ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM SELECTED MEDICINAL PLANTS OF NORTH-EAST INDIA AND A STUDY ON THEIR ANTI *MYCOBACTERIAL* PROPERTIES

A thesis submitted in partial fulfillment of the requirements

for award of the degree of

Doctor of Philosophy

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The Thesis is dedicated to my beloved Parents

Late Shri Hemo Ram Kalita

and

Smt Lily Kalita

and my brother

Shri Jyotirmoy Kalita

Abstract

Mycobacterium is an important genus that includes two of the dreaded pathogenic species responsible for causing leprosy and tuberculosis in human. The often referred *Mycobacterium tuberculosis* complex comprises of *M. africanum, M. microti, M. bovis, M. bovis* BCG *M. canettii* and *M. tuberculosis* (Cole 2002). Of these species, *M. tuberculosis, M. africanum* and *M. bovis* cause the tuberculosis disease. In human however the most common pathogen responsible for tuberculosis (TB) is *Mycobacterium tuberculosis*. TB is one of the leading killer diseases of the world infecting around more than two billion people. A series of drugs comprising of isoniazid, rifampicin, pyrazinamide and ethambutol- called together as he First Line drugs, have been conventionally used against TB since the 1950s in a rather long treatment period of six to eight months with remarkable success.

However the scenario changed alarmingly during the last three decades with the emergence of new resistant strains of *M. tuberculosis* and the pandemic of HIV infection. Patient non compliance of the long treatment period with the first line antibiotic drugs is primarily responsible for the emergence of the resistant strains. Moreover the first line drugs are not without side effects and are known for toxicity on prolonged use. The resistant strains that have emerged are i) The Multi Drug Resistant (MDR) strain and ii) The Extensively Drug Resistant (XDR) strain.

The MDR strain is resistant to the first line series of drugs- isoniazid and rifampicin while the XDR strain is resistant to both the first line drugs and to fluoroquinolone and any one of the injectable second line series of drugs like kanamycin, amikacin and capreomycin. The MDR and the XDR strains have brought in its wake the revival of the tuberculosis threat worldwide to such an extent that the World Health Organization (WHO) was compelled to declare tuberculosis as a global public health emergency in 1993.

There is a great urgency in discovering new chemical entities to address the issues raised above. What is required now are new molecules with greater efficacy against the

resistant strains through shorter treatment period and which have no side effect and toxicity.

Plants have been utilized as medicines for thousands of years and still are important sources of new chemical entities. The North East of India lies in the Indo-Burma region, which is one of the eight biodiversity hotspots of the world. There are many different species of medicinal plants which are used in traditional medicine by the local healers.

Mycobacterium smegmatis, a saprophytic species allied to the pathogenic *Mycobacterium tuberculosis* is considered as a model system because of several advantages offered by this non pathogenic cousin of *M. tuberculosis*. In the present study all experiments were carried out in *M. smegmatis*.

Chapter I cover in detail the Introduction of the problem of Mycobacterial infections with special focus on tuberculosis caused by *M. tuberculosis* and the emergence of drug resistant strains. The chapter also explains the requirement of new drugs and various approaches to drug discovery and the need for development of sustained release drug delivery vehicles. The importance of exploring plant metabolites on the basis of traditional knowledge is also highlighted in the special context of the rich plant biodiversity of North East India.

Chapter II is on Review of Literature and presents comprehensive review on the antimycobacterial drug discovery from medicinal plants during the last fifteen years. The review is divided into antimycobacterial plant extracts, antimycobacterial alkaloids, antimycobacterial flavonoids and other plant metabolites as antimycobacterials. The Chapter also reviews various *in silico* approaches like docking, Quantitative Structure Activity Relationship studies (QSAR) and Adsorption Distribution Metabolism Excretion-Toxicity (ADME-TOX) studies and concludes with a final discussion on antituberculosis drug delivery approaches and the use of microcrystalline cellulose as a pharmaceutical excipient in drug delivery.

Chapter III details the different plant species investigated and the different microbiological, biochemical, analytical and *in silico* methods used in the study.

Chapter IV is on the Results and Discussions. The chapter presents a critical analyses of the results obtained in the studies. Of the ten plant species screened five were

found to possess antimycobacterial activity and two were selected for further analysis of the active molecules. Secondly, the compounds luteolin-6C-glucosyl-2′′-O-glucoside and gallocatechin gallate were isolated from *Camellia sinensis* and the fatty acids linoleic and oleic acid were identified from *Mesua ferrea.* Thirdly the hypothesis were generated through the *in silico* studies relating to drug target identification, QSAR analysis, ADME-TOX. Finally the results of the investigations relating to drug release using microcrystalline cellulose were analysed.

Chapter V presents the conclusions of the work and proposes further future works. The last part of the thesis contains the references cited in the thesis.

DECLARATION BY THE CANDIDATE

I hereby declare that the thesis **"Isolation and Characterization of Compounds from selected Medicinal Plants of North- East India and a study on their anti *Mycobacterial* Properties"**, submitted to **Department of Molecular Biology and Biotechnology, Tezpur University**, Tezpur, Assam in partial fulfillment for the award of the degree of Doctor of Philosophy in Molecular Biology and Biotechnology and it has not been previously considered for the award of any degree, diploma, associateship, fellowship or any other similar title or recognition from any University, Institute or other organizations.

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All helps received from various sources have been duly acknowledged.

No part of this thesis has been submitted elsewhere for award of any degree.

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This is to certify that the thesis entitled **"Isolation and Characterization of Compounds from selected Medicinal Plants of North- East India and a study on their anti *Mycobacterial* Properties"** submitted by **Mr. Ranjan Dutta Kalita, Department of Molecular Biology and Biotechnology, School of Science** to **Tezpur University** in partial fulfillment for the award of the degree of **Doctor of Philosophy** in **Molecular Biology and Biotechnology** has been examined by us on 27.09.13 and found to be satisfactory.

The committee recommends for the award of the degree of Doctor of Philosophy.

Supervisor	**External Examiner**
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Date: 27.09.13	**Date:** 27.09.13

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(Ranjan Dutta Kalita)

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Abbreviations and Symbols

**	QSAR Descriptor
χ	Electronegativity
$v(\vec{r})$	External Potential
°C	Degree Celsius
μ	Chemical Potential
µg	Micro gram
µM	Micro Molar
1D	One Dimensional
2D	Two Dimensional
3D	Three Dimensional
4D	Four Dimensional
Å	Angstrom
ACP	Acyl Carrier Protein
ADME-TOX	Absorption Distribution Metabolism Excretion-Toxicity
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
BCG	Bacille of Calmette and Guerin
bp	Base pair
C	Cytosine
DARQ	Diarylquinolines
DFT	Density Functional Theory
DMEM	Dulbecco's Modified Eagle Media
DMSO	Dimethyl sulfoxide
DOTS	Directly Observed Therapy shortcourse
DPPH	2, 2-Diphenyl-1-picrylhydrazyl
DSC	Density Scanning Calorimetry
E	Total Energy
ETH	Ethambutol
FAME	Fatty Acid Methyl Ester
FASII	Fatty Acid Synthase II
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infra Red
G	Guanine

GC-MS	Gas Chromatography-Mass Spectrophotometry
GC-MS	Gas Chromatography–Mass Spectrometry
Glft2	Galactofuranosyltransferase
h	Hour
HCl	Hydrochloric Acid
HIV	Human immunodeficiency virus
HPLC	High Pressure Liquid Chromatography
IC	Inhibitory Concentration
INH	Isoniazid
InhA	Enoyl- ACP Reductase
KatG	Catalase Peroxidase Hemoprotein
KBr	Potassium bromide
KCl	Potassium Choride
KH_2PO_4	Potassium Dihydrogen Phosphate
LCMS	Gas Chromatography–Mass Spectrometry
LD	Lethal Dose
M	Molar
M	*Mycobacterium*
mA	Milli ampere
MAG	Mycolyl Arabinogalactan
mAGP	Mycolyl- arabinogalactane- peptidoglycan
MCC	Microcrystalline Cellulose
MDCK	Madin-Darby Canine Kidney
MDR-TB	Multi Drug Resistant Tuberculosis
mg	Milligram
MIC	Minimum Inhibitory Concentration
ml	Milliliter
mM	Milli Molar
MR	Molar Refractivity
MS	Mass Spectroscopy
mtKasB	Beta-ketoacyl acyl carrier protein synthase II
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
MVD	Molegro Virtual Docker
N	Normality
N	No of electrons
η	Chemical Hardness
Na_2HPO_4	Disodium Hydrogen Phosphate
NADH	Nicotinamide Adenine Dinucleotide

NaOH	Sodium Hydroxide
nm	Nanometer
NMR	Nuclear Magnetic Resonance
OADC	Oleic Acid – Albumin- Dextrose- Catalyst
PBS	Phosphate Buffer Saline
PDB	Protein Data Bank
POA	Pyrazinoic Acid
PZA	Pyrazinamide
Q- TOF	Quadrupole-Time-of-Flight
QSAR	Quantitative Structure Activity Relationship
RDR	Resistant Determining Region
RIF	Rifampicin
rRNA	Ribosomal Ribonucleic Acid
S	Svedberg Unit
SE	Standard Error
SEM	Scanning Electron Microscope
SIF	Simulated Intestinal Fluid
TB	Tuberculosis
TGA	Thermo Gravimetric Analysis
TK	Traditional Knowledge
TMS	Tri Methyl Silane
UV-VIS	Ultra Violet- Visible Spectrophotometry
WHO	World Health Organization
XDR-TB	Extensively Drug Resistant Tuberculosis
XRD	X-Ray Diffractogram
λ	Absorption
ω	Electrophilicity Index

Chapter I
Introduction

1.1 Mycobacterium Biology

The genus *Mycobacterium* comprises of more than 70 species. Out of these, *M. tuberculosis, M. leprae, M. ulcerans* are responsible for mortality and morbidity in human. Some of the other species like the *M. avium* complex, *M. kansasii, M. chelonae, M. abscessus, M. xenopi* and *M. fortuitum* are also responsible for opportunistic lethal infections. The majority of the other species in the genus is harmless and is present in soil and water as saprophytes. Though the genus is a Gram positive organism yet they are characterized by the more important parameter of acid fastness. The genus is also characterized by the distinctive cell wall constituents made up of mycolic acids. The cell wall constituent structures are hydrophobic, less permeable and are responsible for protecting the organisms from different antimicrobial agents (Dworkim, et al, 2006).

Pathologically *M. tuberculosis* is the most important species amongst all other species of the genus. It is responsible for the disease tuberculosis (TB) which is accountable for maximum mortality worldwide. The *M tb* genome was worked out in 1998. It is a circular genome comprising of 4,411,529 bp and has a G+C (guanine +cytosine) content of 65.6%. There are 4006 protein coding genes and 50 stable RNAs. All these genes are classified into 11 broad functional groups. Of these 52% have been assigned as genes with putative functions while the remaining 48% genes are functionally still unknown (Cole et al, 1998).

TB, an infectious disease, is identified as one of the leading causes of death among human and is one of the three leading causes of death among women in particular. The World Health Organization in its update on tuberculosis in 2009 reported that more than 2 billion people or one third of the total population of the world is infected with the TB bacilli. It is believed that one out of every ten infected people would develop active tuberculosis in their lifetime and every person with active TB will infect on an average 10-15 people every year. In 2011 alone, there were nearly 8.7 million new TB cases, of which 3.6 million are women and 1.1 million cases included HIV infected people (WHO Report 2011). Also 1.7 million

people died of TB in 2009 that includes 3, 80,000 women and the same number of HIV infected individuals, thus killing on an average of 4700 people daily. (WHO Report 2010).

M. tuberculosis can spread from person to person through aerosol droplets. This disease has been in existence for centuries with widespread infection of not only humans but also cattle. Historical information available sheds light into the earliest infections from tuberculosis. The earlier term describing Tuberculosis was '*Phthisis*' in the Greek literature. Hippocrates, the ancient Greek physician first described *Phthisis* as almost always fatal and that was widespread. Benjamin Marten, an English physician in 1720 was the first to report that TB could be caused by some '*wonderfully minute living creatures*'. It was only in 1882 that Robert Koch first identified the bacterium- *Mycobacterium tuberculosis* using staining techniques. Koch was awarded the Nobel Prize in 1905, for this discovery (Prescott, et al, 2005).

M. tuberculosis is an obligate pathogen found only in human and has no environmental reservoirs. Another mycobacterium causing tuberculosis in human as well as in cattle is *M. bovis*. However it has a natural reservoir present in ruminants. Both species along with *M. africanum, M. cannettii, M. bovis BCG* and *M. microti* belong to the *Mycobacterium tuberculosis* complex (Cole 2002). Though *M. africanum* is also known to cause tuberculosis rarely, *M. microti* does not cause human tuberculosis. Also other less virulent species of the *Mycobacterium avium* complex, *Mycobacterium avium* and *Mycobacterium intracellulare* can also sometimes pose serious threats to patients with weak immune system as witnessed in patients with AIDS. Both are opportunistic pathogens and are environmental bacteria found in water, soil, air, food and animals (Pieters 2002, Nguyen 2006). The mycobacteria are generally straight or slightly curved non motile rods, non spore forming and are approximately 0.2-0.6 um wide and 1.0-10um long.

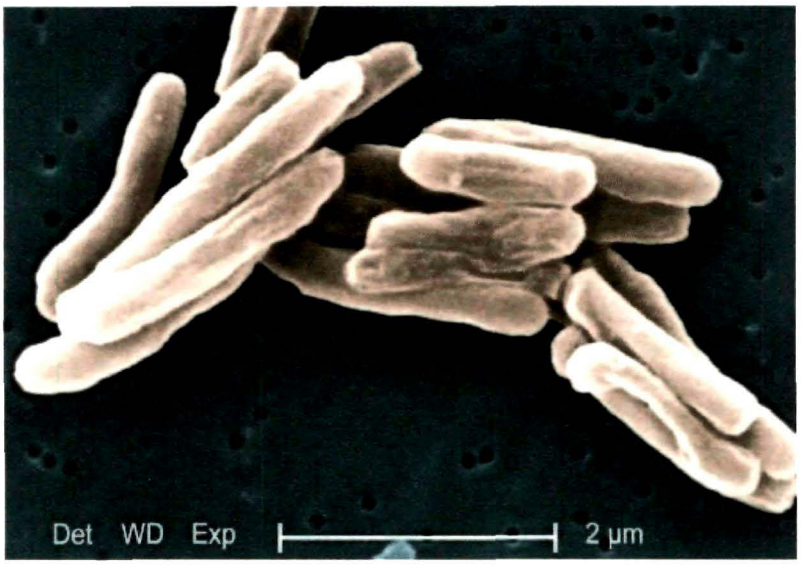


Fig 1.1: SEM micrograph showing *M. tuberculosis*
(http://www.cdc.gov/nchhstp/newsroom/DiseaseAgents.htm)

The mycobacterial cell wall is divided into two segments- Upper and Lower. The peptidoglycan is located beyond the membrane and is attached covalently to the arabinogalactan. The arabinogalactan in turn is attached to the mycolic acid fraction. This entire constituent comprising of the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex is known as the cell wall core (Brennen, 2003). The complex cell wall is responsible for the low permeability of small hydrophillic molecules into the organism and also for the development of resistance of the baciterium to antibiotics, dehydration and other forms of chemical injury (Barry, 2001). Thus the cell wall envelope acts a first line of defence against any form of toxic molecules produced by the host (Neyrolles, 2011).

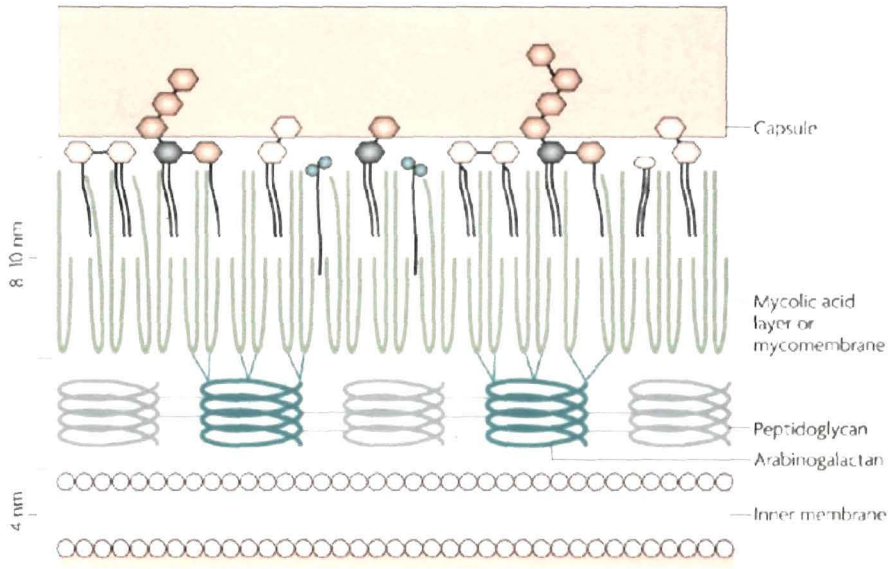


Fig1.2: The Mycobacterial cell envelope (Courtesy: Abdallah, et al, Nature reviews microbiology, 5, 883-891, 2007)

M. tuberculosis infection in human occurs as a result of inhalation of droplets containing the bacilli released into the air by infected individuals (Kaufmann 2001). Inside the body, the bacilli enters the lungs and are then internalized by the alveolar macrophages through phagocytosis. These alveolar macrophages with the appropriate stimuli effectively transfer the phagocytosed *M. tuberculosis* to the lysosomes. However, some of these bacilli escape the process and thereby survive within the macrophage. These infected macrophages either remain in the lungs or are transferred to some other organs of the body. Only a small percentage of the infected people (about 10%) develop active tuberculosis while in majority of the healthy individuals the immune defence system of the host is competent enough to check the bacilli so that the disease does not develop (Pieters 2008). Generally the mycobacterial outgrowth within the host is controlled by the innate and adaptive responses (Pieters 2002).The subsequent tuberculosis infection, latency of the bacilli, activation of the disease in suitable environments and finally spread of the disease is attributed to the safe enclosure of the bacteria within the pathogen friendly phagosome, which do not mature into the phagolysosome (Russel et al 2002). Once the bacilli are successfully established in the host macrophage, the latent bacilli can cause severe disease in the

host if the immune system is compromised under conditions of malnutrition, poor health or when infected by the HIV in which case the host becomes severely immunocompromised (Pieters 2002).

The interactions between *M. tuberculosis* and the host depend upon the following: the ability of the mycobacteria to prevent phagosome-lysosome fusion, suppression of host immunological detection and defense mechanism by *M. tuberculosis,* effects of the products unique to the pathogen on the host (Chua et al, 2004).

1.2 *Mycobacterium smegmatis* as a model organism for study of *Mycobacterium tuberculosis*

Mycobacterium tuberculosis is a well known pathogen and therefore calls for very stringent safety requirements. However *Mycobacterium smegmatis* which is a avirulent species, has a shorter regeneration time and is a fast growing species shares significant commonalities with *M. tuberculosis* (Huang et al, 2013, Shiloh et al, 2009). *M. smegmatis* can be subjected to genetic manipulation with relative ease (Altaf et al., 2010). The aforesaid attributes of *M. smegmatis* make it an attractive working model for conducting research relating to the discovery of new drugs to combat *M. tuberculosis* and for investigation of TB biology.

While there are certain striking dissimilarities between *M. smegmatis* and *M. tuberculosis* relating to their growth rate and pathogenicity, there are also similarities between the two species in terms of some of their biological functions. For eg, it has also been reported that the hypoxia response of *M. smegmatis* is similar to that of *M. tuberculosis* (Dick et al, 1998, Mayuri et al, 2002, O'Toole et al, 2003). *M. smegmatis* is also used for study of the stationary phase survival of *M. tuberculosis* (Smeulders et al, 1999).

A comparative study of the genomes of *M. tuberculosis* H37Ra, *M. smegmatis* MC2 155 and *M. bovis* BCG revealed that 2816 (69.8%) of the 4034 protein coding genes of *M. tuberculosis* H37Ra have orthologues in both *M. smegmatis* and *M. bovis* BCG (Altaf et al, 2010). An additional 1083 of the *M. tuberculosis* H37Ra genes (26.9%) have been found in *M. bovis* BCG. However these genes are absent in *M. smegmatis*. In the context of the discussion above it is not surprising that there are

different opinions regarding the use of the *M. smegmatis* for research leading to drug discovery to treat *M. tuberculosis* infection.

However a comprehensive quantitative comparative analysis by Altaf et al (2010) surmised that *M. smegmatis* can be effectively used for detecting inhibitors against *M. tuberculosis* in order to forward potential antitubercular drug molecules. For eg, One of the promising anti-tubercular drugs diarylquinoline TMC 207 was identified from a high throughput screening using *M. smegmatis* (Andries et al, 2005). *M. smegmatis* is the model system for studying the gene regulatory mechanism of *M. tuberculosis* (Snapper et al, 1990). A conserved transcriptional repressor- KstR belonging to the TetR family controls the expression of 74 genes in *M. tuberculosis* and about 83 genes in *M. smegmatis* (Yang et al, 2011).

It has also been found that the secretory apparatus is functionally conserved between the pathogenic mycobacteria and *M. smegmatis* (Flint et al, 2004).

1.3 Chemotherapy against *M. tuberculosis*

The present chemotherapy against tuberculosis includes a multidrug regimen which is administered over a long period of time usually from six to nine months. A multidrug regimen is given as single drug may rapidly give rise to drug resistant bacilli (Pepper et al, 2007).

1.3.1 First line drugs: The first line drugs are the most important and effective antibiotics that are prescribed to TB patients. These drugs include isoniazid (INH), rifampicin (RIF), ethambutol (ETH) and pyrazinamide (PZA). Although streptomycin was the first drug to be used against tuberculosis, the use of streptomycin has been discontinued due to development of fast resistance against it.

1.3.2 Second line drugs: The second line drugs are administered when the first line drugs fail to contain the infection in the infected patients, *i.e.*, when the mycobacteria become resistant to the known first line drugs. Some of the second line drugs are fluoroquinolones, kanamycin, amikacin *etc.*

The chemotherapy for TB includes treatment using the first line drugs for a period of six months with the following treatment regimen. In the first two months the patient is treated with isoniazid, rifampicin, pyrazinamide and ethambutol followed by four months of treatment with rifampicin and isoniazid alone (Balganesh et al, 2008).

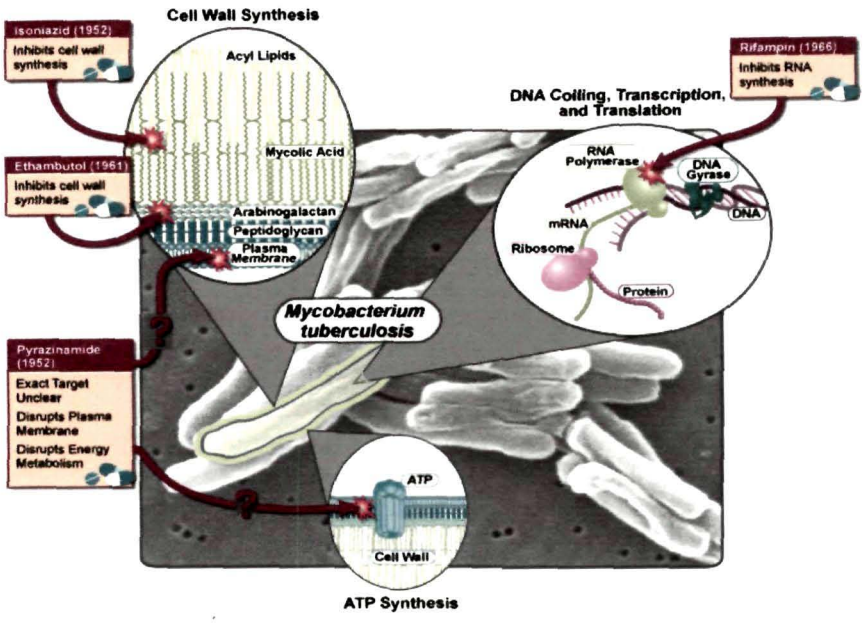


Fig 1.3: The First Line drugs and their targets

(Courtesy: National Institutes of Health, USA)

1.3.3 The First line drugs, their mode of action and mechanism of resistance to the drugs

1.3.3.1 Streptomycin

Streptomycin was the first antibiotic identified from aminoglycosides. It has a molecular formula of $C_{21}H_{39}N_7O_{12}$ and a molecular weight of 581.57.

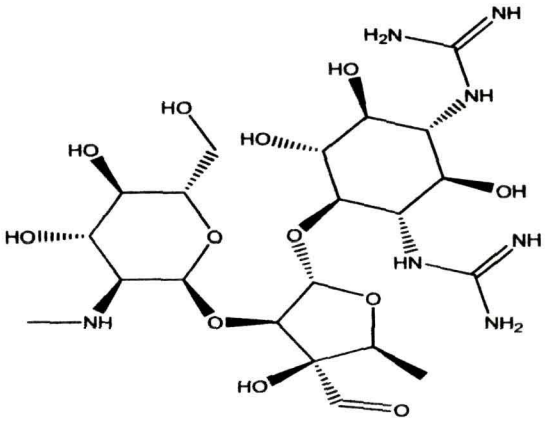


Fig 1.4: Structure of Streptomycin

Mode of action: It inhibits protein synthesis by binding tightly to the conserved A site of the 16S rRNA in the 30S ribosomal subunit.

Drug resistance mechanism: Changes in the ribosome at the 16S rRNA and the ribosomal protein S12 are the major factors contributing to the resistance mechanism of *M. tuberculosis* against streptomycin. (Chen, et al, 2003, Perri and Bonora, 2004)

1.3.3.2 Isoniazid

Isoniazid was first used clinically in 1952 though it was synthesized way back in 1912. It enters the cell of the bacilli through passive diffusion and starts killing only the dividing bacteria. However it does not kill the bacteria when they are in the stationary phase or when growing under anaerobic conditions. The drug acts as bacteriostatic for the first twenty four hours of treatment after which the action turns bactericidal (Vilcheze, et al, 2006).

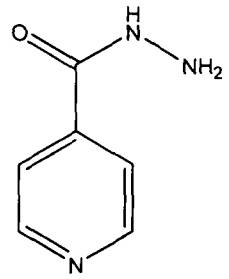


Fig 1.5: Structure of Isoniazid

It has the molecular formula of $C_6H_7N_3O$ and molecular weight of 137.14.

Mode of action: Isoniazid is a prodrug that is activated by KatG (catalase peroxidase hemoprotein). It inhibits InhA, a nicotinamide adenine dinucleotide (NADH)- specific enoyl-acyl carrier protein (ACP) that is involved in fatty acid synthesis.

