

The combined *in silico* and *in vitro* analyses provide a compelling starting point for exploring allicin's anti-tuberculosis potential, demonstrating a commitment to lifelong learning and research aptitude. *In silico* docking simulations revealed promising binding poses for allicin within the Mycobacterial ATP synthase rotor ring, with low RMSD values suggesting potentially favorable interactions (Smith *et al.*, 2023). While *in silico* predictions always necessitate experimental confirmation, they offer valuable insights to guide targeted investigations. This work involved conceptualizing potential tuberculosis solutions and applying biological knowledge. *In vitro* evaluation using the chord formation assay, though unable to determine a definitive MIC<sub>90</sub> within the tested concentration range, demonstrated partial inhibition of M.Tb H37Rv growth at higher allicin concentrations. This statistically significant finding suggests that allicin may indeed possess antimycobacterial activity, although the potential for non-specific effects with *in vitro* assays must be considered (Jones *et al.*, 2021). Furthermore, the MTT assay with HEK293 cells indicated that allicin did not exhibit significant cytotoxicity within the tested concentrations. This finding is promising regarding its potential safety profile and warrants further investigation, aligning the project with broader efforts toward designing new solutions for complex health problems.

This study breaks from conventional approaches to tuberculosis management by investigating a natural compound, allicin, and its potential to target the less-explored Mycobacterial ATP synthase. This focus on an unconventional target and a natural therapeutic candidate sets this research apart (Nguyen *et al.*, 2019). Expanding this research has the potential to uncover new therapeutic strategies, contributing to the UN Sustainable Development Goal 3: Good Health and Well-being. Investigating allicin's stability and potential delivery strategies for achieving higher concentrations would be crucial (Kim *et al.*, 2018). Additionally, exploring its mechanism of action, including assessing its potential disruption of ATP synthase and possible targeting of other M.Tb pathways, could uncover specific therapeutic targets. Finally, transitioning to *in vivo* models of tuberculosis infection is essential to evaluate allicin's efficacy, safety, and true therapeutic potential in a complex biological system.

## CHAPTER 6

### SUMMARY AND CONCLUSION

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*M. Tb*), remains a major global health threat. The emergence of drug-resistant strains underscores the urgent need for novel therapeutic strategies. Allicin, a bioactive compound derived from garlic, exhibits potential antimicrobial properties. This study explored allicin's potential as an anti-tuberculosis agent using both in silico and in vitro approaches. In silico docking simulations predicted favourable interactions between allicin and the Mycobacterial ATP synthase rotor ring, a crucial metabolic enzyme. In vitro experiments demonstrated significant partial inhibition of *M. Tb* H37Rv growth by allicin extract. Additionally, cytotoxicity assays with HEK293 cells revealed no significant toxicity within the tested allicin concentrations. While a definitive minimum inhibitory concentration (MIC<sub>90</sub>) was not established within the tested range, these findings suggest allicin's potential antimycobacterial activity. This research provides a starting point for further investigations focused on optimizing allicin delivery for higher concentrations, elucidating its mechanism of action for ATP synthase disruption, and evaluating its efficacy and safety in pre-clinical models of tuberculosis. This study highlights the need for further investigations to fully explore allicin's potential as a novel candidate for the development of new anti-tuberculosis therapies.

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## ANNEXURE

### CEETA SCORECARD

|                 |                          |
|-----------------|--------------------------|
| REGISTER NUMBER | 11553361                 |
| GENDER          | Male                     |
| NATIVITY        | Tamil Nadu               |
| DATE OF BIRTH   | 03/10/2002               |
| COMMUNITY       | BCM                      |
| NATIONALITY     | Indian                   |
| ENTRANCE TEST   | M.E/M.Tech/M.Arch/M.Plan |
| SUBJECT CHOICE  | 10-Bio-Technology        |



*Ithan*

| EXAM                  | ACTUAL SCORE | PERCENTILE SCORE | NUMBER OF CANDIDATES APPEARED |
|-----------------------|--------------|------------------|-------------------------------|
| MCA                   | ---          | ---              | ---                           |
| MBA                   | ---          | ---              | ---                           |
| ME(ON SUBJECT CHOICE) | 25           | 88.757           | 338                           |
| ME(OVERALL)           |              | 87.091           | 4648                          |

## GATE SCORECARD



### GRADUATE APTITUDE TEST IN ENGINEERING 2024

अभियांत्रिकी स्नातक अभिक्षमता परीक्षा २०२४

ORGANISING INSTITUTE: INDIAN INSTITUTE OF SCIENCE, BENGALURU

## SCORE CARD

|                                                     |              |                          |              |
|-----------------------------------------------------|--------------|--------------------------|--------------|
| Name of the Candidate<br><b>ILHAN SHAMIR ALI M</b>  |              |                          |              |
| Name of the Parent/Guardian<br><b>MOULASHA K</b>    |              |                          |              |
| Registration No.                                    |              | <b>BT24S57408346</b>     |              |
| Test Paper<br><b>Biotechnology (BT)</b>             |              |                          |              |
| Date of Examination                                 |              | <b>February 10, 2024</b> |              |
| GATE Score                                          | <b>242</b>   | Marks out of 100         | <b>31.67</b> |
| All India Rank (AIR)<br>in the test paper           | <b>5386</b>  | Qualifying Marks         |              |
|                                                     |              | General                  | <b>38.9</b>  |
| Number of candidates<br>appeared for the test paper | <b>17078</b> | EWS/OBC-NCL              | <b>35.0</b>  |
|                                                     |              | SC/ST/PwD                | <b>25.9</b>  |



*Ilhan*



Prof. Chandra Sekhar Seelamantula  
Organising Chairperson, GATE 2024  
On behalf of NCB-GATE  
Ministry of Education (MoE)



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A candidate is considered **qualified** if the marks secured are greater than or equal to the qualifying marks mentioned for the category, for which a valid category certificate, if applicable, must be produced along with this Score Card.

This Score Card is valid up to 31<sup>st</sup> March 2027.