Drug resistance mechanism: Mutations arising in the target gene (inhA) and in the activating enzyme KatG helps in the bacilli developing resistance against this drug. (Cohen-Gonsaud, et al, 2002)

1.3.3.3 Rifampicin

It was first discovered in the late 1960's. It is one of the leading drugs against tuberculosis which when used in combination with the other drugs helps in reducing

the period of treatment to six months from the initial eighteen months. It has the molecular formula of $C_{43}H_{58}N_4O_{12}$ and a molecular weight of 822.94. (Aristoff 2010).

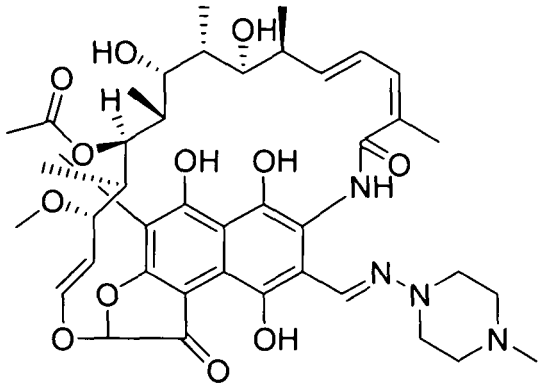


Fig 1.6: Structure of Rifampicin

Mode of action: Rifampin inhibits the *rpoB* gene product of the B subunit of DNA dependent RNA polymerase and acts early in transcription. Thus rifampin is believed to block the transit of the growing RNA chain after 2-3 nucleotides have been added.
Drug resistance mechanism: It has been observed that 97% of the mutants occur in the RIF- resistant determining region (RDR). (Huitric et al, 2006)

1.3.3.4 Pyrazinamide

Pyrazinamide was first synthesized in 1936 and is one of the four first line drugs used in the first two months of antituberculosis therapy. It is an unusual drug given that it is not active against *M. tuberculosis* under normal culture conditions or at neutral pH. It has the ability to kill the bacteria in an hypoxic and acidic environment. Its inclusion has thus made it possible for the current therapy to be shortened to six months from the initial nine months (Dover and Coxon, 2011).

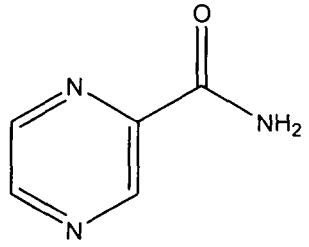


Fig 1.7: Structure of Pyrazinamide

It has the molecular formula of $C_5H_5N_3O$ and molecular weight of 123.11

Mode of action: It is believed that pyrazinoic acid (POA), the active part of pyrazinamide (PZA) inhibits *M. tuberculosis* at an acidic pH. Various evidences suggest that PZA diffuses into the *M. tuberculosis* and gets converted into POA by pyrazinamidase (PZAase). After the conversion of POA, a portion of it exits the cell when the media is acidic and on protonation reenters which then is believed to disrupt the membrane potential of the mycobacteria thereby killing the pathogen. (REF)

Drug resistance mechanism: The resistance to pyrazinamide is believed to be due to the loss of pyrazinamidase activity by the *M. tuberculosis* strains (Somoskovi et al, 2001).

1.3.3.5 Ethambutol

It is the fourth drug in the armament of the first line drugs used in the treatment of tuberculosis. It was first inducted for the treatment of TB in 1966. It has the Molecular formula of $C_{10}H_{24}N_2O_2$ and molecular weight of 204.31. It is used in the treatment of tuberculosis in combination with the other drugs in the first two months of the treatment.

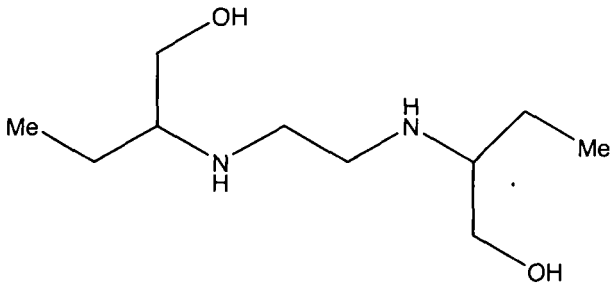


Fig 1.8: Structure of Ethambutol

Mode of action: Ethambutol inhibits arabinosyl transferase that is involved in the biosynthesis of the cell wall.

Drug resistance mechanism: Resistance to ethambutol is observed when mutations occur in the *M. tuberculosis embA* or *embB* gene. (Belanger et al, 1996)

1.4 Directly Observed Therapy Shortcourse (DOTS)

Patient non compliance of the long drug regimen is a major factor in development of drug resistance A new dimension was added in the treatment of tuberculosis with the incorporation of the Directly Observed Therapy Shortcourse (DOTS) strategy Through this programme it is ensured that the patient takes the drugs for treatment of tuberculosis under the direct observation of the Health Care Workers DOTS ensure that the patient does not skip the daily drug regimen and consequently the chances of getting cured increases while development of resistance is lessened (Ahmad et al, 2003) Though the DOTS therapy is demanding for patients, labor intensive for Health Workers and even compromised with poorly available or inaccessible health services, the therapy is rigorously promoted by the WHO The therapy has been promoted as the backbone of TB control and treatment programmes throughout the world (Balganesh etal , 2008 and www msf or jp / info / pressreport / pdf / TBpipelineE pdf) A major achievement under the DOTS and Stop TB strategy is that nearly six million lives were saved and forty one million people have been successfully treated since 1995 around the world Also 5 8 TB cases were notified through DOTS programme in 2009 globally (www who int/tb/publications/2010/factsheet_tb_2010 pdf)

1.5 Vaccines for TB

Vaccination against tuberculosis has proved to be elusive The vaccine(s) must be able to drive the cellular arm of the body immune system which is a challenging task The first vaccine against TB was developed in 1921 by Calmette and Guerin at the Pasteur Institute in France This vaccine is commonly known as the BCG (Bacillus of Calmette and Guerin) vaccine The vaccine is administered in many countries of the world including India The vaccine has been successful in giving protection to children only against severe forms of TB However the BCG cannot prevent the establishment of latent TB or reactivation of pulmonary TB in adults BCG being a live vaccine also has a chance to cause severe complications in the immune compromised individuals Several attempts are currently employed to develop non-viable novel vaccines replacing the present BCG vaccines (Grange et al, 2011) It is very urgent and important that new and effective vaccines against tuberculosis is urgently developed (Ottenhoff and Kaufmann, 2012)

1.6 HIV and TB

A major area that requires thrust which is a priority of the WHO is the identification, recognition and efficient management of TB in the HIV infected individuals. The problem is alarming as individuals suffering from both HIV and TB experience higher rates of complications from both the diseases. Also treatment with the drugs relates to toxic effects in the individual. (Dharmadhikari et al, 2009). The dual pandemic of HIV and TB cause considerable morbidity as well as mortality. In 2000, nearly 11 million people were coinfected with HIV and TB globally. Approximately 40 million people worldwide are infected with HIV and 2.9 million people died in the year 2006. TB hastens the progression of HIV infection to AIDS by accelerating viral replication while the HIV increases the risk of TB incidence. Besides the patients, mostly belonging to the developing countries has to bear the brunt of expensive chemotherapy, drug toxicities and drug-drug and drug-disease interactions(Dharmadhikari et al, 2009).

1.7 Drug resistant TB

Although the first line series of drugs can be effectively used to treat TB, the treatment period is too long-upto about six months. This often leads to the non-compliance of the prescribed treatment by the patient. This is one of the major factors responsible for the emergence of resistant strains. At present two types of *M. tuberculosis* resistant strains are encountered *viz.*, - Multidrug Resistant Tuberculosis (MDR-TB) and Extremely Drug Resistant Tuberculosis (XDR-TB).

1.7.1 MDR-TB

Multidrug resistant TB is a form of tuberculosis which is resistant to atleast two of the most effective first line drugs viz. isoniazid and rifampicin. MDR-TB is spread in the same way as the drug responsive strain of TB. In 2008, there were nearly 4, 40,000 MDR-TB cases in the world with nearly 1, 50,000 deaths. In 2009, it has been estimated that nearly 3.3% of all new TB cases are positive for MDR-TB (WHO factsheet, 2010). In parts of Northwest Russia, the occurrence of MDR- TB percentage is the highest with 1 MDR case in every four reported TB cases. The highest numbers of MDR-TB cases are reported from China and India, sharing 50% of the total MDR-TB infections in the world (WHO MDR/XDR TB datasheet 2012).

Resistance to anti-tuberculosis drugs develops either due to mismanagement of the drug regimen. Some factors that contribute to such resistance include the following:

i) Patient non-compliance to the prescribed drug regimen, because of the very long treatment period (06 months).

ii) Prescription of wrong dose, wrong treatment or wrong treatment period.

iii) Inadequate supply of drugs

iv) Low/poor (spurious) quality of drugs.

1.7.2 Treatment of MDR-TB

The treatment for MDR-TB is long and expensive. It takes nearly two years for the treatment and the drugs belonging to the second-line drugs are expensive. Moreover these drugs have high toxicity.

1.7.3 XDR-TB

Extensively drug resistant tuberculosis (XDR-TB) is a form of multidrug resistant TB which is resistant not only to the first line drugs but also to the best second line medications such as fluoroquinolones and atleast to any of the three injectable drugs – amikacin, kanamycin and capreomycin.

1.7.4 Treatment of XDR-TB

Patients with XDR-TB are left with very little options for treatment as no medication is available and even those which are used are ineffective with severe side effects. As such, this form of tuberculosis is virtually incurable with the present available drugs.

1.8 The need for new Drugs

The lack of new drugs with the last drug rifampicin discovered way back in 1960's and the emergence of resistant strains of tuberculosis, call for an urgent necessity to discover new TB drugs.

1.8.1 Traditional Knowledge (TK) in Drug Discovery

Since time immemorial plants have been the basis of life and strength for the human race. The *Rigveda*, one of the four Vedas, which dates back to 4500-1600 BC is one of the oldest documentary evidences embodying a repository of human knowledge on the use of medicinal plants in the Indian subcontinent (Jamal, et al, 2012). Besides, among the many tribal societies across the world, there has been the practice of using different traditional knowledge driven medicinal plants as herbal drugs for generations. This is noted as an important strategy in the conservation of biodiversity and in the discovery of new drugs. This in turn helps in the upliftment of the quality of life of the common people in the distant rural locations where access to modern medicine and healthcare is still a distant dream (Moshi, et al, 2012). Two third of the total population in the developing countries believe and use traditional medicines and other herbal plant based drugs for the initial primary treatments of several diseases. Due to the easy availability of the plant based traditional medicines, its belief and acceptance among the local people, traditional medicine encompassing ecological, cultural and traditional values has been in use for generations (Kunwar et al, 2010). Information on the use of medicinal plants through folklore has been an important tool in revealing numerous plants with medicinal properties (Maregesi et al, 2007).

1.8.2. Natural products from plants as a source of drugs

Plants have also been the sources of drugs used in modern medicine (Maregesi et al, 2007). Natural products are indeed in the forefront of drug discovery. In the last decade itself natural product derived molecules contributed to nearly half of all the small molecules that are approved as drugs. Ethnobotanical approach and the traditional herbal medicines provide the much required drug discovery platform for consideration in the development of new drugs (Patwardhan and Mashelkar, 2009). Various medical breakthroughs are based on natural products. Uses of natural products have been found to be the most successful strategy in the process of discovery of new and novel medicines.

Several strong justifications have been put forward in favour of the natural products as the potential source for future drug discovery.

- Most of the presently available different classes of drugs contain either natural products or have natural products as their original leads
- Natural product compounds generally possess unique biological properties as well as challenging structural complexity (Beghyn et al, 2008)
- Mode of action of many synthetically derived leads has been identified in natural products.
- Of the 974 new small chemical entities reported as drugs during 1981-2006, 331 were of natural origin either extracted or hemisynthetic (Newmann and Cragg, 2007).
- The isolation and identification of natural products have been growing at more than 10,000 molecules per year.

As such screening of natural products is a useful and important strategy to find different lead molecules (Tulp and Bohlin, 2002). Plant based drug discovery resulted mainly in the development of anti-infectious agents and continues to contribute to the new leads in clinical trials (Saklani et al, 2008). Plant based medicines were initially dispensed in the form of crude drugs such as, tinctures, teas, poultices, powders, and other herbal formulations and now serve as the basis of novel drug discovery (Jachak et al, 2007).

Assam, in North-East India, with its vast and unique plant resources has huge potential and possibilities in the field of drug discovery. Assam lies in the North East India which falls in the Indo-Burma region and has been identified as one of the eight hotspots for biodiversity in the world depending on five factors that include Endemic plants (Myers et al, 2000). Of the three thousand odd plant species found in the state, some 950 species are known to possess definite medicinal properties. Some of these species have been used in the ayurvedic, unani, and other traditional alternative systems since time immemorial. This huge body of traditional knowledge is an invaluable asset for the process of drug discovery. Many of the native plant species of Assam are traditionally known to have antibacterial activity. These plants need to be investigated for validating their antibacterial properties. Therefore drug discovery efforts can justifiably be focused on these species for identifying the much needed new potent antimycobacterial molecules.

1.9 Virtual screening in aid of drug discovery

Natural product extraction and purification for drug discovery is often a difficult process on account of the very large numbers of molecules present in plants and secondly the structural complexities present in these molecules. Through *in silico* approaches many of the natural products can be screened avoiding wastage of pure compounds. Virtual screening is therefore very advantageous in that it often results in reduction of time in searching for new potential active chemical entities, reduces the resources required to screen a library of compounds and therefore is inexpensive and saves labor (Oprea, 2002).

Drug discovery is a ligand based approach using Docking, Quantitative Structure Activity Relationships (QSAR) studies, Absorption Distribution Metabolism Excretion and Toxicity (ADME-TOX) studies and other pharmocophores related parameters. In such studies a model is generated using designed software. Such models enable optimization of antibacterial activity against a particular target and also in finding out a new hit to lead molecule (s) (Ekins et al, 2011).

Virtual screening of new drug candidates as well as existing compounds is an emerging strategy which is increasingly used in the drug discovery process. Such screening processes in conjugation with Structural Biology enhance the success and increase in the number of leads for drug discovery. Molecular docking is an essential part of the virtual screening which is a receptor based computational method. In docking a ligand (usually, a small molecule) is effectively docked into a target protein (enzyme) producing a definite score based on the fitness of the ligand to the target binding site (Lyne, 2002). Such studies using small molecules in virtual screening help in identification of the hits (Kitchen et al, 2004). In any drug discovery process the identification and development of active ligands, specifically for a target; generally a protein (enzymes) is the primary goal (Reddy et al, 2007). Therefore there are two major aspects in docking i) accurate structure modelling and ii) correct activity prediction. (Kitchen, 2004).

Quantitative structure activity relationship (QSAR) is another widely used tool employed in drug discovery process. The QSAR method is generally used to establish a relation between the electronic and the structural properties of various putative drug molecules and the affinity of binding of these molecules against macromolecular target(s), which is either a protein or an enzyme.

The Absorption, Distribution, Metabolism, Elimination and Toxicity (ADME-TOX) studies in drug discovery are required to identify the compounds/molecules with favorable pharmacokinetics and toxicological properties. Such studies on the molecules using standard *in silico* softwares help in saving time and expenditure and enable screening out large numbers of undesirable molecules in a relatively very short period of time (Naez and Rodriguez, 2002).

1.10 Drug delivery and sustained drug release

The efficacy of a drug depends to a great extent on its effective release without which the disease may aggravate further with serious biomedical implications. An example of this is witnessed in the case of the first line drugs of tuberculosis. Tuberculosis (TB) patient is prescribed multiple oral administrations of the first line drugs (e.g. isoniazid) daily for at least six to eight months. This makes patient compliance of the prescribed drugs difficult and therefore precludes effective treatment of the disease. This is one of the main reasons attributed to the recent emergence of Multidrug Resistance and Extensive Drug Resistance in *Mycobacterium tuberculosis*. It is pertinent to point out that tuberculosis has emerged as a major challenge with nearly one third of the total world population, roughly about more than two billion people infected by the pathogen (WHO 2011-12 report). There is urgency therefore in not only discovering new drug molecules but also for developing an effective drug delivery system. Such a system should have a longer release time to facilitate the release of the drug over a longer period of time so that multiple daily oral administration of the drug is not required (Ain et al, 2003).

Microcrystalline cellulose (MCC) based drug delivery system has been proposed with its implicit advantages of targeted delivery in the pathogen infected cells enhancing its therapeutic value and sustained release minimizing frequent drug dosing issues(Clemens et al. 2012). The present study entails an investigation on developing suitable MCC based drug delivery vehicle for use in treatment of *M. tuberculosis*.

1.11 Objectives

The work embodied in this thesis proceeded with the following objectives which are identified against the broad perspectives of the urgent necessity to forward new molecules as potential drugs to cure tuberculosis described in the preceding introduction

General Objective:

1 To explore local traditional knowledge pertaining to treatment of respiratory diseases

2 To generate hypothesis on drug target interaction relating to treatment of tuberculosis

3 Validation of the traditional knowledge

Specific Objective:

1 Screening of medicinal plants traditionally used for treatment of respiratory diseases like tuberculosis

2 Isolation and characterization of active compounds from selected plant parts

3 *In silico* investigation of phytochemicals against selected *M tuberculosis* target proteins

4 Development of drug delivery vehicles for sustained release

Chapter II
Review of Literature

2.1 *Mycobacterium tuberculosis*

Tuberculosis, caused by the bacillus *Mycobacterium tuberculosis* is at present one of the most successful human pathogens The microbe has been successful in evading the host immune system and has developed resistance to almost all the known first line drugs used in the treatment of the disease Nearly 8 7 million new cases were reported in 2011 Also in 2011, 1 4 million people died from TB which includes 4, 30,000 HIV positive people (WHO Global TB Report, 2012) 3, 10,000 cases of MDR-TB were reported in 2011 with majority of the cases reported from Brazil, China, India, South Africa and the Russian Federation Nearly 84 countries in the world have reported atleast one case of XDR-TB (WHO MDR TB Report, 2012)

The tubercle bacillus has some basic characteristic features which set aside the microbe from others Some such features include slow growth, complex cell envelope, dormancy (latency), genetic homogeneity and intracellular pathogenesis (Cole, et al, 1994) The generation time of *M tuberculosis* is about 24 hours, which perhaps contribute towards the complex pathogenesis of the disease Cell mediated immune response to the bacterial infection contains the infection without eradicating it This results in the shutdown of all the metabolic activities in the bacteria and in setting in dormancy or latency Such dormant or latent bacteria reactivate again when the immune system of the person is compromised (Cole, et al, 1994)

The genome of *M tuberculosis* is GC rich The presence of high amounts of lipids, glycolipids and polysaccharides as an additional layer beyond the peptidoglycan is an important characteristics of mycobacterial cell envelope (Cole, et al, 1998, Kolattukudy, et al, 1997) Several pathways contribute to the generation of mycolic acid, lipoarabinomannan, arabinogalactan, mycocerosic acid and phenolthiocerol These components are responsible for the mycobacterial pathogenesis, resistance to various drugs and its adaptive ability to survive within the host overcoming all the defense mechanism of the body (Cole, et al, 1998)

The first line drugs used for the treatment of TB were developed a few decades ago with rifampicin in the 1960's which incidentally is the last of the developed drugs. At present the resistant strains- MDR-TB and XDR-TB, have emerged against all the known first line and also second line series of drugs. The development of new anti-TB drugs therefore is the need of the hour.

Natural products and their derivatives have been playing an important role in the development of new drugs for many ailments. During 2005-2007, 13 natural products related drugs were derived (Butler, 2008). Different natural products have been reported to have anti-TB activity as reported in a number of literatures (Tripathy, et al, 2005, Nayyar and Jain, 2005).

2.2 Medicinal Plant Use

The history of use of plants in treatment of various ailments is buried in deep antiquity. The use of medicinal plants in various parts of the world is well documented. One of the most comprehensive ancient documents is undoubtedly the *Ayurveda,* from India. The multitude of ethnic groups across the world has their own experiences with medicinal plants. Generally such experiences and wisdom are undocumented and are referred to as 'Traditional Knowledge' (TK). These are invaluable treasure troves for discovering new drug molecules. Some of the recent attempts to document TK on the use of medicinal plants may be pertinent to mention here.

The use of 53 plant species is reported of which 50 are used regularly for treatment of various human diseases in Mozambique (Ribeiro, et al, 2010). Plant resources are known to be used against various ailments by the local people of Churu district in Rajasthan (Parveen, et al, 2007). A study on 243 medicinal plants belonging to 76 different families from the Sitamata Wild life sanctuary in Rajasthan, in India was carried out. All the plants have been found to have use in traditional medicine in the state (Jain, et al, 2005). Assam, in North East India is well known for its vast biodiversity. Various medicinal plants from the state have been used in traditional medicine since long. A report on the use of 65 different species of plants with diverse medicinal use has been published (Purakayastha and Nath, 2006). A study on 66 species of medicinal plants from the Terai forest of Western Nepal has been reported. These belonged to 60 genera in 37 families of angiosperms. Of these, 41 are used in

treatment of gastro-intestinal disorders. 53% of these species were herbs while trees constituted only about 23% (Singh, et al, 2012). 39 medicinal plant species belonging to 35 genera and 27 families were reported to be used as medicinal plants by tribal practitioners, mainly by the Jaintia tribes inhabiting the North Cachar Hills district of Assam, India (Sajem and Gosai, 2006).

2.3 Natural products and their antimycobacterial activities

Natural products are secondary metaboilites which are known for beneficial effects on human health. These molecules are produced in platns with distinct biological properties (Garcia, et al, 2012, Kishore, et al, 2009). It is believed that nearly two third of the world population today still relies on medicinal plants as their primary sources of medicine (Mc Chesney, et al, 1995). Natural products from plants and their various derivatives were reported in various literatures to inhibit *M. tuberculosis* and other *Mycobacterium* species. A brief review of medicinal plants having antimicrobial activity is presented below.

2.3.1 Resolving Plant Extracts for Antimycobacterial Agents

From 118 different plant species, 230 organic extracts were evaluated for their antimycobacterial activity (Cantrell, et al, 1998). All the extracts were screened for activity against *M. tuberculosis* at a concentration of 100 μg/ml. 95% inhibition was observed in 24 extracts while activity against *M. avium* was recorded in 10 other extracts. *Borrichia frutescens, Solidago arguta* and *Inula helenium, Erigeron strigosus* and *Magnolia acuminata* showed 100% inhibition of *M. tuberculosis* while *Euthamia leptocephala, E. strigosus* and *M. acuminata* exhibited 100% inhibition of *M. avium*. The hexane and methanol extracts obtained from the plant *Juniper excelsa* showed moderate activity against *Mycobacterium tuberculosis* $H_{37}Rv$ with a MIC of 15.5 μg/ml and 17.0 μg/ml respectively (Topcu, et al, 1999). The chloroform extracts and the physalin containing fractions from the plant *Physalis angulata* exhibited antimycobacterial activity against *M. tuberculosis, M. avium, M.kansasii* and *M. malmoense* at different concentrations (Pietro, et al, 2000). Extracts from 43 different plants were assayed for antimycobacterial activity (Newton, et al, 2002). The methanol extracts of 3 different plants *Commiphora mukul, Psoralea corylifolia* and *Sanguinaria canadensis* were found to have significant activities against

Mycobacterium aurum 4721E with a MIC of 62.5 μg /ml. The hexane extract of *P. corylifolia* was found to be the most active with a MIC of 31.25 μg/ml. Dichloromethane extracts of nearly 270 Peruvian plant samples representing 216 species were investigated for their antimycobacterial property. Of these, *Senna silvestris, Sommera sabiceoides* Schum, *Nectandra hihua* Rowher, *Senna obliqua, Heisteria accuminata* Engler and *Zanthoxylum sprucei* Engler showed MICs of <6.25 μg/ml, <6.25 μg/ml, 10 μg/ml, 10 μg/ml, 10 μg/ml and 15 μg/ml respectively (Graham, et al, 2003). The dichloromethane and the light petroleum extracts from the plant *Hapllopappus sonorensis* exhibited 60% inhibition against *M. tuberculosis* at 100 μg/ml (Murillo, et al, 2003). The hexane extract of the plant *Lantana hispida* showed antimycobacterial activity against *M. tuberculosis* $H_{37}Rv$ with a MIC of 200 μg /ml (Arellanes, et al, 2003). From the forty four different plant species of Turkey, screened for their activity against *M. tuberculosis* $H_{37}Ra$, the petroleum ether extracts from 6 species, *viz., Chelidonium majus, Pinus brutia, Salvia aethiopis, Stachys sylvatica, Ulmus glabra, Urtica dioica* and the chloroform extracts of *Chelidonium majus* and *Salvia aethiopis* showed a MIC of 50 μg/ml (Tosun, et al, 2004). The plant *Pelargonium reniforme* and *Pelargonium sidioides* were investigated for their antimycobacterial activity against *M. aurum, M. smegmatis, M. fortuitum, M. phlei* and *M. abcessus*. The powdered roots of the plants were used for the study and the extractions were done using n-hexane and ethyl acetate. The various fractions subsequently obtained from the above extracts showed different antimycobacterial activity with MICs ranging from 8-512 μg/ml. The major compounds in these fractions were found to be linoleic acid and oleic acid (Seidel and Taylor, 2004). 55 extracts obtained from twenty two species were investigated for their antimycobacterium activities against *M. bovis* BCG at a specific concentration of 100μg/ml. Of the extracts, the methanol extract of the fruit of *Amborella trichopoda* demonstrated the best activity with a MIC of 1-2.5 μg/ml (Billo, et al, 2005). The methanolic and alkaloid fractions from species *Alstonia scholaris* from the Phillipines showed antituberculosis activity against the test strain of *M. tuberculosis* $H_{37}Rv$ (Macabeo, et al, 2005). 5 different solvent fractions from three different plant species- *Inula helenium subsp. turcoracemosa, Usnea barbata* and *Veratrum album* exhibited anti tuberculosis activity with greater than 90 % inhibition at concentrations less than 100 μg/ml (Tosun, et al, 2005). 5 medicinal plants *Artemisia ludoviciana* Nutt.,

Chenopodium ambrosioides L., *Marrubium vulagare* L., *Mentha spicata* L. and *Flourensia cernua* DC from Mexico, were evaluated for their antimycobacterial potential against *M. tuberculosis* H37Rv and *M. tuberculosis* CIBIN:UMF:15:99 (MDR strain resistant to streptomycin, isoniazid, rifampicin, ethambutol and pyrazinamide). Of the five plants, the hexane and acetone extracts of *F. cernua* not only inhibited the bacteria at a MIC of 50 μg/ml and 200 μg/ml. The hexane and acetone extracts were also found to be active against the MDR strain at MICs of 25 μg/ml and 100 μg/ml respectively (Salinas, et al, 2006). 12 different plant species from Brazil, Chile and Argentina were screened for their antimycobacterial activity. 48 different organic extracts were prepared and used in concentrations of 100 μg/ml. Of these, 7 extracts from 4 different plant species tested positive for the antimycobacterial activity against *M.tuberculosis* $H_{37}Rv$ (ATCC 27294). The hexane and dichloromethane extracts from the leaves of *Lantana trifolia, Lippia lacunosa, Lippia rotundifolia* and the methanol extract (methanol: water, 1:1) from the bark of *Vitex cooperi* were found to be positive (Leitao, et al, 2006). The acetone, chloroform and the ethanol extracts of the roots of the plant species *Pelargonium reniforme* was found to exhibit antituberculosis activity against *M. tuberculosis* H37Rv with a MIC of 5mg/ml (Mativandlela, et al, 2006). The ethyl acetate and ethanol extracts from the leaf, bark and roots of the plants, Acacia *nilotica* and *Combretum krausii* inhibited the growth of *M. aurum* A+ (Eldeen and Staden, 2007). The methanol extracts from the roots and leaves of *Leucophyllum frutescens* and the ethyl ether extracts from the roots of *Chrysanctinia mexicana* exhibited antimycobacterial property against *M. tuberculosis* $H_{37}Rv$ ATCC 27294, at MIC's of 62.5, 125 and 62.5 μg/ml (Salinas, et al, 2007). The *Salvia* species, *S. radula*, *S. verbenaca* and *S.dolomitica* showed activity against *M. tuberculosis* $H_{37}Ra$ at an MIC of 100 μg/ml (Kamatou, et al, 2007). The ethyl acetate crude extract of the bark from the plant *Evodia elleryana* exhibited significant inhibition of *M. tuberculosis* $H_{37}Ra$ at a MIC of 50 μg/ml with 95% inhibition (Barrows, et al, 2007). Various solvent extracts of different polarities were separated from the leaves, roots and stem bark of the plant *Diospyros anisandra* and investigated for their activity against *M. tuberculosis* $H_{37}Rv$ and *M. tuberculosis* CIBIN/UMF 15:99. Of all the extracts the hexane extract from the stem bark was found to be the most active against both the strains (Argaez, et al, 2007). 95% ethanol extract from the stem of the plant *Indigofera aspalathoides* inhibited the growth of *M.*

tuberculosis $H_{37}Rv$ at a MIC of more than 100 μg/ml (Rajkapoor, et al, 2007). The dichloromethane extract form the plant *Warburgia salutaris* was found to have activity against *M. tuberculosis* $H_{37}Rv$ at 200 μg/ml and *M. bovis* BCG at a concentration of 100 μg/ml (Madikane, et al, 2007). Of the seven different plant species selected for assessing their antimycobacterial properties, four species *viz.*, *Artemisia afra, Dodoneia angustifolia, Drosera capensis* and *Galenia africana* inhibited the growth of *Mycobacterium smegmatis* MC2 155 at different MIC concentrations of 0.781-6.25 mg/ml. Two of the plants, *D. angustifoli* and *G. africana* also demonstrated activities against *M. tuberculosis* $H_{37}Rv$ at MIC values of 5.0 and 1.2 mg/ml (Mativandlela, et al, 2008). The ethanol extract of the root of *Glycyrrhiza glabra* showed activity against both *M. tuberculosis* $H_{37}Rv$ and $H_{37}Ra$ strains at a concentration of 500 μg/ml. However the ethyl acetate fraction and the chloroform: ethyl acetate fraction showed better antimycobacterial activity at 100-200 μg/ml and 50-120 μg/ml. The purified compound glabridin was found to be more active at a MIC of 29.16 μg/ml (Gupta, et al, 2008). The acetone and ethanol leaf extracts of the plant *Coleonema album* was investigated for their activity against *M. aurum* A+. Almost all the extracts showed moderate activity against the *M. aurum* strain, with a MIC of 3.1 mg/ml (Eldeen and Staden, 2008). Eight medicinal plants traditionally used for treatment of tuberculosis and other respiratory diseases in Nigeria were evaluated for their activity against *M. tuberculosis* clinical isolates and *Mycobacterium bovis* BCG using the methanol extracts. Of these, 4 extracts from *Entada africana, Hymenocardia acida, Sterculia setigera* and *Stereospermum kunthianum* exhibited activity against the *M. tuberculosis* clinical isolates at MICs of 78-1250 μg/ml (Mann, et al, 2008). The methanol leaf extracts of the plants *Persea americana* Mill and *Gymnosperma glutinosum* were investigated for their activity against *M. tuberculosis* $H_{37}Rv$ and *M. tuberculosis* $H_{37}Ra$. The antimycobacterial activity of *P. americana* with a MIC of 125 μg/ml and 62.5 μg/ml against *M. tuberculosis* $H_{37}Ra$ and *M. tuberculosis* $H_{37}Rv$ was found to be better than that of *G. glutinosum* which exhibited a MIC of 250 μg/ml against both the strains. Also the hexane extract of *P. americana* was found to be active and exhibited a MIC of 31.2 μg/ml against both the strains (Flores, et al, 2008). 36 plants from the Atlantic region of Brazil were investigated for their antitubercular activities against *M. tuberculosis* H37Rv. Ethanol extracts of the plants were prepared from the air dried and powdered leaves. Each of the extracts was then subjected to

anti-tuberculosis study at a concentration of 100μg/ml. Of all the extracts, only *Psychotria vellosiana, Pouteria filips, Cedrela fissilis, Plathymenia foliolosa* and *Peschirea affinis* was found to be positive. Extracts obtained from these plants, demonstrated antimycobacterial activity at MICs of 0.2, 0.78, 3.12, 0.78 and 0.2 μg/ml respectively (Ramos, et al, 2008). The crude methanol and aqueous extracts of ten different *Venda* medicinal plants were investigated for their antimycobacterial activity. The plants are traditionally used for treatment of various respiratory diseases. Of the plants tested, three of them, *Securidaca longepedunculata, Syzygium cordatum*, and *Tabernaemontana elegans* were found to possess MIC values of ≤ 1 mg/mL against *M. smegmatis* (Pallant and Steenkamp, 2008). 9 medicinal plants used for treatment of TB and other respiratory diseases in Mexico were subjected to anti-tuberculosis study against *M. tuberculosis* MDR strains. Of the plants, *Nasturtium officinale* showed the best activity with a MIC of 100 μg/ml (Corona, et al, 2008). The methanol extract of the aerial parts of *Thymbra spicata* and *Origanum minutiflorum* were found to exhibit antimycobacterial activity against *M. tuberculosis* $H_{37}Ra$ at a MIC of 196 μg/ml and 392 μg/ml (Askun, et al, 2009). The chloroform and the methanol extracts of *Lantana camara* collected from South West Uganda, were screened for their activity against *M. tuberculosis* $H_{37}Rv$, *M. tuberculosis* TMC 331 and a wild strain of *M. tb*. It was found that the methanol extract has the highest activity against all the three strains with MIC values of 20 μg/ml for $H_{37}Rv$, 15 μg/ml for both the TMC 331 and the wild strain (Kirimuhuzya, et al, 2009). The acetone extracts of *Berchemia discolor, Bridelia micrantha, Terminalia sericea* and *Warbugia salutaris* showed promising inhibitory activities against *M. tuberculosis* $H_{37}Ra$ with MIC ranging from 12.5 μg/ml for *B. discolor* and 25 μg/ml each for the others. Four other plants *Ziziphus mucronatha, Scotia brakepetale, Rhus rogersii and Piper capense* also exhibited antimycobacterial properties with MICs ranging from 50-100 μg/ml (Green, et al, 2010). The methanol extracts of the twigs and leaves of *Treculia obovoidea, T. africana and T. acuminata* were investigated for their antimycobacterial activity. It was found that the leaf crude extracts of all the 3 species and the twigs of the plant *T. africana* could prevent the growth of *M. smegmatis* ATCC 700084 and *M. tubercuosis* $H_{37}Rv$ 27294. Against *M. smegmatis* the lowest recorded MIC was 19.53 μg/ml with the methanol extract of *T. africana* while both *T. africana* and *T. acuminata* showed MIC of 19.53 μg/ml against *M .tuberculosis* (Kuete, et al, 2010).

The methanol extract of the powdered stem bark of the plant *Thecacoris annobonae* was found to possess activity against *M. tuberculosis* $H_{37}Rv$ ATCC 27294 and on *M. smegmatis* ATCC 70084, with MICs of 19.53 μg/ml and 39.06 μg/ml (Kuete, et al, 2010). Of the 78 plant extracts from 70 plants investigated for activity against *M. tuberculosis* $H_{37}Rv$, 38 plant extracts from 36 different species exhibited inhibitory properties. All the extracts were prepared with 80% methanol. Of all the extracts, the leaf extract of *Angiopteris evecta* exhibited the highest activity with a MIC of 400 μg/ml. The stem and flower extracts from *Costus speciosus*, whole plant extract of *Piper sarmentosum,* leaf and flower extracts of *Pluchea indica* and leaf extract of *Tabernaemontana coronaria* also exhibited antimycobacterial activity with MIC of 800 μg/ml each (Mohamad, et al, 2011). The methanol, 80% methanol and chloroform extract of the leaves from the plant *Byrsonima fagifolia niedenzu* exhibited a MIC of 280, 500 and 625 μg/ml against *M. tuberculosis* $H_{37}Rv$ ATCC 27294 (Higuchi, et al, 2011). Of the fifteen medicinal plants from Mozambique tested for activity against *M. tuberculosis* $H_{37}Rv$ and *M. bovis* BCG, the *n*-Hexane extracts of *Maerua edulis* and *Securidaca longepedunculata*, the ethyl acetate extract of *Tabernaemontana elegans* and dichloromethane extract of *Zanthoxylum capense* were found to possess antimycobacterial activity. The MICs of the extracts ranged from 15.6-62.5 μg/ml with the ethyl acetate extract from *Tabernaemontana elegans* being the most potent at 15.6 μg/ml (Luo,et al, 2011). 7 herbaceous plants from South Africa were investigated for their antimycobacterial activities. Of these plants, the ethanol extract of the plant *Knowltonia vesicatoria* and the *Helleborus* (cultivar) exhibited antimycobacterial activity against *M. smegmatis* at MICs of 0.6 and 1.2 μg/ml. Two other species, *Symphytum officinale* and *Ranunculus repens* along with *Knowltonia vesicatoria* and *Helleborus* exhibited MICs of 5mg/ml, 1mg/ml, 0.1mg/ml and 1mg/ml against *M. tuberculosis* H37Rv (Labuschagne, et al, 2012). *Piper nigrum* L among the five medicinal plants tested against *M. tuberculosis* $H_{37}Rv$ was found to exhibit the best MIC at 100 μg/ml (Birdi, et al, 2012). Some of the plant extracts and their antimycobacterial activity against *M. tuberculosis* is being presented below in Table 2.1.

Table 2.1: Antimycobacterial activity of plant extracts

Sl. No	Medicinal Plant	Plant Extract	MIC value (µg/ml)	$H_{37}Rv$/ $H_{37}Ra$	Reference
1	*Juniper excelsa*	Methanol	17.0	$H_{37}Rv$	Topcu et al, 1999
		Hexane	15.5	$H_{37}Rv$	"
2	*P.corylifolia*	Hexane	31.25	$H_{37}Rv$	Newton et al, 2002
3	*Senna silvestris*	Dichloromethane	□6.25	$H_{37}Rv$	Graham, 2003
	Sommera sabiceoides Schum	"	□6.25	$H_{37}Rv$	"
	Nectandra hihua Rowher	"	10	$H_{37}Rv$	"
	Senna obliqua	"	10	$H_{37}Rv$	"
	Heisteria accuminata Engler	"	10	$H_{37}Rv$	"
	Zanthoxylum spruce Engler	"	15	$H_{37}Rv$	"
4	*Haollopappus sonorensis*	Dichloromethane	100	$H_{37}Rv$	Murillo et al,2003
		Light petroleum	100	$H_{37}Rv$	
5	*Lantana hispida*	Hexane	200	$H_{37}Rv$	Arellanes et al 2003
6	*Chelidonium majus*	Petroleum ether	50	$H_{37}Ra$	Tosun et al, 2004
	Pinus brutia	"	50	$H_{37}Ra$	"
	Salvia aethiopis	"	50	$H_{37}Ra$	"
	Stachys sylvatica	"	50	$H_{37}Ra$	"
	Ulmus glabra	"	50	$H_{37}Ra$	"
	Urtica dioica	"	50	$H_{37}Ra$	"
	Chelidonium majus	Chloroform	50	$H_{37}Ra$	"
	Salvia aethiopis	"	50	$H_{37}Ra$	"
7	*Inula helenium subsp. turcoracemosa*	Solvent fractions	100	$H_{37}Rv$	Tosun et al., 2005
	Usnea barbata	Solvent fractions	100	$H_{37}Rv$	"
	Veratrum album	Solvent fractions	100	$H_{37}Rv$	"
8	*F.cernua*	Hexane	50	$H_{37}Rv$	Salinas et al., 2006
		Acetone	200	$H_{37}Rv$	Salinas et al., 2006
9	*Pelargonium reniforme*	Acetone	5000	$H_{37}Rv$	Mativandlela et al, 2006
		Chloroform	5000	$H_{37}Rv$	"
		Ethanol	5000	$H_{37}Rv$	"
10	*Leucophyllum frutescens (root)*	Methanol	62.5	$H_{37}Rv$	Salinas et al, 2007

	Leucophyllum frutescens (leaves)	"	125	$H_{37}Rv$	"
	Chrysanctinia Mexicana (root)	Ethyl ether	62.5	$H_{37}Rv$	"
11	*Evodia elleryana*	Ethyl acetate	50	$H_{37}Ra$	Barrows et al, 2007
12	*Indigofera aspalathoides*	Ethanol	100	$H_{37}Rv$	Rajkapoor et al, 2007
13	*Warburgia salutaris*	Dichloromethane	200	$H_{37}Rv$	Madikane et al., 2007
14	*Byrsonima fagifolia niedenzu*	Methanol	280	$H_{37}Rv$	Higuchi et al, 2008
		80% methanol	500	$H_{37}Rv$	"
		Chloroform	625	$H_{37}Rv$	"
15	*Glycyrrhiza glabra*	Ethanol	500	$H_{37}Rv$, $H_{37}Ra$	Gupta et al, 2008
16	*Entada africana*	Ethanol	78-1250	$H_{37}Rv$	Mann et al, 2008
	Hymenocardia acida	"	78-1250	$H_{37}Rv$	"
	Sterculia setigera	"	78-1250	$H_{37}Rv$	"
	Sterospermum kunthianum	"	78-1250	$H_{37}Rv$	"
17	*P .americana*	Methanol	62.5	$H_{37}Rv$	Flores et al., 2008
			125	$H_{37}Ra$	"
		Hexane	31.2	$H_{37}Ra$	"
	G .glutinosum	methanol	250	$H_{37}Rv$, $H_{37}Ra$	"
18	*Psychotria vellosiana*	Ethanol	0.2	$H_{37}Rv$	Ramos et al., 2008
	Pouteria filips	"	0.78	$H_{37}Rv$	"
	Cedrella fissilis	"	3.12	$H_{37}Rv$	"
	Plathymania folidosa	"	0.78	$H_{37}Rv$	"
	Peschirea affinis	"	0.2	$H_{37}Rv$	"
19	*Nasturtium officinale*	Ethanol	100	$H_{37}Rv$	Corona et al, 2008
20	*Thymbra spicata*	Methanol	196	$H_{37}Ra$	Askun et al, 2009
	Origanum minutiflorum	"	392	$H_{37}Ra$	"
21	*Lantana camara*	Methanol	20	$H_{37}Rv$	Kirimuhuzya et al., 2009
		Chloroform	20	$H_{37}Rv$	"
22	*Berchemia discolor*	Acetone	12.5	$H_{37}Ra$	Green et al, 2010
	Bridelia micrantha	"	25	$H_{37}Ra$	"
	Terminalia sericea	"	25	$H_{37}Ra$	"
	Warbugia salutaris	"	25	$H_{37}Ra$	"
	Ziziphus mucronatha	"	50-100	$H_{37}Ra$	"
	Scolia brakapetela	"	50-100	$H_{37}Ra$	"
	Rhus rogersii	"	50-100	$H_{37}Ra$	"

	Piper capense	"	50-100	$H_{37}Ra$	"
23	*T.africana*	Methanol	19.53	$H_{37}Rv$	Kuete et al, 2010
	T.acuminata	"	19.53	$H_{37}Rv$	"
	Thecacoris annobonae	"	19.53	$H_{37}Rv$	"
24	*Angiopteris evecta*	80%methanol	19.53	$H_{37}Rv$	"
	Costus speciosus(stem and flower)	"	800	$H_{37}Rv$	"
	Piper sarmentosum(whole plant)	"	800	$H_{37}Rv$	"
	Pluchea indica(leaf and flower)	"	800	$H_{37}Rv$	"
	Tabernaemontana coronaria(leaf)	"	800	$H_{37}Rv$	"
25	*Tabernaemontana elegans*	Ethyl acetate	15.6	$H_{37}Rv$	Luo et al, 2011
	Maerua edulis	"	15.6-62.5	$H_{37}Rv$	"
	Securidaca longepedunculata	"	15.6-62.5	$H_{37}Rv$	"
26	*Piper nigrum L*	Ethyl acetate	100	$H_{37}Rv$	Birdi et al., 2012
27	*Symphytum officinale*	Ethanol	5000	$H_{37}Rv$	Labuschagne et al., 2012
	Ranunculus repens	"	1000	$H_{37}Rv$	"
	Knowltonia vesicatoria	"	100	$H_{37}Rv$	"
	Helleborus sp	"	1000	$H_{37}Rv$	"

2.3.2 Alkaloids as Antimycobacterial Agents

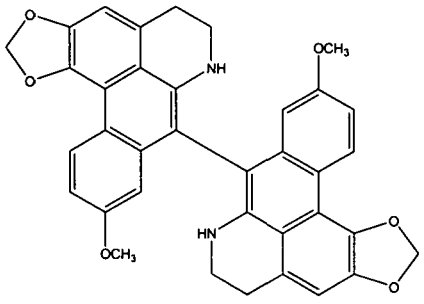
A large number of the reported plant derived natural products with activities against *Mycobacterium* species has been found to belong to the alkaloids. Alkaloids are organic compounds containing a nitrogen atom and are basic in nature. Alkaloids have limited distribution and well defined pharmacological properties.

Cleistopholine (**5**), an alkaloid obtained from the plant *Cleistopholis patens* inhibited the growth of *Mycobacterium intercellulare* at a MIC of 12.5 μg/ml (Peterson, et al, 1992). Vasicine (**8**), isolated from *Adhatoda vasica* and its related semi-synthetic derivatives bromhexine and ambroxol, inhibited the growth of *M. tuberculosis* with MIC values of 6-64 μg/ml (Grange, et al, 1996). The pyrolle alkaloid, solsodomine A obtained from the plant *Solanum sodomaeum* showed

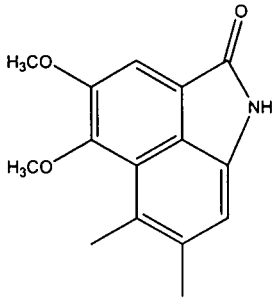
activity against *M. intracellulare* at a MIC of 10 μg/ml (Sayed, et al, 1998). Two pentacyclic indole alkaloids – ibogaine and voacangine extracted from the plant *Tabernaemontana citrifolia,* were found to have activity against *M. tuberculosis, M. avium* and *M. kansasii* with MICs ranging from 50-100 μg/ml (Rastogi, et al, 1998). Tryptanthrin (**7**), an indoloquinazolinone alkaloid obtained from the plant *Strobilanthes cusia,* was found to have remarkable activity against *M. tuberculosis, M. avium* and *M. smegmatis* with MICs of 1, 4 and 6 μg/ml (Mitcher, et al, 1998). Six different alkaloids obtained from the plant *Galipea officinalis* inhibited the growth of *M. tuberculosis* at MICs ranging from 6.25-50 μg/ml (Houghton, et al, 1999). Two alkaloids, sanguinarine and chelerythrine isolated from *S. canadensis* showed antimycobacterial properties against both *M. aurum* and *M. smegmatis* Mc^2 155 (Newton, et al, 2002). Chabamide (**3**) demonstrated a MIC of 0.1-1.6 μg/ml against *M. tuberculosis* $H_{37}Ra$. It is a methylenedioxybenzene alkaloid, isolated from the plant *Piper chaba* (Rukachaisirikul, et al, 2002). Cryptolepine hydrochloride, an indoloquinoline alkaloid salt obtained from the medicinal plant *Cryptolepis sanguinolenta,* exhibited a MIC of 16 μg/ml against *M. fortuitum.* Further investigation of this compound against the fast growing mycobacterial species *M. phlei, M. aurum, M. smegmatis, M. bovis* BCG and *M. abcessus* revealed activities at MICs ranging from 2-32 μg/ml (Gibbons, et al, 2003). An alkaloid, texaline, isolated from *Amyris texana* and *Amyris elemifera,* was found to possess activity against *M. tuberculosis, M. avium, M. kansasii* with a MIC of 25 μg/ml (Giddens, et al, 2005). Six carbazole alkaloids were isolated from the dichloromethane extract of the stem bark of *Micromelum hirsutum.* Of these, micromeline, lansine, 3-formylcarbazole and 3-formyl-6-methoxycarbazole were found to have activity at 31.5, 14.3, 42.3 and 15.6 μg/ml while two other alkaloids- 3-methylcarbazole and methylcarbazole-3-carboxylate had a MIC of more than 128 μg/ml (Ma, et el, 2005). Quinolone alkaloids isolated from the plant *Evodia rutaecarpa* were found to possess activity against the fast growing species- *M. fortuitum, M. smegmatis and M. phlei* at different MICs ranging from 2-32 μg/ml (Adams, et al, 2005). From the roots of *Piper sarmentosum,* four pyrrolidine alkaloids, *viz.,* sarmentine, pyrrolidine, sarmentosine and brachyamide B, and two alkyl amides- pellitorine and brachystamide B were isolated. Of these 6 compounds, pyrrolidine exhibited an MIC of 25 μg/ml while the other 5 exhibited 50 μg/ml against *M. tuberculosis* $H_{37}Ra$ (Tuntiwachwuttikul, et al, 2006).

Piperolactam A and Cepharanone B (**2**), isolated from the leaves and stem of the plant *Piper sanctum,* demonstrated MIC of 8 and 12 μg/ml against *M. tuberculosis* $H_{37}Rv$ (Wirasathien, et al, 2006). From the plant *Pseuduvaria setosa* the compounds N-methylouregidione, liriodenine (**4**) and oxostephanine were isolated which showed activity against *M. tuberculosis* $H_{37}Rv$ at MICs of 100, 12.5 and 25 μg/ml (Wirasathien, et al., 2006). Three different quinoline alkaloids 4-methoxy-2-phenylquinoline, graveolinine and kokusagine (**6**) were isolated from the plant *Lunasia amara* which exhibited antimycobacterial activity against *M. tuberculosis* $H_{37}Rv$ at MICs of 16 μg/ml (Aguinaldo, et al, 2007). The compound bidebiline E (**1**), a dimeric aporphine alkaloid, isolated from the roots of the plant *Polyalthia cerasoides* demonstrated antimycobacterial activity against *M. tuberculosis* with an MIC of 6.25 μg/ml (Kanokmedhakul, et al, 2007). Two other quinoline alkaloids, dictamnine and γ-fagarine isolated from the plant *Zanthoxylum wutaiense* exhibited MICs of 30 μg/ml against *M. tuberculosis* $H_{37}Rv$ (Huang, et al, 2008). Nordicentrine an aporphine alkaloid isolated from the flowers of *Goniothalamus laoticus* was found to have activity against *M. tuberculosis* $H_{37}Ra$ with MIC of 12.5 μg/ml (Lekphrom, et al, 2009). From the roots of *Clausena harnandiana* a carbazole alkaloid, 7-hydroxymukonal was isolated which inhibited *M. tuberculosis* $H_{37}Ra$ at MIC of 25 μg/ml (Thongthoon, et al, 2010).

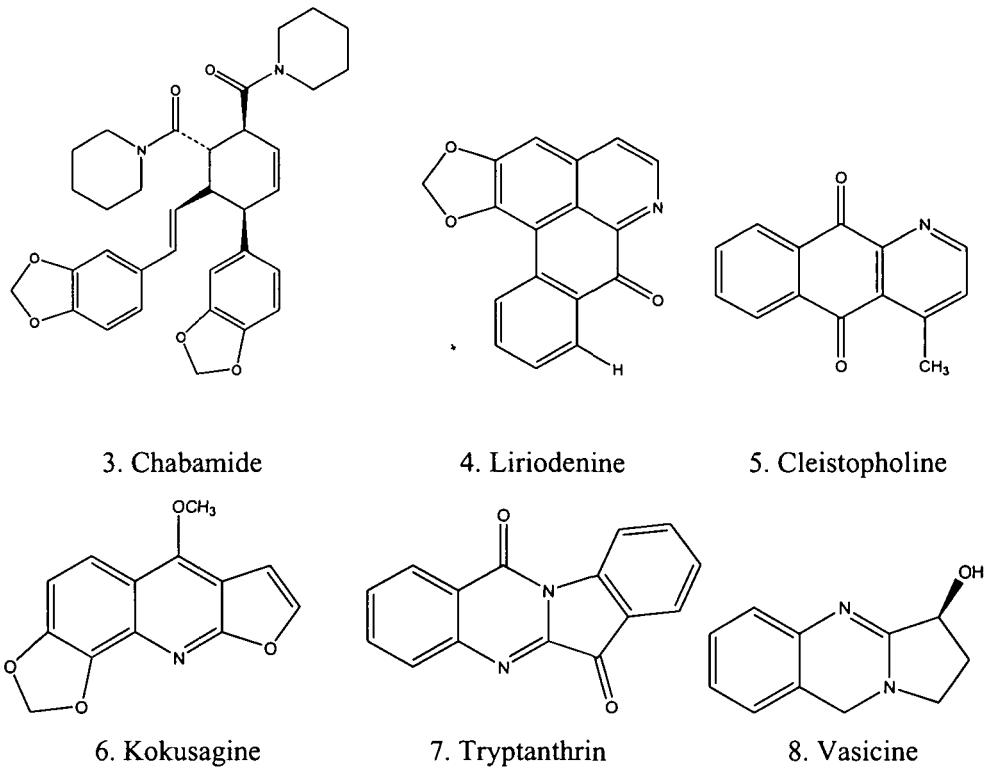
The structures of some of the alkaloids having anti mycobacterial activity are presented in the Figure 2.1:



1. Bidebiline E



2. Cepharanone B



3. Chabamide 4. Liriodenine 5. Cleistopholine

6. Kokusagine 7. Tryptanthrin 8. Vasicine

Fig 2.1: Structures of alkaloids reported to have activity against *Mycobacterium spp*

2.3.3 Flavonoids as Antimycobacterials

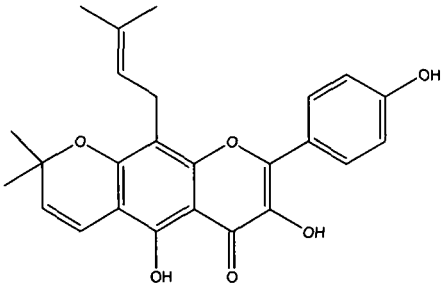
Flavonoids are a group of plant secondary metabolites containing the basic structural component- 2 phenyl chromane. They are responsible for the color of the flowers, fruits and sometimes the leaves. Flavonoids have immense pharmacological significance. A brief review of these compounds which are reported to have antimycobacterial activity is discussed below.

The dichloromethane and the light petroleum extracts from the plant *Hapllopappus sonorensis* exhibited 60% inhibition against *M. tuberculosis* at 100 μg/ml. From both the dichloromethane and light petroleum extracts, the same flavone compound- ermanin was isolated which displayed 98% inhibition at 100 μg/ml (Murillo, et al, 2003). The flavonoids isosakurentin, 4′-hydroxy-5,6,7-trimethoxy flavone, acacetin and luteolin isolated from the flowers of the plant *Chromolaena odorata* exhibited antimycobacterial activity against *M. tuberculosis* $H_{37}Rv$ at MIC of 174.8, 606.0, 704.2 and 699.3 μM (Suksamrarn, et al, 2004). Six known flavonoids, demethoxykanugin, 3, 7 dimethoxy flavone, lacheolatin B, maackiain,

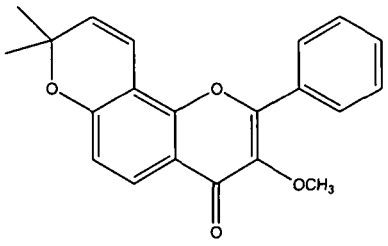
karanjachromene (**2**) and pinnatin (**6**) were isolated from the roots of *Derris indica.* The flavonoids demonstarted growth inhibition of *M. tuberculosis* at different MICs. The first four compounds required MIC of 50 μg/ml and the last two 12.5 μg/ml (Koysomboon, et al, 2006). Bioassay guided fractionation of the acetone extract of *Helichrysum melanacme* led to the isolation of two chalcone molecules- 2,4′,6′-trihydroxy-3′-prenylchalcone and 4′,6′,5′′- trihydroxy-6′′,6′′-dimethyldihydropyrano[2′′,3′′-2′,3′] chalcone. Both the chalcone molecules were found to inhibit *M. tuberculosis* at MICs of 0.05 μg/ml (Lall, et al, 2006). A new homoisoflavanone, identified as 3-(4′-methoxybenzyl)-7,8-methylenedioxy-chroman-4-one obtained form the roots of the plant *Chlorophytum inornatum* exhibited activity against *M. smegmatis, M. aurum, M. fortuitum* and *M. phlei* at various MIC of 256, 32, 128 and 16 μg/ml(O'Donnell, at al, 2006). Two flavonoids-lantanoside and linaroside and an acetylated derivative, acetyl linaroside isolated from the methanol extract of the aerial parts of the plant *Lantana camara* demonstrated activity against *M. tuberculosis* $H_{37}Rv$ at a concentration of 6.25 μg/ml with the acetylated compound showing maximum inhibition percentage of 98% (Begum, et al, 2008). Various flavonoids isolated from the plants *Ficus chlamydocarpa* and *Ficus cordata* exhibited activities against *M. tuberculosis* $H_{37}Rv$. Of the compounds laburnetin (**4**), isolated from *F. chlamydocarpa,* showed the best activity at MIC of 4.88 μg/ml (Kuete, et al, 2008). Pinocembrin (**8**), a flavanone, along with the isoflavones dalparvone and dalparvinene were isolated from *Dalbergia parviflora* which inhibited *M. tuberculosis* $H_{37}Ra$ at MICs of 12.5, 50 and 50 μg/ml (Songsiang, et al, 2009). From the roots of the plant *Eriosema chinense* 5 known flavonoids- lupinifolinol, dehydrolupinifolinol (**1**), flemichin (**5**), eriosemaone A (**3**) and lupinifolin (**7**), were isolated which demonstrated inhibition of *M. tuberculosis* $H_{37}Ra$. The flavonoid lupinifolinol exhibited a MIC of 50 μg/ml while the other four flavonoids exhibited MICs of 12.5 μg/ml (Sutthivaiyakit, et al, 2009). The flavonoids of the plant *Dorstenia barteri* were investigated for their antimycobacterial properties against *M. smegmatis* ATCC 700084 and *M. tuberculosis* $H_{37}Rv$. Of the various flavonoids investigated, isobachalcone showed good antimycobacterial activity at MIC of 10 μg/ml (Kuete, et al, 2010). 5,4′-dihydroxy-7,3,5′-trimethoxyflavone, a flavone compound obtained from the methanol extract of the vines of *Linostoma pauciflorum* was found to possess antimycobacterial activity at MIC of 3.13μM (Navarat, et al, 2011).The

isoflavanone, platyisoflavanone A, isolated from the stem bark of *Platycelphium voense* using dichloromethane: methanol (1:1) as the solvents, was found to possess activity against *M. tuberculosis* at a MIC of 23.7μM (Gumula,et al, 2012). From the methanol extract of the plant *Aralia nudicaulis,* the flavonoids, falcarinol and panaxydol were isolated, both of which displayed MICs of 25.6μM and 36.0 μM against *M. tuberculosis* $H_{37}Ra$ (Li, et al., 2012).

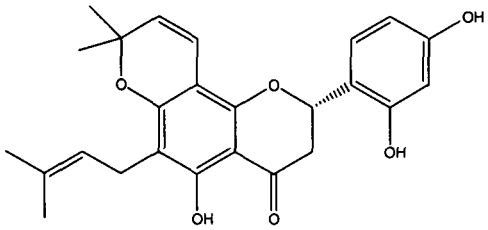
The structures of some of the compounds belonging to the flavonoids are depicted in the Figure 2.3:



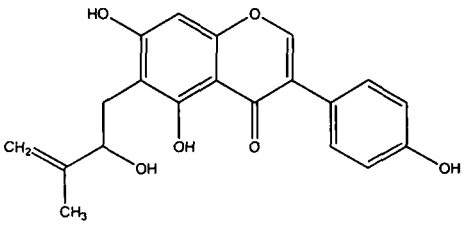
1. Dehydrolupinifolinol



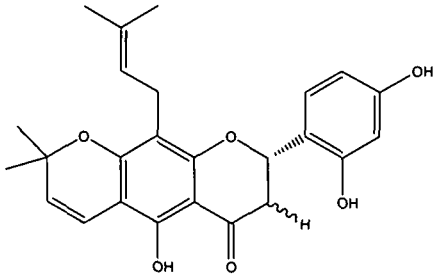
2. Karanjachromene



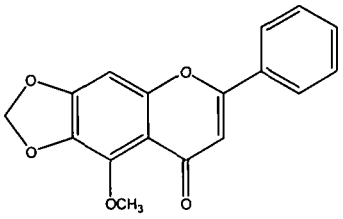
3. Eriosemaone A



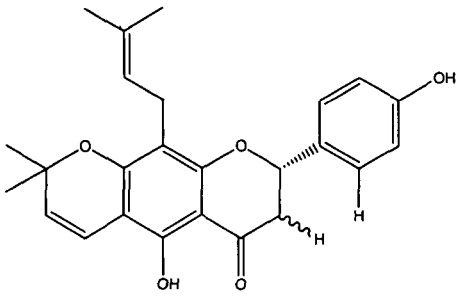
4. Laburnetin

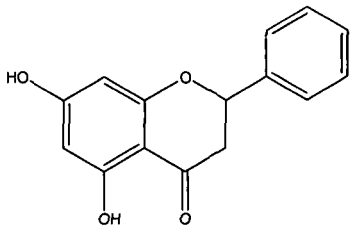


5. Flemichin



6. Pinnatin





7. Lupinifolin 8. Pinocembrine

Fig 2.2: Structures of flavonoids reported to have activity against *Mycobacterium spp*

2.3.4 Other Plant Metabolites as Antimycobacterials

Two polyynes falcarinol and oplopandiol acetate, isolated from the methanol extract of the inner bark of the plant *Oplopanax horridus* exhibited activity against both *Mycobacterium tuberculosis* TMC 107 and *Mycobacterium avium* TMC 724 at a concentration of 20 μg/ml in the disk diffusion assay (Kobaisy, et al, 1997). Cryptolepine, neocryptolepine and biscryptolepine obtained from the plant *Cryptolepis sanguinolenta* showed activity against *M. fortuitum* with MICs of 25, 31 and 6.25 μg/ml (Cimanga, et al, 1998). Jujubogenin, a saponin, obtained from the ethanol extract of the stem of *Colubrina retusa*, was found to inhibit *M. intracellulare* (ATCC 23068) at a MIC of 10 μg/ml. (Elsohly, et al, 1999). An azaanthraquinone metabolite isolated from the plant *Mitracarpus scaber* exhibited activity against *M. intracellulare* at MIC of 6.25 μg/ml (Okunade, et al, 1999). Several plants from Argentina and Chile were screened for their activity against *M. tuberculosis* $H_{37}Rv$. From the plants twenty two triterpenoids were obtained and their MIC values against *M. tuberculosis* determined. Of the 22 compounds, zeorin was the most active at 8μM while ursolic acid, uvaol, betulinic acid, betulin, tormentic acid and pomolic acid acetate exhibited activity at 32μM. The others inhibited at a higher concentration of more than 128 μM (Wachter, et al, 1999). A new compound, juniperexcelsic acid, along with the hexane and methanol extracts obtained from the plant *Juniper excelsa* showed moderate activity against *Mycobacterium tuberculosis* $H_{37}Rv$ at the MIC of 14.4, 15.5 and 17.0 μg/ml (Topcu, et al, 1999). Seven compounds 3,4-seco-olean-12-ene-3,28-dioic acid, 3ά-hydroxyolean-11-en-28,13β-olide, 3 ά-hydroxyoleane-11:13 (18)- dien-28-oic acid, oleanolic acid, oleanonic acid, 3-epioleanolic acid and

epibetulinic acid, the first three being triterpenes, were isolated from the aerial parts of the shrub *Junellia tridens* with dicloromethane:methanol (1:1). All the seven compounds were found to have antimycobacterial activity with MICs of 50,16,16,128,64, 64 and 50 μg/ml (Caldwell, et al, 2000). Rehmannic acid, camaric acid and 3β,25-epoxy-3ά,21ά-dihydroxy-22β-tigloyloxyolean-12-ene-28-oic acid were found to be highly active in inhibiting *M. tuberculosis* $H_{37}Rv$ at MIC concentrations of 32μM, 64 μM and 64 μM. The compounds were isolated from the aerial parts of the plant *Lippia turbinata* (Wachter, et al 2001). Binaphthoquinone, a diospyrin (**5**) obtained from the plant *Euclea natalensis* was found to inhibit *M. tuberculosis* $H_{37}Rv$ strain at an MIC of 100 μg/ml (Lall and Meyer, 2001). Of the nine compounds isolated and purified from the fruits of *Sapium indicum,* seven including two new compounds, both phorbol esters; 12-(2-N-methylaminobenzoyl)-4β,5,20-trideoxyphorbol-13-acetate and 12-(2-N-methylaminobenzoyl) -4ά,5,20-trideoxyphorbol-13-acetate inhibited *M. tuberculosis* $H_{37}Ra$ at a range of MIC from 3.12 to 200 μg/ml (Chumkaew, et al, 2003). Nevadensin and isothymusin, purified from the chloroform extract of the aerial part of the plant *Limnophila geoffrayi,* exhibited activity against *M. tuberculosis* $H_{37}Ra$ with a MIC of 200 μg/ml (Suksamrarn, et al, 2003). Two new compounds- 8,9- secokaurane diterpenes; *ent*-8,9-seco-7ά,11β-diacetoxykaura-8 (14), 16-dien-9,15 dione and *ent*-8,9-seco-8,14-epoxy-7ά- hydroxy-11 β-acetoxy-16-kauren-9,15-dione and another known compound, *ent*-8,9-seco-7ά- hydroxy-11 β-acetoxykaura-8 (14), 16-dien-9,15 dione have been isolated from the dichloromethane extract of the leaves of *Croton kongensis*. The compounds exhibited activity against *M. tuberculosis* $H_{37}Ra$ with MICs of 25.0, 6.25 and 6.25 μg /ml (Thongtan, et al, 2003). Eight out of the fourteen new compounds isolated from the leaf of *Piper sanctum* showed activity against *M. tuberculosis* $H_{37}Rv$ (Mata, et al, 2004). Quinquangulin (**3**) and robrofusarin-two antimycobacterial napthopyrones compounds having a MIC of 12 μg/ml against *M. tuberculosis* (ATCC 27294), were isolated from the methanolic extracts of the stem and fruits of *Senna obliqua* (Graham, et al, 2004). The compounds 1ά-hydroxy-6β-(2ξ-methylbutyryloxy)-10άH-9-oxofuranoeremophilane, 6β-angeloloyloxy- 1ά-hydroxy- 10άH-9- oxofuranoeremophilane and 4′- hydroxyacetophenone isolated from the n-hexane and dichloromethane extract of the roots of the plant *Senecio chionophilus* exhibited mild activity against *M. tuberculosis* $H_{37}Rv$ ATCC 27294 at

MICs of 119, 114 and 121 μg/ml (Gu, et al, 2004). Two new stilbene derivatives, lakoochin A (7) and lakoochin B, isolated from the roots of the plant *Artocarpus lakoocha* were found to have activity against *M. tuberculosis* $H_{37}Ra$ with MICs of 12.5 and 50 μg/ml (Puntumchai, et al, 2004). Three new coloratane sesquiterpene along with nine other known sesquipterpenes isolated from the dichloromethane extract of the stem of *Warburgia ugandensis* were evaluated for their antimycobacterial activity against *M. aurum, M. fortuitum, M. phlei,* and *M. smegmatis.* The compounds showed a range of MICs within 4-128 μg/ml (Wube, et al, 2005). Two known butanolides, isoobtusilactone A and lincomolide isolated from the methanol extract of the stem wood of *Cinnamomum kotoense* demonstrated activity against *M. tuberculosis* 90-221387 at MICs of 22.48 and 10.66 μM (Chen, et al., 2005). From the chloroform root extract of *Euclea natalensis*, six napthoquinones were isolated of which, diospyrin, isodiospyrin, 7-methyljuglone (**1**) and neodiospyrin showed activity against *M. tuberculosis* $H_{37}Rv$ at MICs of 8, 10, 0.5 and 10 μg/ml (Kooy, et al, 2006). T-cadinol, the major compound isolated from 95% ethanol extract of the bark of *Swartzia polyphylla* showed antimycobacterium activity at an MIC of 50 μg/ml (Rojas, et al, 2006). Three constituents, engelhardione, 3-methoxyjuglone and (-)-4- hydroxy-1-tetralone with antimycobacterial activity were isolated from the chloroform extract of the root of *Engelhardia roxburghiana*. The MIC of the compounds against *M. tuberculosis* 90-221387 was found to be 5 μg/ml (Wu, et al, 2007). Of the six new O-acetylated jatrophane diterpenes from the latex of *Pedilanthus tithymaloides,* the compounds; 1ά,13β,14ά-trihydroxy-3β,7β,-dibenzoyloxy-9β,15β-diacetoxy-jatropha-5, 11 E- diene, 1ά,8β,9β,14ά,15β-Pentaacetoxy -3β-benzoyloxy-7-oxo-jatropha-5,12-diene, and 1ά,7,8β,9β,14ά,15β-Hexaacetoxy-3β-benzoyloxy-5β-hydroxy-jatropha-6(7),12-diene possessed activity against *M. tuberculosis* $H_{37}Ra$ with MICs of 12.5,50 and 50 μg/ml (Mongkolvisut, et al, 2007). Three compounds, 3-acetoxy-22-(2-methyl-2Z-butenyloxy)-12-oleanen-28-oic acid, reduced lantadene A and oleanolic acid isolated and purified from the plant *Lantana hispida,* exhibited antimycobacterial activity against *M. tuberculosis* H37Rv at MICs of 50, 50 and 25 μg/ml (Arellanes, et al, 2007). Costunolide and dehydrocostuslactone, the major components of Laurel oil, isolated from the fruits of the plant *Laurus novocanariensis* inhibited rifampicin resistant *M. tuberculosis* $H_{37}Rv$ at MICs of 6.25 μg/ml (Herrera, et al, 2007).Various compounds were isolated from

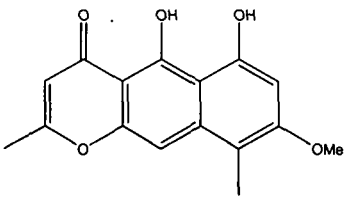
the methanol extract of the roots from *Microtropis fokieniensis.* The compounds 1ά-acetoxy-2ά-hydroxy 6β, 9β, 15-tribenzoyloxy- β –dihydroagarofuran, 2ά-acetoxy-1ά-hydroxy 6β, 9β, 15-tribenzoyloxy-β–dihydroagarofuran, orbiculin G and triptogelin G-2 exhibited activity against *M. tuberculosis* 90-221387 at MICs of 19.5, 15.8, 14.6 and 26 μM (Chen, et al, 2007). Tetrahydroxysqualene (**8**), a new triterpene obtained from *Rhus taitensis,* was found to be active against *Mycobacterium tuberculosis* $H_{37}Ra$ exhibiting an MIC of 10 μg/ml (Noro, et al, 2008). 9Z, 17 octadecadiene-12,14-diyne-1,11,16-triol, 1 acetate and oplopandiol, both polene obtained from the plant *Angelica sinensis* exhibited antimycobacterial activity (Deng, at al, 2008). From the stem bark of *Micromelum hirsutum,* a natural compound micromolide was isolated that showed promising antimycobacterial activity against *M. tuberculosis* $H_{37}Rv$ at a MIC of 1.5 μg/ml (Yuan, et al, 2008). Azadiradione, a limonoid, isolated from the seeds of the plant *Chisocheton siamensis,* was found to exhibit strong inhibitory effect against *M. tuberculosis* $H_{37}Ra$ at MIC of 6.25 μg/ml (Maneerat, et al, 2008). The compounds 15-Acetoxyorbiculin G, celahin C and salasol A isolated from the methanol extract of the stem from *Microtropis japonica* showed activity against *M. tuberculosis* $H_{37}Rv$ with MICs of 39.6, 31.3 and 28.2μM (Chen, et al, 2008). 1-methoxyerythrabyssin II, a new pterocarpons was isolated from the bark of *Erythrina subumbrans,* inhibited the growth of *M. tuberculosis* $H_{37}Ra$ (Rukachaisirikul, et al, 2008).

Three compounds methyl 7-methoxyanodendroate, 7-methoxywutaifuranal and wutaiensal isolated from the cold methanol extract of the roots from *Zanthoxylum wutaiense* showed activity against *M. tuberculosis* H37Rv at various MICs of 35, 35 and 30 μg/ml (Huang, et al, 2008). Three napthoquinones- diospyrone, crassiflorone (**4**) and plumbagin (**2**) isolated from the stem bark of *Diospyros canaliculata* and *Diospyros crassiflora* exhibited activity against *M. smegmatis* ATCC 700084 with MICs of 1.22, 9.76 and 4.88 μg/ml and *M. tuberculosis* $H_{37}Rv$ with MICs of 2.44, 4.88 and 4.88 μg/ml(Kuete, et al, 2009). Three compounds, a sesquiterpene-longifolene and two diterpenes- totarol and trans-communic acid were isolated from the n-hexane extracts from the roots and aerial parts of the plant *Juniperus communis.* Of all the three compounds totarol showed best activity against *M. tuberculosis* $H_{37}Rv$ with a MIC of 73.7μM. Both totarol and longifolene were active against the rifampicin resistant variants and trans-communic acid was found to be active against

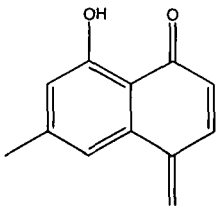
M. aurum at MIC of 13.2 μM (Gordien, et al, 2009). The essential oils viridiflorene and 1,8- cineol from the plants *Achyrocline alata* and *Swinglea glutinosa* were found to be most active at MICs of 62.5±0.1 μg/ml and 100±3.6 μg/ml (Sanchez, et al, 2009). From the methanol extract of the leaves and twigs of *Callicarpa pilosissima,* the compounds 12-Deoxy-11, 12-dihydro-*seco*-hinokiol methyl ester, callicarpic acid B and ά-tocopherol trimer B were purified which showed activity against *M. tuberculosis* $H_{37}Rv$ at MICs of 38, 63.6 and 31.2 μM respectively (Chen, et al, 2009). Four compounds goniotriol, (+)- altholactone, howiinin A and (-)-nordicentrine, isolated from the flowers of *Goniothalamus laoticus*, were found to exhibit activity against *M. tuberculosis* $H_{37}Ra$ with MIC's of 100, 6.25,6.25 and 12.5 μg/ml (Lekphrom, et al, 2009). The compound 3,4′-di-*O*-methylellagic acid 3′- *O*-β-D-xylopyranoside isolated from the plant *Terminalia superba,* inhibited the growth of *M. smegmatis* ATCC 700084 with a MIC of 4.88 μg/ml (Kuete, et al, 2009). A labdane diterpene dialdehyde- labda-8(17), 12-diene- 15, 16-dial, isolated from the chloroform extract of the rhizomes of *Curcuma amada* was found to exhibit activity against *M. tuberculosis* $H_{37}Rv$ with a MIC of 500 μg/ml (Singh, et al, 2010). Aristolochic acid obtained from the methanol extract of the powdered stem bark of the plant *Thecacoris annobonae* was found to possess activity against *M. tuberculosis* $H_{37}Rv$ ATCC 27294 and *M. smegmatis* ATCC 70084, with MICs of 19.53 μg/ml and 39.06 μg/ml for the crude extract and 9.76 μg/ml and 19.53 μg/ml for the active compound (Kuete, et al, 2010). Isobavachalcone (**6**), a chalcone and scopoletin, a coumarin, isolated from the plant *Fatoua pilosa,* were found to inhibit *Mycobacterium* species at MICs of 17.6 and 42 μg/ml (Chiang, et al, 2010). Three neolignans: licarin A, licarin B and eupomatenoid-7, isolated from the hexane extract of the plant *Aristolochia taliscana,* were found to have activity against various strains of *M. tuberculosis.* Licarin A was found to be the most potent of the three neolignans, with MIC of 25 μg/ml against *M.tuberculosis* $H_{37}Rv$, while it showed more activity against majority of MDR tuberculosis strains with MICs ranging from 12.5-50 μg/ml (Diaz, et al, 2010). Litseakolide L and N-*trans*-feruloylmethoxytyramine obtained from the methanol extract of *Litsea hypophaea* Hay showed activity against *M. tuberculosis* $H_{37}Rv$ with MICs of 25 and 1.6 μg/ml (Pan, et al, 2010). Mauritine M and nummularines H, isolated from the methanol extract of the roots of *Ziziphus mauritiana* demonstarted activity against *M. tuberculsis* $H_{37}Ra$ with MIC of 72.8 and

4.5 μM respectively (Panseeta, et al, 2011). The compound 8-hydroxy- 6-methoxy-pentylisocoumarin obtained from the methanol extract of the stem bark of *Xylosoma longifolia* was able to inhibit *M. tuberculosis* at a MIC of 40.5 μg/ml (Truong, et al, 2011). Globospiramine, isolated from *Voacanga globosa,* displayed activity against *M. tuberculosis* $H_{37}Rv$ at a MIC of 4 μg/ml (Macabeo, et al, 2011). The root and stem of *Pisonia aculeata,* yielded different compounds. Of these pisonin B, pisonivanone, (2S)-2′-hydroxydemethoxymatteucinol, (R)-N-*trans*-feruloyloctopamine and dihydrooroxylin A demonstrated inhibitory activity against *M. tuberculosis* $H_{37}Rv$ with MICs of 25, 12.5, 20, 50 and 50 μg/ml (Wu, et al, 2011). Ethyl *p*-methoxycinnamate purified from the plant *Kaempferia galanga* inhibited *M. tuberculosis* $H_{37}Ra$, *M. tuberculosis* $H_{37}Rv$ and other MDR clinical isolates with MICs ranging from 0.242-0.485 mM (Lakshmanan, et al, 2011). Hyperenone A and isolated from the hexane extracts of the aerial parts of *Hypericum acmosepalum* exhibited antimycobacterial activity against *M. Tb* and *M. bovis* at MIC ranges of 75 μg/ml and 100 μg/ml (Osman, et al, 2012).

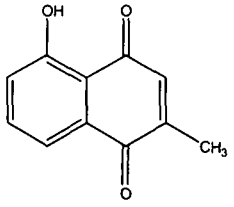
The structures of some of the plant metabolites which inhibit the growth of different *Mycobacterium* spp. are presented in the Figure 2.3:



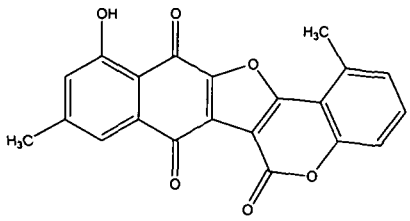
1. 7- Methyljuglone



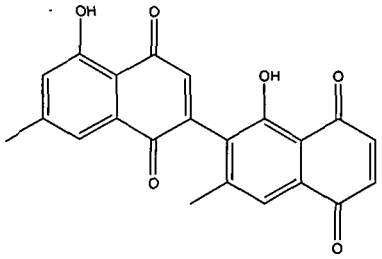
2. Plumbagin



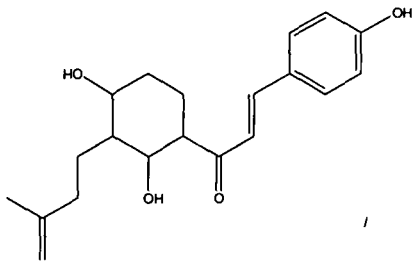
3. Quinquangulin



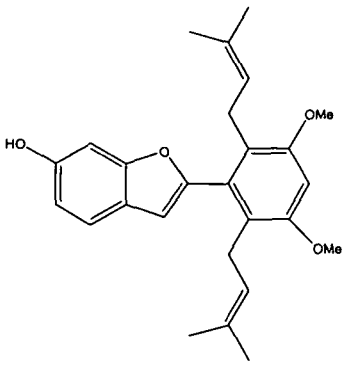
4. Crassiflorone



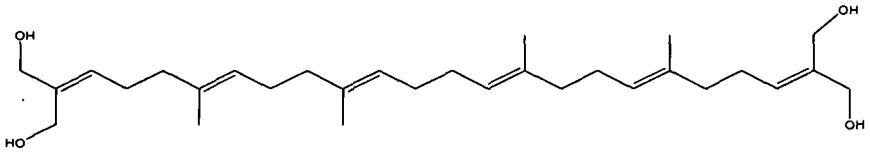
5. Binaphthoquinone



6. Isobavachalcone



7. Lakoochin A



8. Tetrahydroxysqualene

Fig 2.3: Structures of some plant metabolites reported to have activity against *Mycobacterium spp*

2.4 *In silico* Studies

Structure based drug design studies *in silico* represent a strategy of choice in the drug discovery process which is gaining rapid popularity. The strategy enables more rapid hit identification in generation of a hypothesis about the drug target interaction and possible mechanism of drug inhibition of mycobacterial pathogenesis. Molecular docking is such a computer based approach for rapid drug screening against targets of known structure (Kolb, et al, 2009, Izumizono, et al, 2011). A docking process involves the prediction, conformation and orientation of the ligand molecules within the binding site of a target protein. A docking study has 2 objectives, *viz.*, a) accurate structural modeling of the ligand (potential drug) and the target and b) correct activity prediction of the ligand. Docking is a multi step process, each of which unfolds the introduction of a new additional degree of complexity. The first step is the posing of the small molecules within the active site of the target. After this, various degrees of freedoms are performed so that the best conformation matching the structure of the receptor is identified. This enables faster and accurate docking evaluation. Finally, the docking process evaluates the biological activity of the molecules which is given by a scoring function (Kitchen, et al, 2004). Many

computational biologists and chemists have been working to find out novel hit molecules with the help of such docking studies.

Fifteen thousand four hundred and fifty two compounds from a small molecule database were screened against the chorismate dismutase protein of *M. tuberculosis*. Of these compounds, 95 potential molecules were selected on the basis of their docking efficiencies. Further evaluation of the hits using Flex X molecular docking software helped in identifying 15 best compounds which were then subjected to biological study for further validation (Agrawal, et al, 2007). 27 different quinoline and amide derivatives were evaluated for antimycobacterial activity. *In silico* molecular docking studies were conducted using ATP synthase protein of *M. tuberculosis* (Upadhyaya, et al, 2009). Ten cyclic azole substituted diphenyl ether compound derivatives having anti-mycobacterial activity against *M. tuberculosis* $H_{37}Rv$ were docked with the Flex X software against the *E. coli* ENR_NAD^+_triclosan coomplex. Of the 10 compounds, the compound 7b was found to have the highest docking score (Kini, et al, 2009). The *M. tb* purine nucleoside phosphorylase protein was taken as the target protein for various sets of molecules. Of all the molecules 2 methyl adenosine was found to be the lead compound inhibiting the target (Ducati, et al, 2010). Synthesis of many novel cyclic peptides was carried out for development of new drug molecules against *M. tuberculosis*. All the compounds were evaluated for antimycobacterial properties through docking studies (Chandra, et al, 2010).). Five potent molecules from a virtual compound library containing 1, 52,102 compounds were identified on the basis of their docking scores against *M. tuberculosis* Inh A using the GOLD and DOCK software (Izumizono, et al, 2011). Six compounds, derivatives of quinidine-3-carbohydrazide were evaluated for antimycobacterial activity and docked against the *M. tuberculosis* enoyl ACP reductase (Thomas, et al, 2011). 5 different Inh A inhibitors were docked against Inh A which is a NADH- dependent enoyl acyl carrier protein reductase in *Mycobacterium tuberculosis* (Stigliani, et al, 2012). 03 phytochemical compounds from three different plants *Ficus religiosa, Saraca asoca* and *Tylophore indica* were docked against *M. tuberculosis* PKs 18 and Acc D5 protein receptors. Of the three compounds, phytol exhibited the best docking score while the rest of the molecules did not exhibit any scores (Devi, 2012). A series of triazole derivatives were

synthesized which were evaluated for inhibitory activity against Inh A of *M. tuberculosis* $H_{37}Ra$ using Molegro Virtual Docking software (Menendez, et al, 2012).

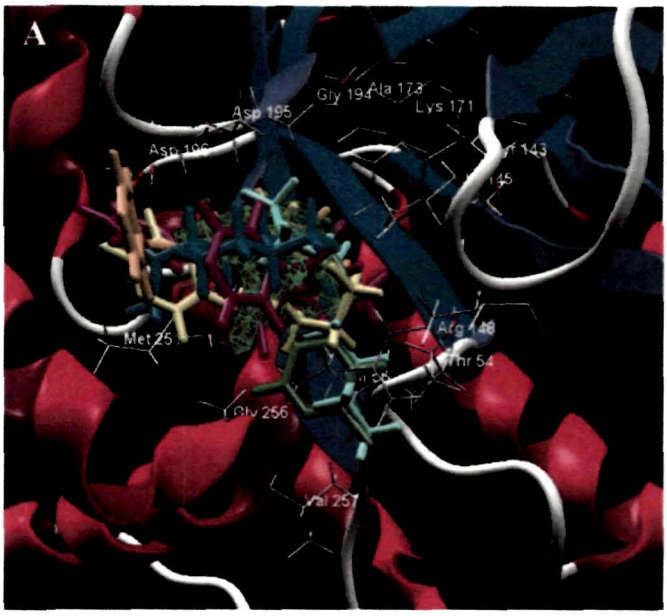


Fig 2.4: An example of a Docking hit of 2--methylheptyl isonicotinate with *M. tuberculosis* DHDPS enzyme (Singh, et al, 2013)

In silico studies of various compounds have been intensively undertaken to understand the important properties of Absorption, Distribution, Metabolism, Excretion and Toxicity (ADME-Tox). The various characteristics of the compounds pertaining to absorption and subsequent distribution in the body cells, their metabolism in the body, the removal of the compound from the body and finally the toxicity of the compounds are studied using ADME (Eddershaw, et al, 2000, Butina, et al, 2002). ADME studies of various quercitin analogues with *Helicobacterium pylori* D alanine: D alanine ligase has been reported (Singh, et al, 2013). ADME-Tox investigation and molecular docking studies of 2-methylheptyl isonicotinate with *M. tuberculosis* DHDPS enzyme has been reported (Singh, et al., 2013).

The first ever Quantitative Structure Activity Relationship (QSAR) studies were reported by Hansch and coworkers (1963). QSAR is the study to determine the quantitative relationships between the biological activities of compounds and their chemical structures (Dan et al, 1989). Many workers have since then used different QSAR applications in drug design for understanding the relationships between the

diverse molecules/ compounds available. The earlier QSAR studies were primarily one dimensional (1D) and focused only on a single physico-chemical property such as solubility, pKa value or by the biological effect of the molecule. The two dimensional (2D) QSAR analysis was based on the studies of the compounds single atoms, their functional groups and the contribution of these to the biological activity. Three dimensional (3D) QSAR studies include the length and width of the molecules. The use of the Comparative Molecular Field Analysis (CoMFA) in 1988 reported for the first time the structure activity relationship study on the 3D structure of a ligand molecule. This was the beginning of the 3D-QSAR analysis. Finally, the fourth dimensional (4D) QSAR analyses represents the use of molecules in different conformations, orientations, stereo-isomers and tautomers (Lill, et al, 2007).

3D QSAR studies were carried out on a set of synthesized pyrrole derivatives to find out the correlation between the chemical structures of the compounds and their anti-TB activity (Ragno, et al, 2000). For a series of compounds based on isonicotinic acid hydrazide, QSAR studies were carried out with multiparameter regression analysis (Bagchi, et al, 2004). 3D QSAR analysis were done for different ring-substituted quinoline compounds with anti-TB activity using CoMFA, Comparative Molecular Similarity Indices Analysis (CoMSIA) and CoMFA alongwith the inclusion of Hydropathy field (HINT) (Nayyar, et al, 2006). 24 compounds corresponding to 4 series of anti-TB ring substituted 2/4 quinolinecarbaldehyde derivatives were synthesized. The compounds were further subjected to 3D QSAR studies (Nayyar, et al, 2006). 25 chalcone molecules were synthesized and QSAR studies were carried out for these molecules (Sivakumar, et al, 2007). Different compound libraries available online including PUBCHEM, were subjected to QSAR analysis. 8 different models were built based on various descriptors (Kovalishyn, et al, 2011). A 3D QSAR study was carried out based on CoMFA and CoMSIA to find out the correlation between the chemical structures and their antimycobacterial activity (Khunt, et al, 2012).

2.5 Antimycobacterial drug delivery

Drug delivery is an important area in TB research which is of central focus at present. Microcrystalline cellulose obtained from cellulose fibers on acid hydrolysis have garnered immense interest in the area of drug release and delivery studies

(Habibi, et al, 2010). MCC's have been widely used in the medicinal, cosmetics, food and pharmaceutical industries. MCC extends advantages as a water retainer, suspension stabilizer, thickening agent and in the reinforcement of tablets. It is used for the direct compression of tablets because of its tablet binding, low chemical reactivity and high compactability. MCC's are also used to modify the release of drugs in tablets and capsules or drug formulations (ElSakhawy and Hassan, 2007).Application of MCCs for slow release of drugs from capsules and tablets is opening up new trends of research in exploring MCCs as drug delivery vehicles. They are also used as a diluent and disintegrating agent for release of oral solid dosages. MCCs possess the characteristics and advantages for use as a constituent of oral dosage forms (Kamel, et al, 2008, Saigal, et al, 2009). MCC is considered as one of the best and most useful fillers because of its excellent compactibility at low pressures, superior disintegration properties and also high dilution potential. The chemical inertness of MCC and its compatibility with most drugs make MCC a highly sought after pharmaceutical agent (Jivraj, et al, 2000). It is also increasingly used in the delivery of TB drugs. Delivery of anti-TB drugs by MCC has various advantages over free drugs. Such advantages include; a) Potentiallity of the drug to target the cells and tissues infected by M.tb, thus increasing the therapeutic efficacy and toxicity and b) the capacity for sustained release of the drug over a longer period of time minimizing the need for frequent drug dosing (Clemens, et al, 2012).

Sodium alginate, a commonly available natural copolymer of guluronic acid and mannuronic acid and is derived from oceanic brown seaweed mainly *Laminaria*. It is the second most abundantly available biopolymer only next to cellulose. Alginates have profound applications in the industry due to its stabilizing and gelling properties. Besides, it is also used as a binding and disintegrating agent in tablets and as a thickening agent in gels, creams and lotions. It is also used as a stabilizer for emulsions (Ahmad and Khuller, 2008). It is used as a drug delivery vehicle. It forms a mesh in the presence of calcium chloride and shrinks at a acidic pH and erodes at alkaline pH. Alginate has found diverse use in the pharma and healthcare industry. They have also been used in immobilization of drugs and also in the sustained release of drugs fro effective treatment of various diseases and wounds (Goh, et al, 2012, Soni, et al, 2010). Alginate is well suited for the entrapment of different kinds of drugs and also other bioactive compounds. It is reported that the drugs encapsulated

could be delivered in a controlled release system at a desired rate. Thus alginate has been found to be used effectively in the field of drug delivery (Ain, et al, 2003, Goh, et al, 2012). Alginates have also been found to be effectively used in the release of various TB drugs for effective treatment of tuberculosis and management (Ain, et al, 2003, Pandey and Khuller, 2004).

2.6 New Drug Candidates

Presently the war against TB has witnessed the emergence of several new drugs with various mechanisms of inhibition. Most of these putative drugs are under clinical trials. Some of the new drugs are mentioned below (Rivers and Mancera, 2008).

1. **SQ 109**: It is a diamine analogue of ethambutol. It contains unsaturated isoprenyl units and a bulky adamentyl unit. It is believed to target the cell wall synthesis in Mycobacteria. It has been found to exhibit *in vitro* activity against both *M. tuberculosis* and also against drug resistant strains. The administration of SQ109 with INH and RIF exhibits *in vitro* synergistic activity (Protopova, et al, 2005).

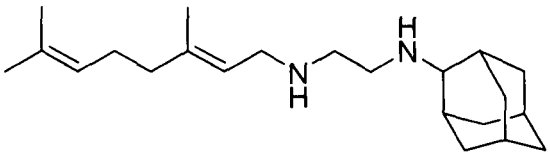


Fig 2.5: SQ109

2. **PA 824**: It is a bicyclic nitroimidazo [2,1-b] oxazine presently under Phase III clinical trials. This drug has not demonstrated any cross resistance to the present known first line drugs. PA 824 has demonstrated *in vitro* anti-TB activity against non replicating *M. tuberculosis*. Its mechanism of action is its ability to inhibit the cell wall lipid and protein synthesis (Stover, et al, 2000).

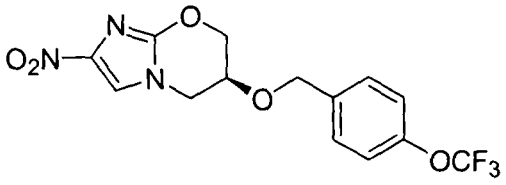


Fig 2.6: PA824

3. **OPC 67683**: It is a 6-nitro-2,3-dihydroimidazo[2,1-b] oxazoles. It has been found to exhibit *in vitro* activity against both drug susceptible and drug resistant strains of tuberculosis and also do not demonstrate any cross resistance to the presently available anti-TB drugs. Its mechanism of inhibition lies in its ability to target the cell wall and inhibiting the methoxy-mycolic acid and keto-mycolic acid synthesis (Sasaki, et al, 2006, Matsumoto, et al, 2005).

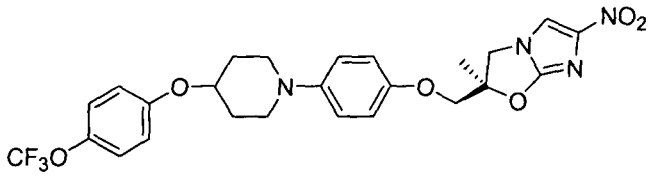


Fig 2.7: OPC67683

4. **TMC 207**: It belongs to the diarylquinolines (DARQs). It has been found to exhibit potent *in vitro* activity against *M. tuberculosis.* TMC 207 exhibits antimycobacterial activity against drug susceptible, MDR and even XDR strains. The drug also do not have any cross resistance to any of the first line anti-TB drugs. TMC 207 has been found to inhibit the *Mycobacterium tuberculosis* membrane bound ATP synthase (Andries, et al, 2005, Huitric, et al, 2007).

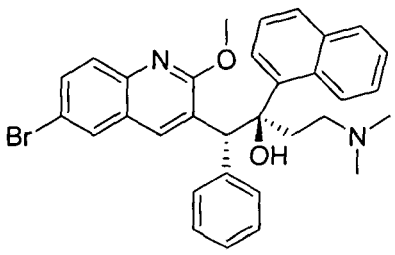


Fig 2.8: TMC 207

Chapter III
Materials and Methods

3.1 Selection of medicinal plants

The selection of the medicinal plants was done keeping in mind the traditional uses of these plants in day to day life and their easy availability. Field visits were made to some of the villages in the districts of Tinsukia (Upper Assam) and Sonitpur (Middle Assam), where traditional healers still prescribe medicinal plants as the first dose of medicine to the common people. Some of the traditional healers were interviewed about their practices. On the basis of the knowledge gained the following plants were collected from the villages located in the vicinity of the Tezpur University campus.

Fig:3.1: Map of Sonitpur District

(http://www.indiaonmap.com/2011/05/sonitpur.html)

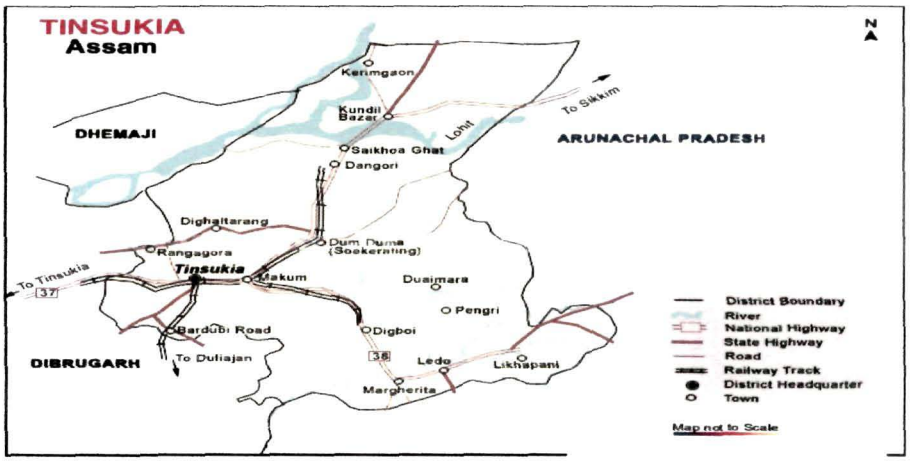


Fig:3.2: Map of Tinsukia District

(http://www.indiaonmap.com/2011/05/tinsukia.html)

The following plants were screened for their antimycobacterial properties.

3.1.1 *Jatropha curcus Linn*

Classification

Family: Euphorbiaceae

Genus: *Jatropha*

Species: *curcus*

It is a small shrub with a soft wood and is deciduous in nature. The trunk of the plant is irregular and the bark is yellowish-brown in colour. The leaves are sub-orbicular, ovate, broadly cordate, shortly acuminate and 3-5 lobed. The petals are united to the middle and the petioles glabrous

It is also known as "*Bongali-bhotora*" is Assamese.

The plant is a native of tropical America and has been cultivated and naturalised throughout the world (Kanjilal and Bor, Vol: 4, 1997).

3.1.2 *Jatropha gossypifolia Linn*

Classification

Family: Euphorbiaceae

Genus: *Jatropha*

Species: *gossypifolia*

It is a shrub, deciduous and characterised with the presence of a soft wood. The leaves are palmately 3-5 lobed. The petals are present with branched stipitate glands. Another characteristic is the presence of free corolla lobes (Kanjilal and Bor, Vol: 4, 1997).

The common Assamese name is "*Bhotera*".

3.1.3 *Xanthium strumarium*

Classification

Family: Asteraceae

Genus: *Xanthium*

Species: *strumarium*

This plant is an annual shrub and grows almost in the entire state of Assam. The leaves are broadly triangular ovate, often lobed, acute, scabrid or hispid. The stem is hispidilose or strigillose. Flowers are unisexual in single (Kanjilal and Bor, Vol: 3, 1997).

In Assam, the plant is known as "*Ogora*"

3.1.4 *Plumeria acuminata*

Classification

Family: Apocynaceae

Genus: *Plumeria*

Species: *Acuminata* Aiton

This small tree is deciduous and is characterised by soft wood. The barks are fissured and with herbaceous branches. The leaves are 6-12 inches long and fleshy with an intramarginal nerve. The flowers are white with an outer purple colour and yellow in the center with fragrance. The bracts are deciduous and corolla salver shaped. The anthers are obtuse and stigma bilobed (Kanjilal and Bor, Vol: 3, 1997).

The local name of the plant is "*Gulanchi* or *gulancha*"

3.1.5 *Euphorbia antiquorum*

Classification

Family: Euphorbiaceae

Genus: *Euphorbia*

Species: *Antiquorum*

The plant is cactus like with fleshy stem and spines (Kanjilal and Bor, Vol: 4, 1997). It is known as "*Siju*" in Assamese.

3.1.6 *Solanum torvum*

Classification

Family: Solanaceae

Genus: *Solanum*

Species: *torvum*

The plant is a shrub attaining a height in between 8-12 feet is sparingly armed and sparsely stellate. Leaves are ovate, serrate or lobed, shortly acuminate and membranous. Flowers are white. The calyx is unarmed and corolla stellate with lobes spreading. The berries are globose and seated on a persistent calyx (Kanjilal and Bor, Vol: 3, 1997)..

The common Assamese name is "*bhit tita*".

3.1.7 *Camellia sinensis*

Classification

Family: Theaceae

Genus: *Camellia*

Species: *sinensis* var. *assamica*

It is a large evergreen shrub with glabrous branchlets. The leaves are obovate, elliptic-oblong, acute or acuminate and often slightly pilose along and near the midrib beneath. Petiole is flattened above and slightly margined. The flowers are white, solitary, sometimes a few together. Petals are long and broadly obovate and connate at the base. Stamens are numerous and filaments glabrous connate (Kanjilal and Bor, Vol: 1, 1997).

The plant is known as "*Cha*" or "*Chah pat*" in Assamese.

3.1.8 *Aegle marmelos*

Classification

Family: Rutaceae

Genus: *Aegle*

Species: *marmelos* Correa

The tree is full of straight and axillary spines. The leaves are normally 3-foliolate however sometimes digitately 5-foliolate. The flowers are greenish white and sweet scented. Petals about 4-5 in numbers, imbricate and fleshy. Fruits are with 10-15 celled with a strong clear mucus within a mass of sweet aromatic mealy pulp (Kanjilal and Bor, Vol: 1, 1997).

It is known as "*Bel*" or "*Bilbo*" in Assamese.

3.1.9 *Nyctanthes arbor tristis*

Classification

Family: Oleaceae

Genus: *Nyctanthes*

Species: *arbor tristis* Linn

This is a small deciduous tree which is bushy in nature. The branchlets are rough pubescent and 4-angular. Leaves are ovate, shortly acuminate, distantly toothed with 4-6 lateral nerves on either half. Flowers sweet scented, sessile. Flowers arranged in cymes or cymose panicles. The bracts are elliptical and pubescent. Anthers sub sessile. Seeds are roundish and compressed (Kanjilal and Bor, Vol: 3, 1997).

The vernacular name of this plant is "*Sewali*"

3.1.10 *Mesua ferrea*

Classification

Family: Callophyllaceae

Genus: *Messua*

Species: *ferrea*

This is a middle sized evergreen tree with the presence of a dense conical crown at the pole stage. The bark is grey and smooth. The leaves are opposite, decussate, thinly, shin above and red in colour when young and tender. Flowers are solitary and generally terminal, sometimes axillary. Stamens are many and filaments slender. Ovary is ovoid and anthers golden yellow. Cotyledons are fleshy with inferior radicle (Kanjilal and Bor, Vol: 1, 1997).

It is known as "*Nahor*" in Assamese.

Pictorial representation of the selected plants

Solanum torvum

Aegle marmelos

Plumeria acuminata

Jatropha gossypifolia

Jatropha curcas

Camellia sinensis

Euphorbia antiquorum

Xanthium strumarium

Mesua ferrea

Nyctanthes arbor tristis

Fig 3.3: Pictorial Presentation of the Selected Plants

3.2 Plant material collection and extraction

The plant materials were collected from within the University and the villages adjacent to the University. The plants were chosen on the basis of their uses among the local people for different medicinal purpose. After collection, the leaves, fruits and roots were washed until clean and then dried in shade. The dried parts were then finely grounded into fine powder using a grinder. The extraction of the crude extracts was carried out by using three different solvent systems. 20 grams of the fine plant powder was taken in a beaker and then 100 ml of solvent was added into it. The extraction was carried out after twenty-four hours. The solvent was passed through Whatman filter paper No 1. The filtrate was then dried in vacuum using a Rotary vapor (Hahn Shin Scientific, Korea). The dried crude extracts were then kept in small aliquots and kept in the refrigerator at 4 °C until use. For isolation of the oil from the seeds of *Mesua ferrea,* extraction was done using the soxhlet apparatus with hexane as the solvent. The solvent was evaporated using a Rotavapor and the oil dried over anhydrous sodium sulfate to remove any moisture content.

Table 3.1: List of plants and their various parts taken for the study

Sl No	Plant Name	Plant parts used	Solvent used
1	*Jatropha curcus*	Leaves, Roots, Stem, Latex	Methanol, Ethyl Acetate and Hexane
2	*Jatropha gossypifolia*	Latex, Leaves, Fruits	Methanol, Ethyl Acetate and Hexane
3	*Camellia sinensis**	Leaves, Green Tea, Assam Orthodox Tea	Methanol, Ethyl Acetate and Hexane
4	*Xanthium strumarium*	Leaves, Stem, Roots	Methanol, Ethyl Acetate and Hexane
5	*Aegle marmelos*	Roots	Methanol, Ethyl Acetate and Hexane, Ethanol
6	*Plumeria acuminata*	Flowers, leaves	Methanol, Ethyl Acetate and Hexane
7	*Solanum torvum*	Fruits and	Methanol, Ethyl Acetate and

		Flowers	Hexane
8	*Nyctanthes arbor tristis*	Leaves and Flowers	Methanol, Ethyl Acetate and Hexane
9	*Euphorbia antiquorum*	Leaves	Methanol, Ethyl Acetate and Hexane
10	*Mesua ferrea*	Seed	Hexane

*The green tea and the Assam orthodox tea samples were collected commercially, while fresh tea leaves were collected from a nearby tea estate.

3.3 Bacterial strain

The bacterial strain used in the present work was *Mycobacterium smegmatis* ATCC 14468. *M. smegmatis* offers various advantages for which it was considered as a model organism. *M. smegmatis* is safe and nonpathogenic against human for which it is increasingly used as a model organism. The mycolic acid synthesis pathway in *M. smegmatis* is similar to that of *M. tuberculosis* (Newton et al, 2006).

3.4 Culture of bacterial strain

The mycobacterial strain was grown in Middlebrook 7H9 broth (Himedia, India) medium supplemented with 0.2% Glycerol and 10% Oleic acid-Albumin-Dextrose-Catalase media as prescribed in the manufacturer's instruction. The strain was stored for further use by preparing glycerol stock and kept -86 °C. Before each experiment the strain is checked for any contamination using the Acid Fast staining procedure.

3.5 Antimycobacterial activity of the crude plant extracts

The antibacterial assay was done by using the Agar Well Diffusion method. Bacterial culture was adjusted to the McFarland standard No. 0.5 before the tests. The media used for the assay was Mueller Hinton Agar. 1% DMSO was taken as the negative control and Isoniazid (1 mg/ml) (Sigma) was taken as the positive control. A stock for the various extracts (20 mg/ml) was prepared from which 50µl was added to each well. The test was performed in triplicates.

3.6 Biochemical Analysis of the plant extracts

The positive extracts were further evaluated for their biochemical constituents.

3.6.1 Total phenols

Total polyphenolic content was determined according to the method of Chumark et al. (2008) with a little modification using gallic acid (Sigma) as the standard. To 5 mg of the dried powder 10ml of 40% ethanol was added and sonicated for 15 minutes. The extract was vortexed for about two min and allowed to stand at room temperature for one hour. The extract was filtered through Whatman filter paper No. 1. 100 µL of the filtrate was taken in a 15 ml centrifuge tube and 6 ml of MilliQ water was added to it. The contents were mixed by swirling and 0.5 ml of Folin Ciocalteu's phenol reagent was added and then mixed again. After about five minutes, 1.5 ml of 20% sodium carbonate solution was added. The contents were mixed and the final volume was made upto 10ml by adding water. The final extract sample was vortexed. The solution was left at room temperature for about two hours. The absorbance was taken at 760nm. Various concentrations of gallic acid (50, 100, 200, and 400µg) were used as standards.

3.6.2 Total Flavonoids

Total flavonoid assay was done according to the protocol taken from protocol of Siddique et al, (2010). 0.5 ml solutions of the various extracts at different concentrations were prepared in methanol. To this 0.1 ml of 10% aluminium chloride, 0.1 ml of 1M potassium acetate and 2.8 ml of MilliQ water were added. The mixture was incubated at room temperature for about 40 minutes. The absorbance of the reaction mixture was measured at 415nm in a spectrophotometer (Thermo Fischer). Quercitin was taken as the standard.

3.6.3 Antioxidant activity

Antioxidant activity was determined by the DPPH assay according to Sharififar et al, (2007). The assay uses DPPH radical as a reagent. When DPPH reacts with antioxidant compound, it is reduced. The change in color (from deep violet to lightyellow) is then measured (Miliauskas et al., 2004). Fifty microliter of various concentrations of the samples (1mg/ml-62.5µg/ml) in methanol was added to 5ml of

0.004% methanol solutions of DPPH. Five different (20, 40, 60, 80, 100 µl) concentrations of the essential oil from *Mesua ferrea* seed were taken. The absorbance were read at 517nm in a spectrophotometer (Thermo Fischer) after 30 minutes incubation in dark at room temperature. Inhibition of DPPH free radical in percentage (I %) was calculated using the following formula.

I% = (A blank- A sample/A blank) * 100,

Where, 'A blank' is the absorbance of the control reaction containing all the reagents except the extract. 'A sample' is the absorbance of the extract, i.e., the test compound. The concentration of the extract that provided 50% inhibition was calculated from the graph plotting inhibition percentage against the extract concentration. All the tests were carried out in triplicates. Ascorbic acid was taken as the standard.

3.6.4 Haemolysis assay

Haemolysis test was carried out according to Nair et al, (2007). Mammalian blood from goat was collected in a vial containing 4% trisodium citrate. It was centrifuged at 750 *g*. The supernatant was discarded and the precipitate containing the erythrocytes was washed with PBS (pH 7.4) twice at 750 *g* for 10 mins. 48.5 ml of PBS was added to 1.5 ml of the erythrocyte suspension to make the final concentration of the erythrocyte 3%. 1.9 ml of this suspension was added to a 2ml centrifuge tube. To the tube 100µl of the extracts was added at a concentration of 1mg/ml. The extract-suspension mix was incubated at 37 °C in an incubator. After the incubation, the tubes were centrifuged at 750 *g* for 10 mins. 200µl of the supernatant was collected in a fresh tube and to it 2.8 ml of PBS was added and its absorbance was measured at 415 nm in a spectrophotometer (Thermo Fischer). PBS was taken as the negative control and Triton X 100 (Sigma) was taken as the positive control.

3.7 Purification of Bioactive compounds

Two of the plant extracts were purified for resolution and identification of the active compound(s).

3.7.1 Green tea

3.7.1.1 Column chromatography

Column chromatography was carried out by using silica gel (230-400 mesh size) chromatography. Initially, 25 g of silica gel was taken in a beaker and 100 ml of hexane was added to it. The mixture was stirred in a magnetic stirrer at about 100 rpm and then degassed. Silica gel was poured into the column using a Pasteur pipette. This was allowed to stand for approximately 6 hours. Now 500 mg of green tea methanol extract was added to the top of the column. Hexane, 90% Ethanol, methanol and water were used as the eluents. For 90% ethanol as eluent 4 different fractions (F1-F4) were collected. The fractions were collected in 50 ml tubes and evaporated in a rotary evaporator (Hahn Shin). The partially purified fractions were stored in the refrigerator at 4°C until further use.

3.7.1.2 High Pressure Liquid Chromatography (HPLC)

The ethanol fraction was further purified using High Pressure Liquid Chromatography (HPLC) using a Waters HPLC system with a binary 515 Pump system and a 2489 UV-VIS detector. A 250mm semi-preparative reverse phase HPLC column Symmetry C18 (7.8μ x 7) was used for the separation and purification of the compounds. The purification was carried out using a gradient system of elution with water (pump A) and acetonitrile (pump B) as the solvent systems. The gradient method used for the elution is as follows: 100% A for the first 5 mins, followed by 70% A and 30% B for the next 20 mins, 55%A and 45%B for the next 7 mins, 35%A and 65%B for the next 5 mins, 20%A and 80% B for the next 4 mins, 100% B for the next 1 min and again 100% A for 2 mins. The flow rate was maintained at 2ml/ min. The detection was carried out at 210 and 280nm respectively.

3.7.1.3 UV Spectroscopy

A Thermo Scientific UV-VIS spectrophotometer was used to determine the λ max of the compounds. The λ max gives a brief idea on the presence of different chromophores (molecules in compounds that absorb light) in the compounds. Water was taken as the blank and the compounds were scanned from 190-800 nm to determine the λ max.

3.7.1.4 Fourier Transform Infra Red (FTIR) Spectroscopy

The FT-IR transmittance spectra were obtained using a Perkin Elmer Spectrum 100 series instrument. The FT-IR spectra were obtained to determine the different functional groups present in the compounds, which enabled partial identification of the compounds. The compounds were first mixed with about 5-7 mg of potassium bromide (KBr) to obtain a fine mixture. This was then taken in a sample holder and then placed under a hydraulic press. The hydraulic press delivers 10 ton pressure to produce a KBr-compound tablet which was then placed in the sample holder for subsequent characterization.

3.7.1.5 Mass Spectroscopy (MS)

MS was performed using a Waters Micromass Q-TOF Acquity LCMS instrument. MassLynx V4.1 software was used to analyze the result. The scan rate was 1scan/ sec. Water was used as the solvent. A mass range of 100-1000 was scanned. Prior to loading, the samples were filtered through a 0.22μ syringe filter.

3.7.1.6 Nuclear Magnetic Resonance (NMR)

Both 1H NMR and 13 C NMR were carried out using JEOL FT 400 MHz NMR. Compounds were dissolved in D2O. Chemical shifts were presented as δ (ppm) and coupling constants (J) as Hz.

3.7.1.7 Minimum Inhibitory Concentration (MIC) of the pure compounds

The MIC activities of the extracts were determined done using the protocol of Rangaswamy, et al. (2007). The MIC activities were checked using 96 well microplates. Stock solutions of the HPLC purified fractions were prepared at a concentration of 2 mg/ml.Various concentrations of the extract were prepared in aliquots by serial dilution from the stock solution. 100μL of various concentrations was added in each of the wells. To this, 100μL of the bacterial inoculum pertaining to 0.5 Mc Farlands Standard was added in each well. Both positive and negative controls were taken. Isoniazid (0.2 mg/ml) was taken as the positive control while DMSO 1% was the negative control. The plates were then incubated at 37°C for 24 h. After the incubation period, 40 μL of MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-

diphenyltetrazolium bromide] solution (0.2 mg/ml) was added to each well and further incubated for 45 min. Change of color to blue indicated bacterial growth while no color indicates a positive result.

3.7.1.8 Cytotoxicity of the Active Compounds

The cytotoxicity of the compounds was assayed against RAW 264.7 macrophage cell lines. Cells lines were cultured in DMEM complete media supplemented with 10%FBS and 100µl of penstrep (Penicillin:Streptomycin) antibiotic reagent (Sigma). Cells were seeded into 96 well plates (NUNC, USA) and allowed to grow until confluency was attained. To the seeded cells, the pure compounds were added in the concentration range of 1mg/ml to3.9 µg/ml. The plates were incubated at 37°C for 12 h. 30µl of MTT was added to the plates after the incubation. Change of colour was used as indicator of toxicity.

3.7.2 Purification of the essential oil

The essential oil (hexane extract of the seed of *Mesua ferrea*) was purified using a solvent mixture of petroleum ether and ethyl acetate using three different ratios: 10:90, 30:70 and 50: 50 in a silica gel (60-120 mesh size) column.

3.7.2.1 Drying of the purified sample

The column chromatography purified oil was dried over anhydrous sodium sulfate and kept in a dessicator vacuum until further use.

3.7.2.2 Preparation of Fatty acid Methyl Esters (FAMEs)

Fatty acid methyl esters were prepared from the seed essential oil. For obtaining the FAMEs, 1ml of the oil was taken in a test tube and mixed with 2ml of chloroform, 4ml of methanol and 500 µl of concentrated sulfuric acid and heated at 100°C for about two hours. The oil-solvent-acid mixture was cooled to room temperature and 3-4ml of hexane was added to it. The hexane fraction was collected and stored at 4°C until further analysis.

3.7.2.3 Gas Chromatography-Mass Spectrometry (GC-MS) analysis of the FAMES

The GC-MS of the FAMEs were carried out using a Varian-3800 GC coupled to a SATURN-2200 Mass spectrophotometer. The temperature of the oven was set as follows. Initial temperature set at 80°C was held for 1 minute, then increased at a rate of 8°C/ minute till and held for 15 minutes. 1 µl of sample was injected for the analysis. Data acquisition was done for the mass range between 50-550 mu and the scan speed was 1scan/sec. The compound identification was done using both the NIST library and the SATURN library available with the instrument.

3.7.2.4 FTIR of Essential Oil

The FTIR of the essential oil was carried out to investigate the functional groups present in the oil and to confirm the presence of fatty acid groups. The FTIR was carried out in a Perkin Elmer Spectrum 100 Series IR spectrophotometer using potassium bromide (KBr) discs. The spectra was taken in the transmittance mode and each sample was scanned 8 times with a scan speed of $4cm^{-1}$/ sec starting from 400 cm^{-1} to 4000 cm^{-1}.

3.7.2.5 NMR of Essential Oil

Both the 1H NMR and 13C NMR was carried out in a 400MHz JEOL FT-NMR. Tri Methyl Silane (TMS) was used as the internal standard. $CDCl_3$ was used as the solvent, while a pulse width of 9.5 µsec and acquisition time of 2.83 sec and pulse 4.75 µsec. A total of 8 scans were given.

3.7.2.6 Antimycobacterial activity of *Mesua ferrea* seed oil

The antimycobacterial activity of the *Mesua ferrea* seed oil was determined using the protocol of Rangaswamy et al, (2007). The activity was checked as described in section 1.7.1.7 with little modification. Stock solution of the essential oil was prepared at a concentration of 20 mg/ml.Various concentrations of the extract were prepared in aliquots by serial dilution from the stock solution. 100µL of various concentrations was added in each of the wells. To this, 100µL of the bacterial inoculum pertaining to 0.5 Mc Farlands Standard was added to each well. Isoniazid (0.2 mg/ml) was taken as the positive control while DMSO 1% was the negative

control. Plates were then incubated at 37°C for 24 h. 40 µL of MTT was added and again incubated. A colour change to blue indicated the growth of bacteria while no change in colour indicated antibacterial effect of the essential oil.

3.7.2.7 Cytotoxicity of the active compounds

The cytotoxicity of the purified essential oil fraction was determined as described in section 3.8.1.8 and was assayed against RAW 264.7 macrophage cell lines with minor modification. Cells lines were cultured in DMEM complete media supplemented with 10% FBS and 100µl of penstrep (Penicillin:Streptomycin) antibiotic reagent (Sigma). Cells were seeded into 96 well plates (NUNC, USA) and allowed to grow until confluency was attained. To the seeded cells, essential oil was added in the concentration range of 5µl, 10µl, 20µl, 40µl and 80µl. The plates were incubated at 37°C for 12 h. 30µl of MTT was added to the plates after the incubation period. Change in colour was indicative of toxicity.

3.8 *In silico* investigation

3.8.1 Molecular Docking

Molecular Docking studies were carried out using Molegro Virtual Docker (MVD) 5.0 (Molegro ApS, Aarhus, Denmark). The scoring functions of the ligands and the H-bonds formed with the amino acids were used in the prediction of the binding affinities, different binding modes and orientation of the compounds in the active site(s) of the target proteins. The water molecules were not taken into account in the study. The cavity detection algorithm in MVD was used for optimizing the potential binding site(s), i.e. it makes it possible to define an approximate location of the most likely interaction sites. A set of 100 runs was given for each docking study using 2000 interactions. Both the Rerank score and the MolDock score were calculated. The best fit score was obtained on the basis of the Rerank score (Singh, et al, 2013).

3.8.2 Selection of the target proteins

The target proteins chosen for the study were downloaded from the Protein Data Base (http://www.rcsb.org/pdb/home/home.do). Three proteins, mtKas B (2GP6), Inh A (2B35) and Glft2 (4FIX) were considered for the study.

3.8.2.1 mt Kas B

The *M. tuberculosis* Kas B is a β ketoacyl acyl carrier protein synthase (ACP) II which is an elongation condensing enzyme present in the fatty acid synthase II (FAS-II) pathway. The Kas B along with Kas A is responsible for the fatty acid elongation in the FAS II pathway. Therefore the Kas B protein is considered as a potential target for anti tuberculosis drug discovery (Sridharan, et al, 2007).

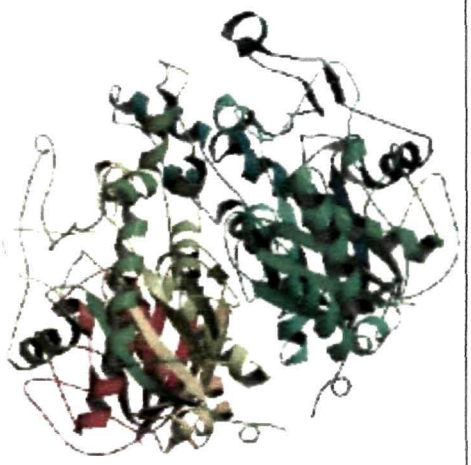
Properties		Protein
mt Kas B		
Resolution: 2.40 Å		
R value: 0.177		
R Free: 0.232		
Space Group: H3		
Unit Cell:		
Length [Å]	Angles [°]	
A=198.68	ά=90	
B=198.68	β=90	
C=71.83	γ=120	

Fig 3.4: Crystal structure of mt Kas B (2GP6) and its properties

3.8.2.2 Inh A

Inh A is the enoyl ACp reductase protein of the mycobacterial type II fatty acid biosynthesis pathway. Inh A is the target of the first line anti-tuberculosis drug isoniazid. Isoniazid is activated by Kat G, a catalase peroxidase enzyme in *M. tuberculosis* to form a INH-NAD adduct which acts as inhibitor of Inh A. As such Inh A is an attractive target for the development of novel anti-tuberculosis drugs.

The Type II fatty acid pathway in *M. tuberculosis* is responsible for the generation of long chain fatty acids, which are the precursors of mycolic acids. Mycolic acids are the most important building blocks of the cell wall of *M. tuberculosis*. Inh A inhibition impairs the cell wall integrity which leads to the death of the cell and finally death of the organism (Sullivan, et al, 2006).

In the present study, Inh A was downloaded from the PDB website (http://www.rcsb.org/pdb/home/home.do). The PDB id of the protein target is 2B35.

Properties		Protein
Inh A		
Resolution: 2.10 Å		
R value: 0.174		
R Free: 0.216		
Space Group: C 222_1		
Unit Cell:		
Length [Å]	Angles [°]	
A=89.81	ά=90	
B=157.51	β=90	
C=91.23	γ=90	

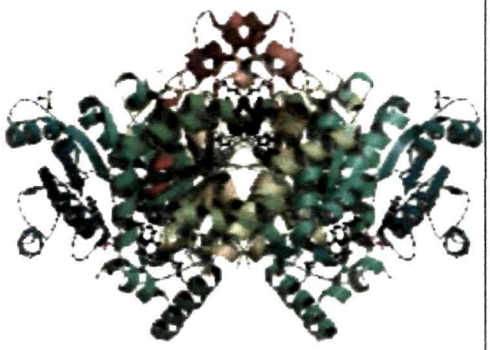


Fig 3.5: Crystal structure of mt Inh A (2B35) and its properties

3.8.2.3 Glf T2

The mycobacterial cell wall depends upon various enzymes for its activities which include several glycosyltransferases. GlfT2 (Rv 3808c) is a polymerizing galactofuranosyltransferase which is involved in synthesis of a variety of the galactan portion of the mycolyl arabinogalactan (MAG) complex. This galactan is the attachment site of three different arabinan domains each of which contains ά- (1→ 5), ά- (1→ 3) and β - (1→ 2) linked arabinofuranose residue (Wheatley, 2012).

Properties		Protein
Glf T2		
Resolution: 2.45 Å		
R value: 0.187		
R Free: 0.222		
Space Group: $P42_12$		
Unit Cell:		
Length [Å]	Angles [°]	
A=150.79	ά=90	
B=150.79	β=90	
C=148.02	γ=90	

Fig3.6: Crystal structure of mt Glf T2 (4FIX) and its properties

3.8.3 Absorption Distribution Metabolism Excretion-Toxicity (ADME-TOX) Prediction

ADME-TOX prediction study was carried out to investigate the various pharmacological properties of the purified molecules having antimycobacterial activity. QikProp (Schrodinger 2012) was used in the prediction of ADME-TOX properties of the compounds. QikProp is also used in the prediction of different physical descriptors and pharmaceutical properties of organic molecules. The QikProp descriptors and properties such as IC50 value for HERG K+ (QPlogHERG), MDCK cell permeability (QPPMDCK), Permeability of Caco-2 cells, the blood brain coefficient (QPlog BB), oral absorption in humans (%human oral absorption) and aqueous solubility were predicted for the compounds. The ACD-iLab 2.0 (Advanced Chemistry Development, Inc 1994) software was used in the prediction of the Lipinski's rule of 5 (Lipinski, et al., 1997) and prediction of LD50 value in both mice and rat.

3.8.4 Quantitative Structure Activity Relationship (QSAR) Study

The QSAR equations were generated using the multiple linear regression study and the Density Functional Theory and Molecular Mechanics based descriptors. The ligands selected for the study belonged to the identified compounds from the plant as well as compounds from the plants which have already been characterized. The compounds were drawn using the CHEM Office software from Cambridge Softwares and saved as mol files. The DFT based reactivity descriptors for the compounds were calculated using the Gaussian 09 programme. The compounds were subjected to full geometry optimization in using 6-311g** basis set in combination with the B3LYP functional software. The physiochemical parameters, *viz.*, hydration energy, logP, surface area (approx), refractivity and polarizability, for each of the compound were computed using the Hyperchem software. These parameters were used to generate various QSAR equations and to calculate the cytotoxity (pIC_{50}) of the compounds.

3.8.4.1 *QSAR calculation*

Multiple regression analysis was used to generate the QSAR model equations. In all equations, R^2 is the squared correction coefficient, SE is the standard error of the estimates, F is the Fisher significance ratio and N is the number of cases used in the analysis. In each model, several cases were selected randomly from the experimental data for an internal validation to examine the predictability of the QSAR model.

3.8.4.2 Computational details

The electronic chemical potential of a molecular (including atomic or ionic) species is defined as the infinitesimal change in energy when electronic charge of the system is changed. In theoretical chemistry, the chemical potential (μ) is defined as the negative of the electronegativity (χ) by Iczkowski and Margrave (1961) and defined as

$$\mu = -\chi = \left(\frac{\partial E}{\partial N}\right)_{v(\vec{r})} \qquad (1)$$

From this viewpoint, the electronegativity of a species is the drop in energy when an infinitesimal amount of electronic charge enters the system. It is a measure of how hospitable an atom or ion, or a molecule, is to the ingress of electronic charge which fits. The chemical hardness (η), (Iczkowski and Margrave, 1961) of an electronic system is defined as the second derivative of total energy (E) with respect to the number of electrons (N) at constant external potential, $v(\vec{r})$.

$$\eta = \frac{1}{2}\left(\frac{\partial^2 E}{\partial N^2}\right)_{v(\vec{r})} = \frac{1}{2}\left(\frac{\partial \mu}{\partial N}\right)_{v(\vec{r})} = -\frac{1}{2}\left(\frac{\partial \chi}{\partial N}\right)_{v(\vec{r})} \quad (2)$$

The hardness of a species is the amount by which its electronegativity, its ability to accept electrons, decreases when an infinitesimal amount of electronic charge is added to it.

Using the finite difference approximation, chemical hardness and chemical potential can be approximated as

$$\eta = \frac{IP - EA}{2} \quad (3)$$

$$\mu = -\left(\frac{IP + EA}{2}\right) \quad (4)$$

where, IP and EA are the first vertical ionization potential and electron affinity, respectively, of the chemical system.

Further, using Koopmans' theorem (Laidlaw and Birss, 1964), the above parameter can be expressed as:

$$\mu = \frac{E_{LUMO} + E_{HOMO}}{2} \quad (5)$$

$$\eta = \frac{E_{LUMO} - E_{HOMO}}{2} \quad (6)$$

where, E_{LUMO} is the energy of the lowest unoccupied molecular orbital and E_{HOMO} is the energy of the highest occupied molecular orbital.

Parr, et al. (1999) introduced the global electrophilicity index (ω) in terms of chemical potential and hardness as:

$$\omega = \frac{\mu^2}{2\eta} \quad (7)$$

According to this definition, ω describes the electrophilic power of a molecule.

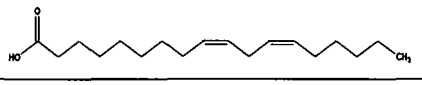
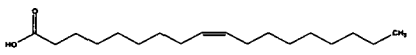
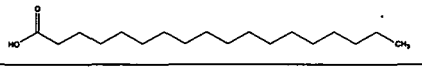
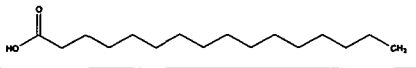
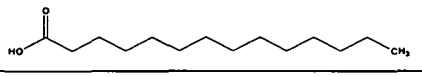
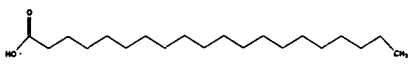
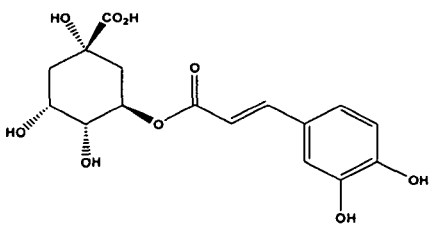
logP (octanol-water partition coefficient), Hansch showed a correlation between biological activity of phenoxy acetic acid with octanol-water partition coefficient (Hansch et al., 1963).

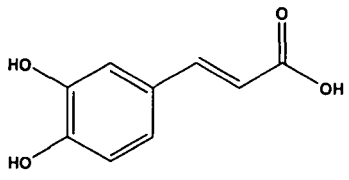
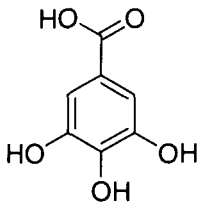
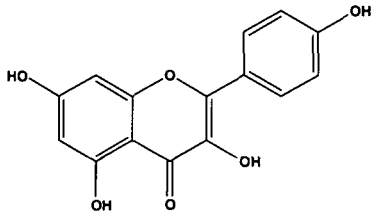
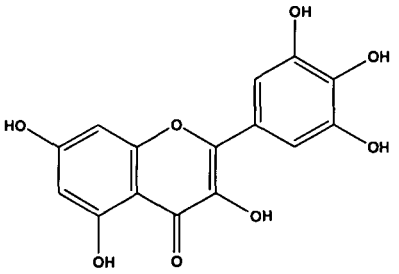
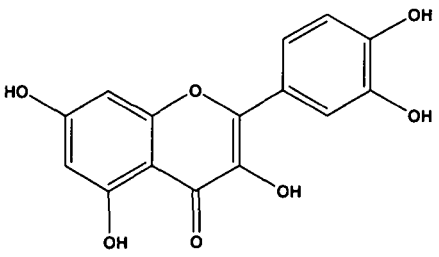
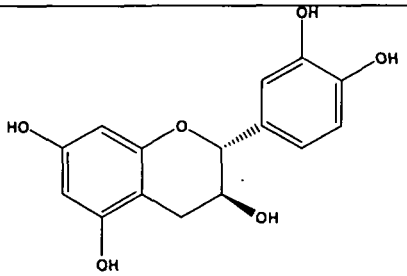
Molar refractivity (MR) index was suggested by Viswanadhan, et al. (1989) in combination with logP. It belongs to molecular descriptor and was tested to estimate the atomic contribution to octanol-water partition coefficient.

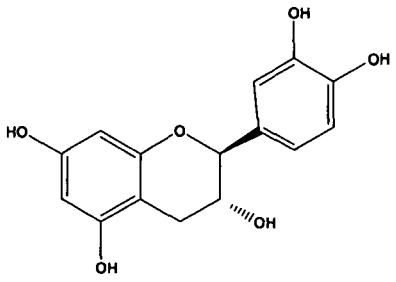
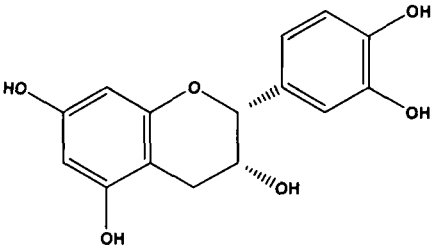
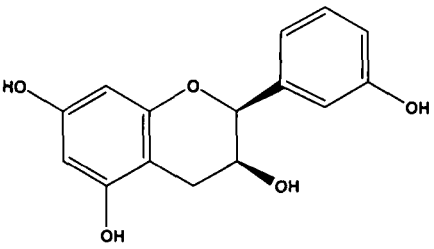
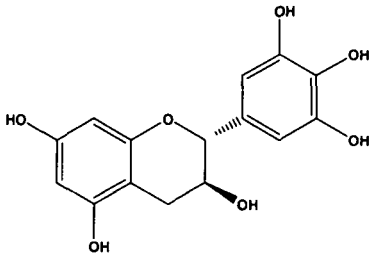
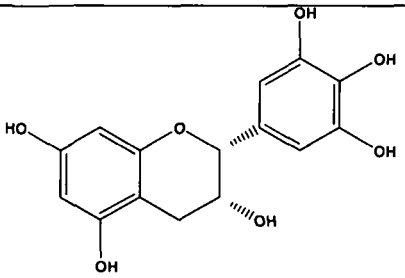
Hydration energy was put forwarded by No, et al, (1994) for the study of the gas-phase proton transfer energies of several amino acids.

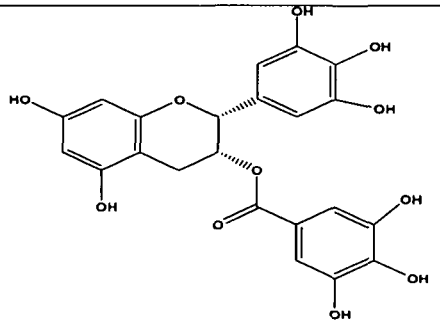
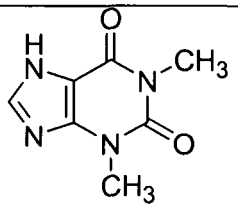
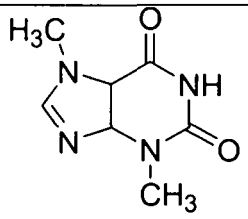
Surface area (approx) descriptors have been defined by Stanton, et al, (1990) in terms of the solvent-accessible surface area of each atom and the atomic charge computed from the atomic electro negativity or with a quantum chemistry method.

Table 3.2: The selected ligands taken for the QSAR study

Sl No	Ligand Name	Structure	Formula	Mol Weight	KEGG Database ID
1	Linoleic acid		C18H32O2	280.4455	C01595
2	Oleic Acid		C18H34O2	282.4614	C00712
3	Stearic acid		C18H36O2	284.4772	C01530
4	Palmitic acid		C16H32O2	256.4241	C00249
5	Myristic acid		C14H28O2	228.3709	C06424
6	Arachidic acid		C20H40O2	312.5304	C06425
7	Chlorogenic acid		C16H18O9	354.31	C17147

8	Caffeic acid		C9H8O4	180.16	C01197
9	Gallic acid		C7H6O5	170.12	C01424
10	Kaempferol		C15H10O6	: 286.23	C05903
11	Myricetin		C15H10O8	318.2351	C10107
12	Quercetin		C15H10O7	302.2357	C00389
13	+- catechin		C15H14O6	290.2681	C06562

14	(-)-catechin		C15H14O6	290 2681	C14079
15	(-)-epicatechin		C15H14O6	290 2681	C09727
16	+- epicatechin		C15H14O6	290 2681	C09728
17	+- gallocatechin		C15H14O7	306 2675	C12127
18	(-)-Epigallocatechin		C15H14O7	306 2675	C12136

19	Epigallocatechi 3-gallate		C22H8O11	458.3717	C09731
20	Theophylline		C7H8N4O2	180.164	C07130
21	Theobromine		C7H8N4O2	180.164	C07480

3.9 Extraction and preparation of MCC

3.9.1 Extraction of alpha cellulose and MCC

The protocol for extraction of MCC was that of Bhattacharya, et al. (2008) with some modifications. The grass samples were first cut into small pieces and then washed thoroughly with water before drying them at room temperature. The dried samples were treated with NaOH (4%) and boiled at 80°C in water bath for 4 hrs. The samples were washed with water until neutral pH and then bleached with a solution of sodium hypochlorite and hydrogen peroxide in 1:1 ratio for 40 minutes at 100° C. The samples were rewashed with water until neutral pH yielding the α-cellulose fibers. For extraction of MCC, 10 grams of the α-cellulose was acid hydrolyzed using HCl (2.5N) at 100° C for 30min. The extracted MCC was washed thoroughly with water for complete removal of HCl.

3.9.2 Characterization of MCC

3.9.2.1 Scanning Electron Microscopy (SEM)

MCC was submitted to SEM for morphological study. A small quantity of the MCC was fixed to a carbon stud. A platinum coating was laid on the stud with a JEOL auto fine coater. The SEM study was carried out in a JEOL 6390 instrument.

3.9.2.2 Fourier Transform Infra Red (FTIR)

FT-IR study was conducted to determine the functional groups present in the MCC. The MCC was mixed with potassium bromide (KBr) and prepared as tablet discs using a Hydraulic press with 10 tons pressure. The discs were then scanned in the spectral range of 400-4000 cm^{-1} to find out the percentage transmittance (%T) of the MCC. The study was done in a Perkin Elmer spectrum 100 series instrument.

3.9.2.3 Thermal Gravimetric Analysis (TGA)

TGA studies were carried out using a Perkin Elmer STA 6000 instrument. In this study a small amount of the sample was placed in a pan inside the instrument in a controlled environment. The TGA spectra were recorded in an ambient nitrogen atmosphere at a heating rate of 20°C/ min. The TGA was carried out to determine thermal stability of the MCC and also to compare the degradation pattern of the α-cellulose and the MCC

3.9.2.4 Density Scanning Calorimetry (DSC)

The DSC analysis was carried out in a Shimadzu DSC 60 instrument. DSC is carried out to find out the phase change during application of temperature. In DSC the change is indicated by the ability of the material to absorb heat. Endothermic heat is produced in such cases and endothermic peaks are observed. The reading was taken in a nitrogen atmosphere at a rate of 30ml/min. The temperature range was from 25° C to 300° C with a 10° C/min rate of change of temperature.

3.9.2.5 X-Ray Diffraction (XRD)

XRD study was carried out to find out the crystallinity of the compound. RIGAKU miniflex instrument using Ni filtered Cu K λ radiation (λ =1.5406 Å) was

employed for the study. The operating voltage and current was 30 KV and 15mA respectively. Crystallinity index has been calculated using various methods as described previously (Keshk and Haija, et al. 2011).

3.9.3 Drug release study

3.9.3.1 Preparation of beads for drug release study

MCC (250 mg, 500 mg) and isoniazid (250 mg) were added separately to 5 % of sodium alginate prepared in a beaker and stirred with a magnetic stirrer until complete mixing took place. The resulting gelatinous solution was taken in a syringe and was dropped down in a 0.2M calcium chloride solution leading to formation of small beads. The beads were filtered through Whatman filter paper No 1 and allowed to dry in a hot air oven at 37°C. The average size of the beads was 1 mm approximately

3.9.3.2 Characterization of the beads

The beads were characterized by using the same procedure as was done for characterizing the microcrystalline cellulose.

3.9.3.2.1 Scanning Electron Microscope (SEM)

SEM morphological study was carried out in the same instrument as was done for the MCC study. However in this case 2/3 numbers of beads were placed in the carbon stud and then coated with gold using a JEOL auto fine coater. The beads were observed with magnification from 100-4000X

3.9.3.2.2 Thermal Gravimetric Analysis (TGA)

TGA studies were carried out similarly as before. In this case 5/7 beads of all the compositions were taken separately in the pan and then taken for the study. All other conditions remained same during the study

3.9.3.2.3 Density Scanning Calorimetry (DSC)

DSC studies were carried out as was done for the MCC in the same instrument and maintaining the same conditions.

3.9.3.2.4 Fourier Transform Infra Red (FTIR)

FT-IR studies were carried out using the same instrument. However in this case 5-7 numbers of beads were crushed with KBr and then taken in a sample holder and placed in a hydraulic press. A 10 ton pressure was applied to obtain fine transparent discs which were then taken in the plate holder and placed in line of the infra-red rays to take the transmittance data.

3.9.3.2.5 Preparation of simulated intestinal fluid (SIF) as buffer for the study

SIF was prepared using the following procedure. In 400ml of distilled water 4g of sodium chloride (NaCl), 100mg potassium chloride (KCl), 720mg disodium hydrogen phosphate (Na_2HPO_4) and 120mg of potassium dihydrogen phosphate (KH_2PO_4) were added. The final volume was made upto 500ml and the pH was maintained at 7.4. The absorbance was taken at 262 nm for SIF. The release study was performed by placing the beads (50mg) in dialysis tube (Sigma) in a beaker containing the buffer solution. Constant stirring of 100 rpm was maintained for the release studies for 24 hours.

3.9.4 Antioxidant assay

Antioxidant assay was performed as mentioned before using 2, 2-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with slight modification in case of the sample to be tested. DPPH solution was prepared by adding 4mg of DPPH in 100ml of methanol. MCC at two different concentrations of 10 and 50mg were considered for the assay. The beads containing a) only sodium alginate b) sodium alginate and isoniazid c) sodium alginate, isoniazid and MCC (250 mg) and d) sodium alginate, isoniazid and MCC (500 mg) were also taken for the study. The tubes were incubated at room temperature in the dark for 40 minutes after adding 3ml of the DPPH solution to each tube. The absorbance was taken at 517 nm. The test was done in triplicates with methanol as blank. The percentage of radical scavenging (I %) was measured following the relation,

$I\% = (A_{blank} - A_{sample}/A_{blank}) * 100,$

where, A_{blank} is the absorbance of the control reaction containing all the reagents except
the beads. A_{sample} is the absorbance of the samples. Ascorbic acid was taken as the positive control. All the tests were carried out in triplicate.

3.9.5 Haemolysis test

Haemolysis test was carried out according to Nair et al (2007). Mammalian blood sample from goat was collected in a vial containing 4% trisodium citrate. It was centrifuged at 750 *g*. The supernatant was discarded and the precipitate containing the erythrocytes was washed with PBS (pH 7.4) twice at 750 *g* for 10 mins. 48.5 ml of PBS was added to 1.5 ml of the erythrocyte suspension and was equally divided in 2 ml tubes. Beads containing two different composition of MCC and weighing 10mg each were added to tubes containing 2 ml of the erythrocyte suspension and incubated for 2 hrs at 37 °C. After the incubation, the tubes were centrifuged at 750 *g* for 10 mins. Now, 200µl of the supernatant was collected in a fresh tube and to it 2.8 ml of PBS was added. The absorbance was taken at 415 nm. PBS was taken as the negative control and Triton X 100 was taken as the positive control.

Chapter IV

Results and Discussions

4.1 Screening of Medicinal plants and biochemical investigation of the important plant metabolites

An extensive search was made on the available traditional knowledge relating to use of plants for treatment of respiratory diseases in general and tuberculosis in particular amongst the various tribes through field visits undertaken in a few villages of Assam, in the Sonitpur and Tinsukia District. On the basis of these field surveys a few species were selected for comprehensive antimycobacterial, biochemical and analytical investigations. The basic objectives of these studies were to validate the traditional knowledge about the medicinal values of these species with respect to the curative or palliative properties in the treatment of respiratory diseases and more specifically tuberculosis. From the field surveys it appeared that some of the species which have been traditionally used for treatment of tuberculosis may have some palliative effect and these may provide lead molecules with potent antimycobacterium properties.

4.1.1 Antibacterial Assay

The antimycobacterial activity of ten plants was investigated in the *M. smegmatis* model system. From the results presented in Table 4.1, it can be seen that five of the species, *viz., Jatropha curcus* (latex), *Camellia sinensis* (Green tea and Assam Othodox Tea), *Xanthium strumarium* (Leaves), *Aegle marmelos* (Root) and *Messua ferrea* L.(Seed oil) was found to be active against *M. smegmatis* ATCC 14468 at the stock concentration tested for the assay. Promising among these were the latex of *J. curcus,* Green tea leaves, Assam orthodox tea leaves, *X. strumarium* and *M. ferrea* seed oil which showed activities measuring zone of inhibitions 14mm,16mm,15mm,14mm and 14mm (Table 4.1).

Table 4.1: Antibacterial activities of various plant extracts

Sl No	Plant name	Plant part	Methanol (20 mg/ml)	Ethyl acetate (20 mg/ml)	Hexane (20 mg /ml)	Isoniazid (1mg/ml)	DMSO (1%)
1	*Jatropha curcus*	Roots	NA	NA	NA	27	NIL
		Leaves	NA	NA	NA	"	NIL
		Stem	NA	NA	NA	"	NIL
		Latex	14	NA	NA	"	NIL
2	*Jatropha gossypifolia*	Latex	NA	NA	NA	"	NIL
		Leaves	NA	NA	NA	"	NIL
		Fruits	NA	NA	NA	"	NIL
3	*Camellia sinensis**	Green Tea Leaves	16	11	8<	"	NIL
		Assam orthodox tea, leaves	15	8	8<	"	NIL
		Fresh Leaves	NA	NA	NA	"	NIL
4	*Aegle marmelos*	Roots	12	NA	NA	"	NIL
5	*Plumeria acuminata*	Flowers	NA	NA	NA	"	NIL
		Leaves	NA	NA	NA	"	NIL
6	*Solanum torvum*	Fruits	NA	NA	NA	"	NIL
		Flowers	NA	NA	NA	"	NIL
7	*Nyctanthes arbor tristis*"	Flowers	NA	NA	NA	"	NIL
		Leaves	NA	NA	NA	27	NIL
8	*Euphorbia antiquorum*	Leaves	NA	NA	NA	"	NIL
9	*Mesua ferrea*	Seed oil	----	----	14	"	NIL
10	*Xanthium strumarium*	Leaves	14	10	11	"	NIL
		Roots	NA	NA	NA	"	NIL
		Stems	NA	NA	NA	"	NIL

4.1.2 Antioxidant Activity Assay

The antioxidant activity of the plant extracts showing antimycobacterial activity was determined (Fig 4.1). It was found that the methanol extract of the green tea leaves had the highest antioxidant activity at 1mg/ml scavenging 95.30 ± 0.01 % of DPPH free radical while Assam orthodox tea at 1mg/ml inhibited 95. 12 ± 0.01 % of DPPH. The essential oil from *Messua ferrea* was found to have antioxidant activity at all the concentrations tested with activity ranging from 91.34 ± 0.02 % for 20ul concentration to 92. 02 ± 0.01 % for 100ul concentration. The *Jatropha curcus* latex extract demonstrated mild antioxidant scavenging activity at 57.248 ± 0.02% while the *Xanthium strumarium* leaf methanol extract did not have any antioxidant activity. The results further support the antioxidant activity of tea which is important for the health. However the antioxidant activity of *M. ferrea* and the other plants has not been previously reported.

4.1.3 Total polyphenolic content

The total polyphenolic content of the positive crude extracts was determined (Table 4.2). It has been found that both the green tea and the Assam orthodox tea had high amount of phenolic content. *Jatropha curcus* latex also possesses a good amount of phenolics. The *Xanthium strumarium* leaf crude extracts exhibit very less amount of phenolic content.

4.1.4 Total flavonoid content

The total flavonoid content was carried out to determine the amount of flavonoid content in the crude extracts (Table 4.3). It has been found that the flavonoid content in both the green tea and the Assam orthodox tea was high as compared to the other two extracts. The J.curcas latex methanol extract was found to be lowest among all the four extracts.

It is well known that *Mycobacterial* infection induces production of reactive oxygen species through activation of phagocytes which also in turn induces tissue injury and inflammation although ROS production is a strategy of host defense against the infection (Beers and Sizer, 1952; Reddy, et al, 2004). Reddy et al (2003) also reported lower antioxidant capacity and higher oxidative stress in TB patients *viz-a-viz* in healthy human. Moreover malnutrition with reduced levels of Vitamin A, Vitamin C and Vitamin E among TB patients have been reported (Plit, et al, 1998, Rwangabwoba et al, 1998,

Dubey, et al, 1985). In the context of these it was considered relevant to study the antioxidant activity of the selected plant extracts with potentials of possessing metabolites with antimycobacterium activity. There has been an emphasis on determining the antioxidant capacities of carious medicinal plants. The high phenolic content is therefore considered an important factor that determines the antioxidant potentials of plants (Cai, et al, 2004, Tang, et al, 2004). The high phenolic content of Green tea leaves, Assam orthodox tea leaves and *Jatropha curcus* latex (879.16 ± 0.08 μg/ml, 680.04 ± 0.06 μg/ml and 395.83 ± 0.08 μg/ml) are in conformity with these reports.

Flavonoids consist of polyphenolic secondary metabolites which are quite common in the plants (Koes, et al, 1994). These polyphenolic compounds are based on a flavan skeleton, made up of 2 aromatic rings connected by a 3- carbon atom heterocyclic ring (Tasdemir, et al, 2006). There are more than 6, 400 flavonoids many of which have antibacterial, antiprotozoal, anti inflammatory and dietary antioxidant, vascular and estrogenic acitivities which are attributed to the inhibition of oxidases and NADH usage (Cos et al, 1998, Harborne and Williams, 2000). Epigallocatechin gallate and related flavonoids which are abundantly present in tea have been found to be inhibitors of β-hydroxyacyl-ACP reductase and enoyl ACP reductase of *Escherichia Coli* (Zhang and Rock, 2004). A number of flavonoids are reported to inhibit β-hydroxyacyl-ACP dehydratase of *Plasmodium falciporum* (Tasdemir et al, 2006). Brown et al (2007) reported for the first time the antimycobacterial activities of 4 flavonoid compounds namely butein, isoliquirtigenin, 2,2,4-Trihydroxychalcone and fisetin which were shown to interfere with mycolic acid biosynthesis.

4.1.5 Assay for Haemolytic activity

The haemolytic activity of the plant extracts was carried out to determine their toxicity against the erythrocyte blood cells (Fig 4.2). It was found that none of the extracts had any cytotoxic activity against the cells. The activity of the cells was at par with the negative control.

None of the extracts which were showing antimycobacterial activity against *M. smegmatis* were found to cause damage of the erythrocyte cells.

Table 4.2: Total phenolics content

Total Phenolics µg/ml GAE per 1mg of fresh weight	
Green tea methanol extract	879.16 ± 0.08
Assam orthodox tea methanol extract	680.04 ± 0.06
Xanthium strumarium leaf methanol extract	91.66 ± 0.09
Jatropha curcus latex methanol extract	395.83 ± 0.08

Table 4.3: Total flavonoid content

Total Flavonoids µg/ml QE per 1mg of fresh weight	
Green tea methanol extract	32.64 ± 0.012
Assam orthodox tea methanol extract	33.96 ± 0.010
Xanthium strumarium leaf methanol extract	14.08 ± 0.010
Jatropha curcus latex methanol extract	7.29 ± 0.060

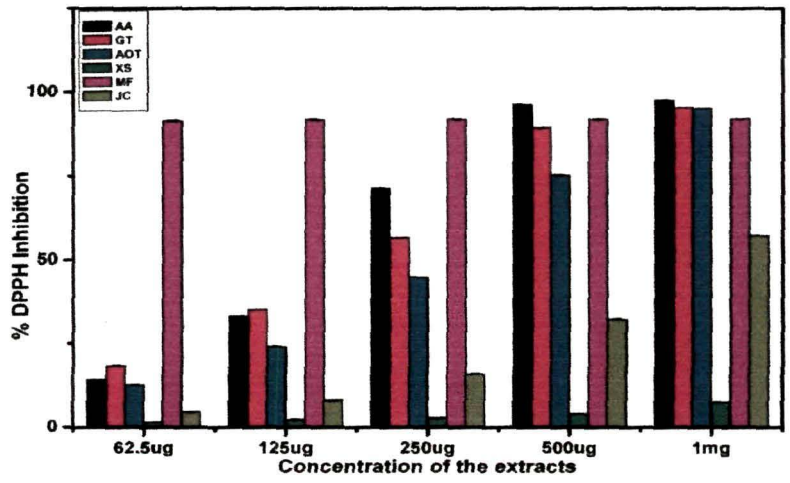


Fig 4.1: DPPH radical scavenging activity of the crude extracts.

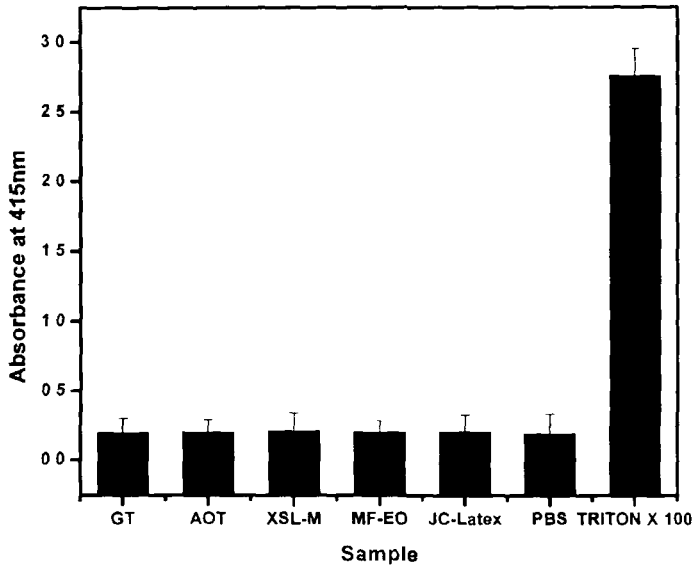


Fig 4.2: Haemolytic assay of the plant extracts

From the results obtained, it has been found that the plants showing antimycobacterial activity showed the presence of flavonoid, phenolics and antioxidant potential. Of all the plants studied, *C. sinensis* demonstrated the highest amount of flavonoids, phenolics and antioxidant activity. *X. strumarium* and *J. curcus* exhibited the lowest amount of phenolics and flavonoids. *M. ferrea* seed oil was found to exhibit high amount of antioxidant activity. Both *C. sinensis* and *M. ferrea* demonstrated more than 90% DPPH scavenging activity. Apart from flavonoids, essential oils have been reported to have antimycobacterial properties (Sanchez, et al, 2009).

The results obtained from the studies relating to the antimycobacterial, antioxidant activities of the screened plants are therefore indicative of their potentials as sources for lead molecules against *Mycobacterium smegmatis.*

4.2 Isolation and Characterization of active compounds

4.2.1 Purification of *Camellia sinensis* extracts (Green tea variety)

Several workers have isolated different flavonoid and alkaloid compounds from *Camellia sinensis*. Goto, et al, (1996) reported the isolation of different catechin and caffeine compounds from Japanese green tea. The use of Mass spectrometry for identification and quantification of various compounds in green tea have been illustrated (Miketova, et al, 2000, Kazuno, et al, 2005). Wu, et al, (2012) reported the use of HPLC and Mass spectrometry in determining different flavonols and catechins from various Chinese tea varieties. In the present study, investigations were made in the Assam green tea.

4.2.1.1 Column Chromatography

The solvent systems hexane, 90 % ethanol, methanol and water were used in the purification of the green tea methanol extract. The fractions were collected separately. The highest elution was obtained with 90 % ethanol with four different fractions collected (F1-F4). The fractions were combined in two groups, *viz.*, F1 + F2 and F3 +F4 which finally yielded two fractions (F1 and F2). All the fractions were evaporated using a Rotary Evaporator (HahnShin Scientific, South Korea). The dried fractions were again investigated for antimycobacterial activity which was shown only by the first fraction (F1) of the 90 % ethanol and with a zone of inhibition of 15mm. The fraction was further purified with HPLC.

4.2.1.2 HPLC

Reverse phase-HPLC method coupled to a UV-Vis detector was used to separate and identify the different compounds present in green tea.The HPLC of the F1 fraction was done using Water: Acetonitrile as the solvent system in a gradient elution method using a symmetry semi preparative HPLC column. The F1 fraction was mixed in methanol and then filtered through a 0.22μ filter assembly. 500 μl of the filtered fraction was continuously injected using a Hamilton syringe into the injection port of the instrument. The flow rate was maintained at 2ml/min. The purification led to the resolution of the F1 fraction into 06 different fractions as shown by the appearance of different peaks (Fig 4.3). The first peak was very broad and divided into two parts when collected. All together 7 different fractions were collected which were further evaporated using a Rotary evaporator and then stored in the refrigerator

at 4°C until further use. All the fractions were brown in color. The first two fractions collected from the broad peak were crystalline while the other fractions were amorphous. All the fractions were soluble in water and methanol. The fractions were again investigated for their antimycobacterial activity against the target strain. The second fraction of the broad peak and the fifth peak showed antimycobacterial activity. Both these fractions were then further characterized using different biophysical methods, *viz.*, UV, FTIR, MS and NMR.

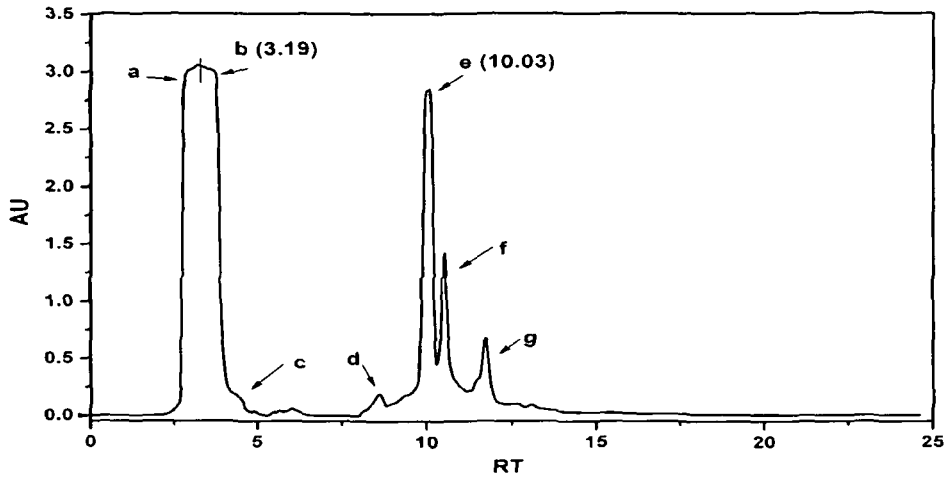


Fig 4.3: HPLC peaks of *Camellia sinensis* (Green tea)

4.2.1.3 UV-Vis Spectroscopy

The resolved compounds were analyzed with the help of a UV-Vis Spectrophotometer (Thermo-Fischer) to obtain the λ_{max}. Presence of different chromophores were predicted using the data obtained from the spectra. Both the compounds were dissolved in methanol and scanned from 190-800 nm to obtain the λ_{max} The λ_{max} for the first and the second compounds were 271.6 and 272.7 respectively (Fig 4.4).

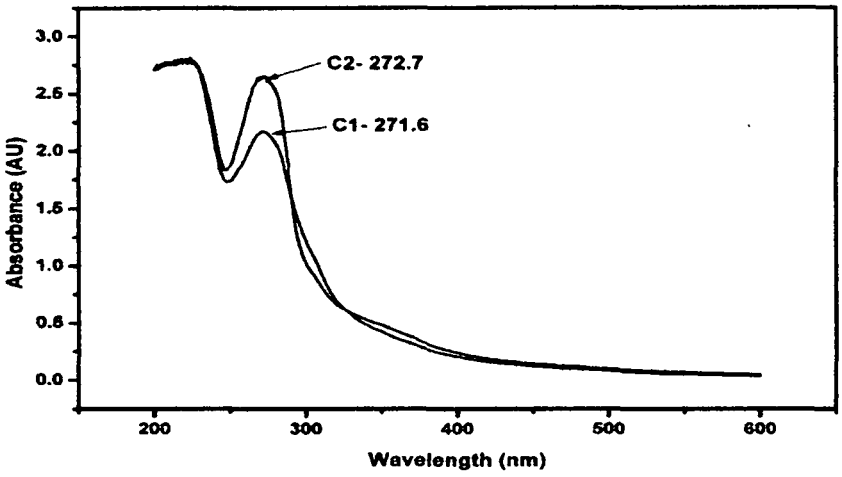


Fig 4.4: λ_{max} value of the purified compounds

4.2.1.4 FT-IR Spectroscopy

The FTIR spectroscopy for the HPLC resolved compounds were carried out to identify the functional groups present. Both the compounds were mixed with KBr powder and then prepared in fine tablet discs and placed in the sample holder for IR characterization. Both the compounds exhibited numerous transmittance peaks suggesting the presence of different functional groups. The peaks indicative of the presence of alkane, alkene, hydroxyl, carbonyl, phenyl substituted bands, aromatic rings in the compounds. However the FTIR data is only for providing complementary data towards characterization of the structure of the compounds (Fig: 4.5)

Table 4.4: FTIR transmittance peaks of purified compounds from *Camellia sinensis* (Green tea)

C1	C2
459.27, 601.47, 767.23, 1122.63, 1196.97, 1396.69, 1674.50, 2967.22, 3193.63, 3327.31	466.32, 607.50, 741.73, 1123.82, 1403.15, 1663.66, 2958.63, 3203.94, 3321.90

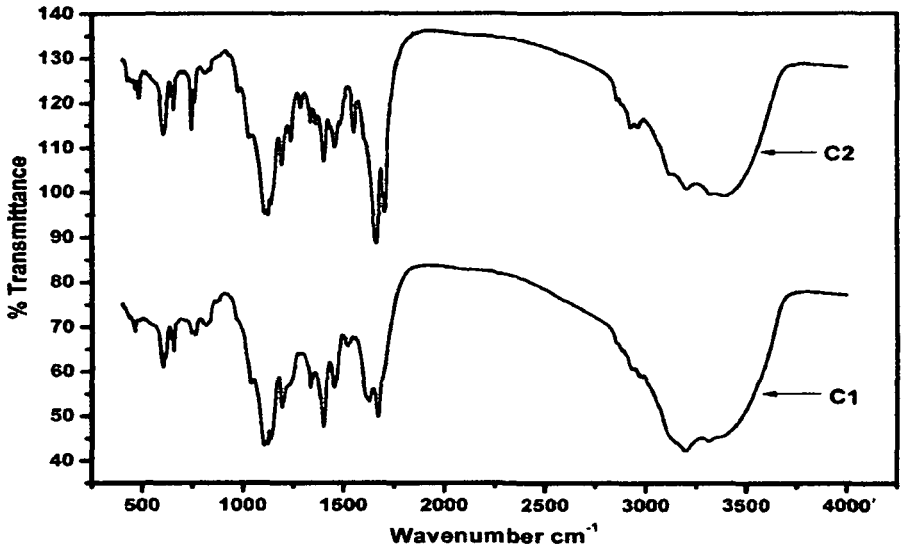
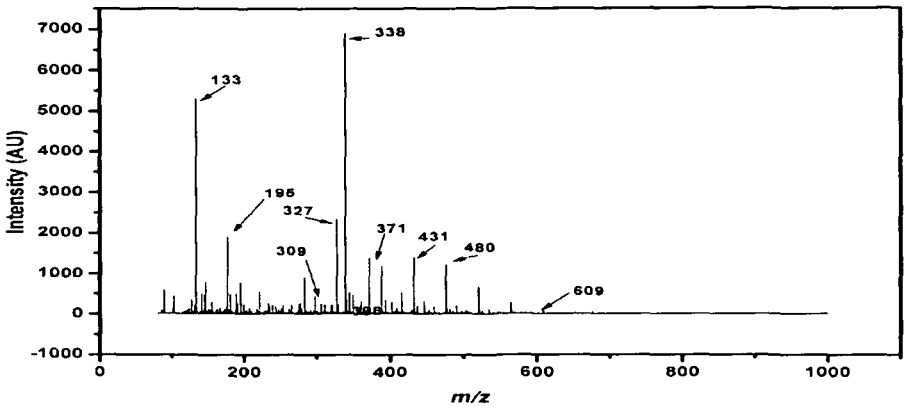


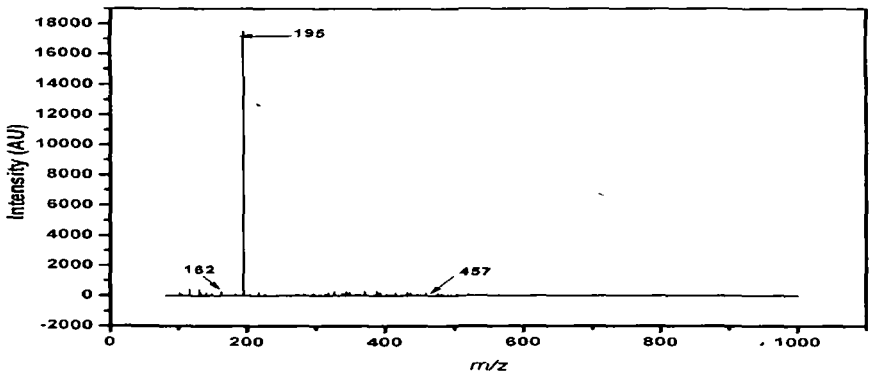
Fig 4.5: FTIR transmittance spectra of the purified compounds

4.2.1.5 Mass Spectroscopy:

Mass spectrometry related studies have been found to be indispensible for identification and quantification of different compounds. The MS spectrum of compound 1, shows the molecular ion peak at *m/z* 609, base peak at *m/z* 338 and other prominent fragments at *m/z* 480, 431, 371, 327, 309 with some other unidentified prominent fragments at *m/z* 133. The fragment at *m/z* 195 represents a signature ion of the catechin family belonging to the flavonoids. In compound 2, the molecular ion peak was observed at *m/z* 457 while the base peak was seen at *m/z* 195 and another fragmented ion peak at *m/z* 162. On the basis of the result obtained and subsequent analysis with reference to the data from other literatures published on *Camellia sinensis*, compound 1 was identified as Luteolin-6C-glucosyl-2′′-O-glucoside and compound 2 as Gallocatechin gallate (Fig 4.6).



A



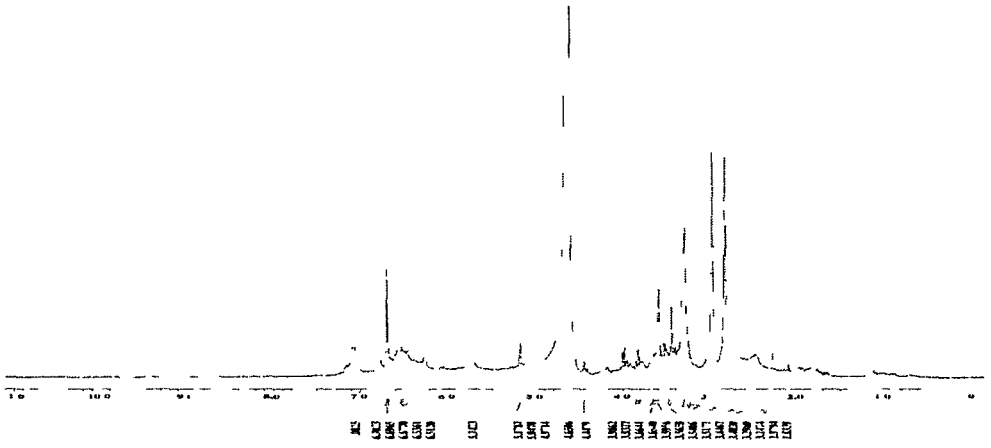
B

Fig 4.6: Mass Spectra of A) Compound 1, B) Compound 2

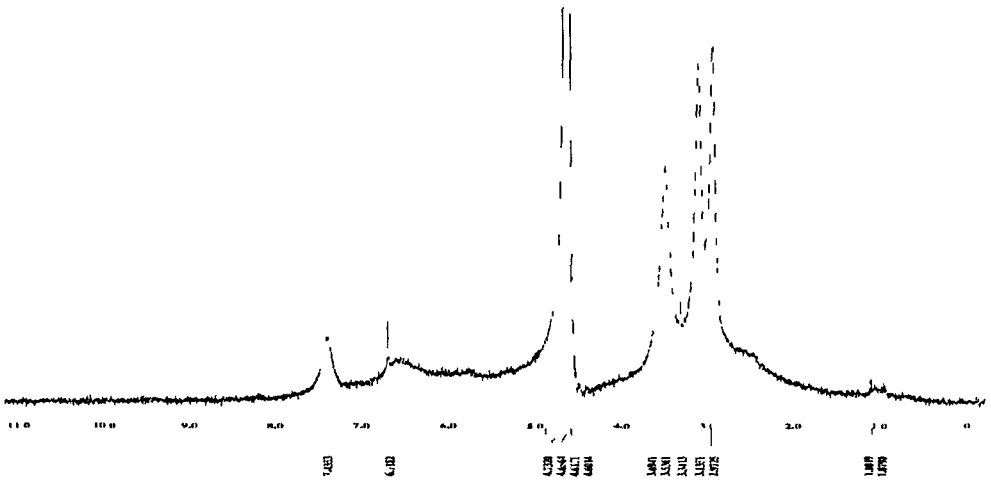
4.2.1.6 Nuclear Magnetic Resonance Spectroscopy:

NMR spectroscopy is an important tool of choice in structure elucidation of unknown compounds. Both ^{1}H and ^{13}C experiments were carried out. 20, 000 scans were given for each run of the samples. However it was observed that the NMR spectrum for the compounds obtained from tea were not distinct, though some faint peaks were observed (Fig: 4.7, Fig: 4.8)

4.2.1.6.1 ^{1}H NMR



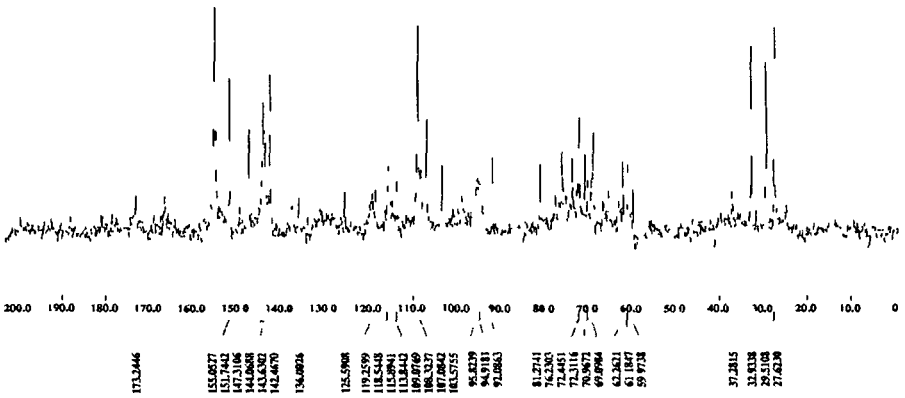
A



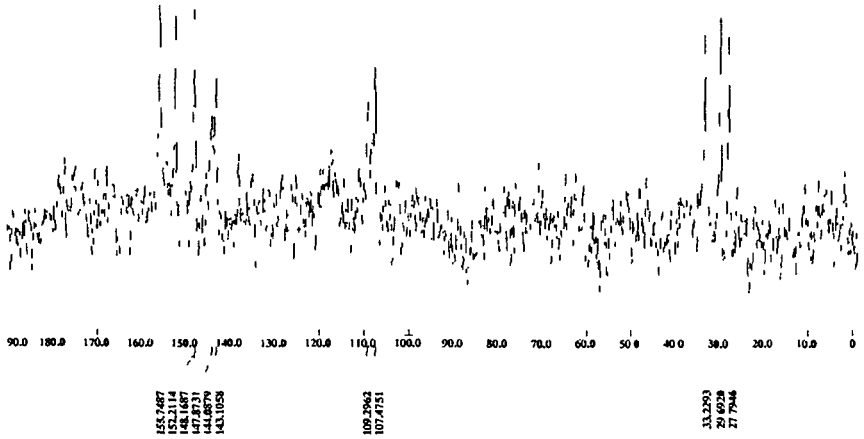
B

Fig 4.7: ^{1}H NMR Spectra of A) Compound 1 and B) Compound 2

4.2.1.6.2 ^{13}C NMR



A



B

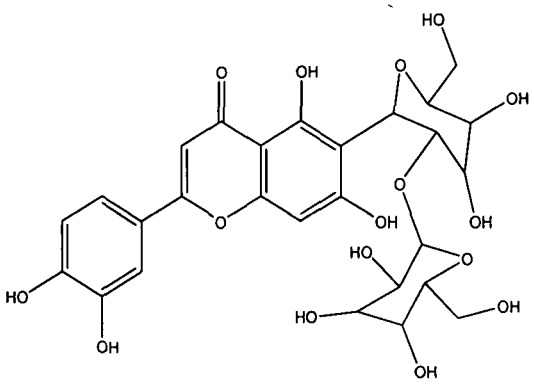
Fig 4.8: ^{13}C NMR Spectra of A) Compound 1 and B) Compound 2

Table 4.5: ^{1}H NMR and ^{13}C NMR spectral data for compounds C1 and C2 from *Camellia sinensis*

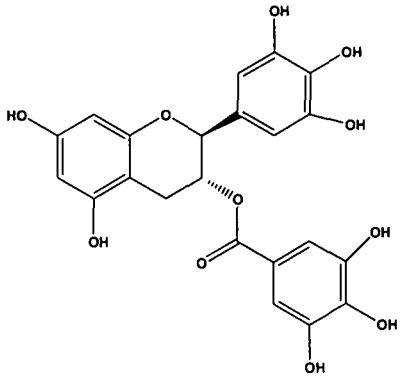
C1- ^{1}H NMR (δ_H)	C2- ^{1}HNMR (δ_H)	C1- ^{13}CNMR (δ_C)	C2- ^{13}CNMR (δ_C)
2.83	1.07	27.6	27.79
2.97	1.1	29.5	29.69
3.24	2.97	32.9	33.22
3.29	3.13	37.2	107.47
3.4	3.34	59.97	109.29
3.44	3.53	61.18	143.1
3.51	3.69	62.26	144.08
3.54	4.6	69.09	147.87
3.56	4.61	70.96	148.16
3.59	4.65	72.31	152.21
3.65	4.73	72.44	155.74
3.66	6.71	76.23	
3.83	7.43	81.27	
3.98		92.08	
4.44		94.91	
4.65		95.82	
4.97		103.57	
5.04		107.08	
5.17		108.32	
5.74		109.07	
6.51		113.84	
6.53		115.89	
6.67		118.54	
6.69		119.25	
6.7		125.59	
7.08		136.01	
		142.46	
		143.63	
		144.63	
		147.3	
		151.74	
		155.05	
		173.24	

4.2.1.7 Structure of the Compounds

The identified molecules were analyzed for inferring their structures through MS analysis of the data and relevant data from the work of Algamdi et al, 2011, Zhao et al, 2011. The compounds are predicted as Luteolin-6C-glucosyl-2′′-O-glucoside and Gallocatechin gallate (Fig: 4.9)



Luteolin-6C-glucosyl-2′′-O-glucoside



Gallocatechin gallate

Fig 4.9: Structures of the Purified compounds

4.2.2 Purification of the extract from *Mesua ferrea* seed

4.2.2.1 Column Chromatography:

The *Mesua ferrea* L seed hexane extract was purified using different solvent ratios of petroleum ether and ethyl acetate. Maximum amount of the oil was eluted in the petroleum ether: ethyl acetate 10:90 solvent medium. The elution was divided into three fractions (F1-F3). Each of the fractions was again tested for their antimycobacterial activity using the MIC activity test. It was found that the fraction F2 possessed antimycobacterial activity while fraction F1 showed limited activity and F3 no activity. The fraction F2 was further analyzed using GC-MS to identify the compounds present.

4.2.2.2 GC-MS

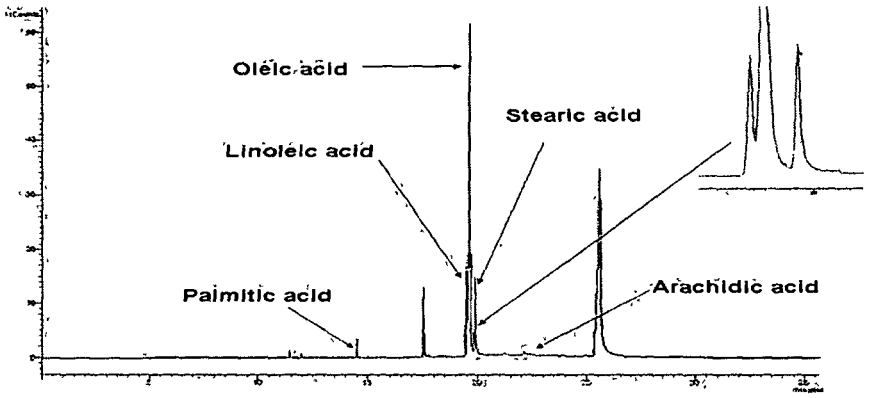
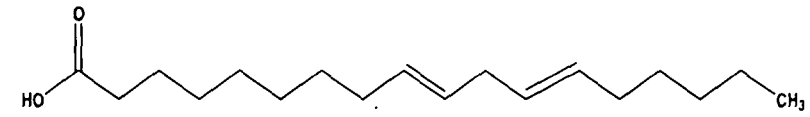
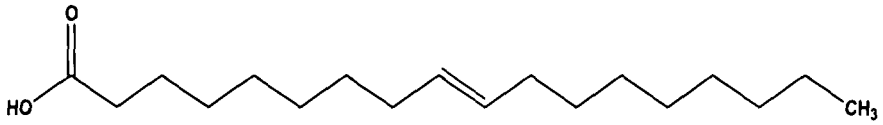


Fig 4.10: GC-MS of seed hexane extract from *M. ferrea*



Linoleic Acid



Oleic Acid

Fig 4.11: Structures of Linoleic acid and Oleic acid present in the essential oil fraction

The F2 fraction obtained from column chromatography was converted into fatty acid methyl esters (FAMEs). 1 µl of the FAMEs was injected into the GC-MS and analyzed to investigate the presence of the fatty acid components. The fatty acid components: palmitic acid, linoleic acid, oleic acid, stearic acid and arachidic acid were found to be present in the F2 fraction, along with some other unidentified compounds (Fig 4.11). From the results it can be inferred that the compounds like linoleic acid and oleic acid may be accountable for antimycobacterial activity of the

fraction. Both the essential fatty acids have been mentioned in the literature (Seidel and Taylor, 2004) for their antimycobacterial activity. The fraction F2 was further analyzed using FT-IR and NMR.

4.2.2.3 FT-IR of the hexane extract

The FT-IR of the essential oil extracted from the seeds of *Mesua ferrea* was carried out to characterize the essential oils. The absorbance at 1379, 1582, 1747 and 1750 are due to the presence of symmetric and asymmetric C=O stretching vibration attributed to the carboxylic acids. The absorbance at 726 and1623 is due to the presence of alkene group. The absorbance at 3007 and 3467 is attributed to the hydroxyl groups present in the fatty acids (Fig 4.12).

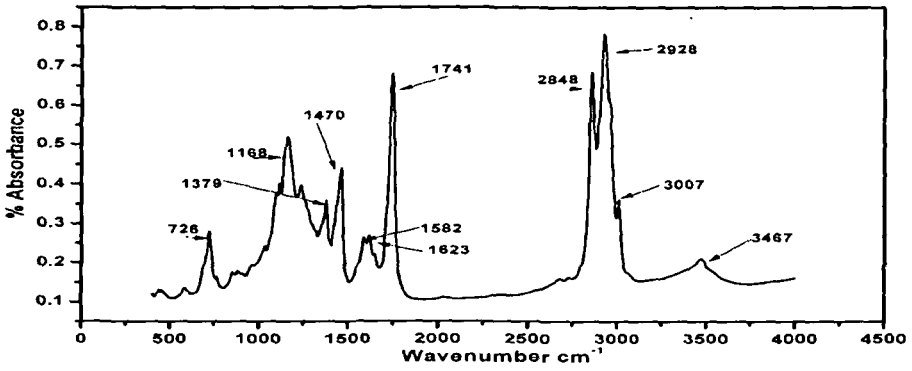


Fig 4.12: FTIR of seed hexane extract from *M. ferrea*

4.2.2.4 NMR of the hexane extract

4.2.2.4.1 ^{1}H NMR

In ^{1}H NMR, the triplet signal at δ_H 0.8545 is due to the presence of terminal methyl resonance. The signal at δ_H 2.28 is attributed to the presence of methylene groups adjacent to carbonyl group. The presence of unsaturated compounds with olefinic methines and allylic and bis allylic methylenes could be seen at δ_H 5.32, 2.01 and 2.76. All these resonances are characteristics of essential oils (Fig 4.13).

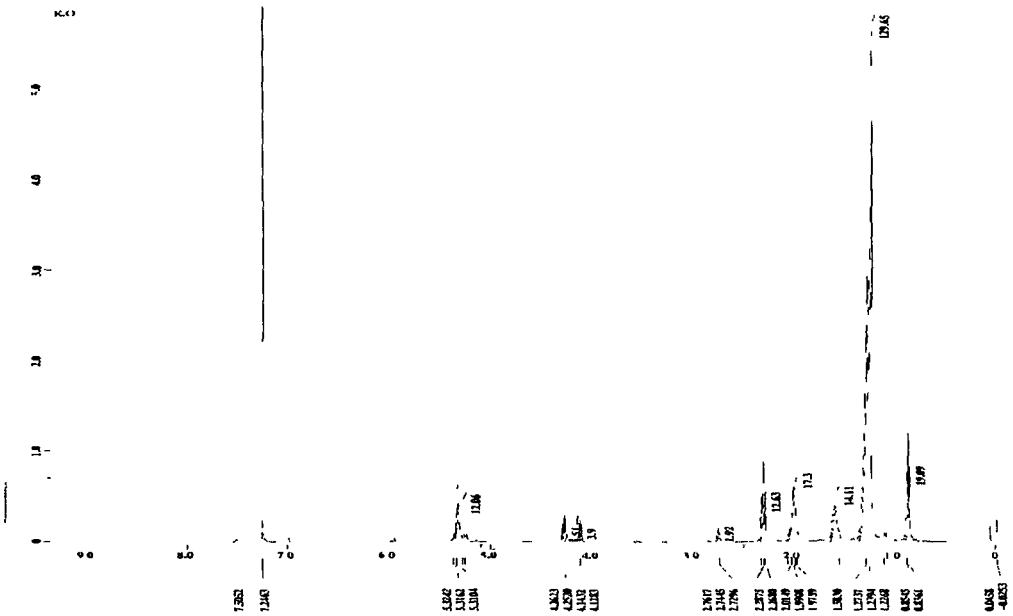


Fig 4.13: ^{1}H NMR spectra of Essential oil

4.2.2.4.2 ^{13}C NMR:

The δ_C at 24.8, 25.7 and 27.3 are due to the presence of the saturated monoene and diene chain, total number of diene chains and twice the total number of monoene and diene chains. The δ_C at 129.76 and 129.78 is due to the presence of the C_{10} and C_9 of the oleic chain while the smaller signals at δ_C 130.09, 129.9, 128.1 and 127.9 was due to the C_{13}, C_9, C_{10} and C_{12} of linoleic chain present in the essential oil component (Fig 4.14).

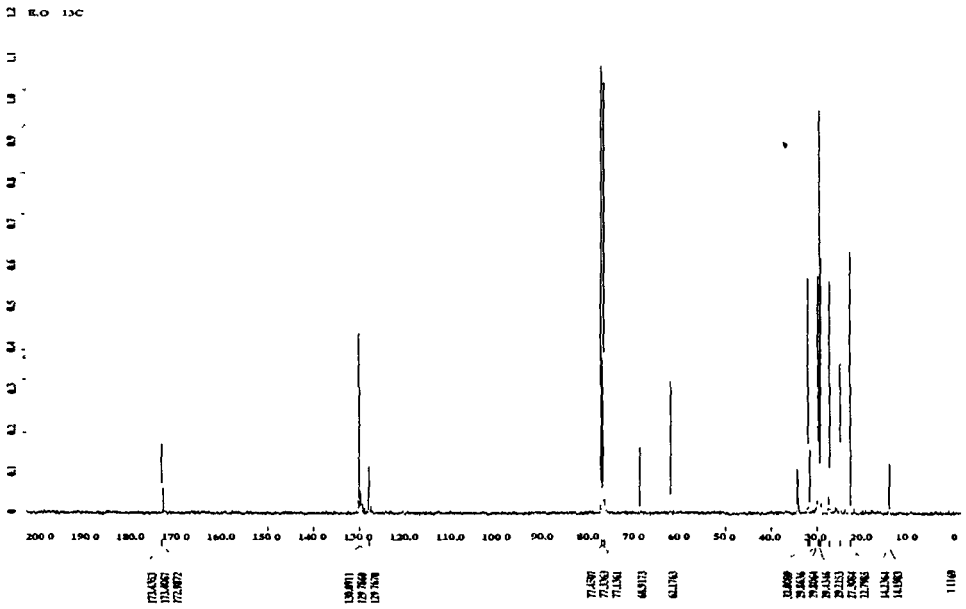


Fig 4.14: ^{13}C NMR spectra of Essential oil

4.2.2.5 MIC of the purified compounds

The MIC of the compounds revealed the minimum concentration at which the bacteria were inhibited by the compounds and essential oil fraction. It was found that Luteolin-6C-glucosyl-2′′-O-glucoside and gallocatechin gallate, obtained from green tea, inhibited *Mycobacterium smegmatis* ATCC 14468 at a concentration of 675μg/ml and 225μg/ml (Table: 4.6). For the essential oil containing fraction of *Mesua ferrea* the MIC at which the bacteria was inhibited was found to be 78μg/ml. Thus it is seen that the essential fraction had better inhibitory properties than the flavonoid compounds. It has been reported that the inhibitory properties of both linoleic and oleic acid are due to their detergent like properties which helps in killing the mycobacterium species (Seidel et al, 2004). Luteolin previously isolated from the flowers of the plant *Chromolena odorata,* was found to be active against *M. tb* $H_{37}Rv$ at a concentration of 699.3 μM (Suksamrarn et al, 2004).

Table 4.6: MIC value of the compounds and the essential oil fraction

Plant Name	Compound	MIC
Camellia sinensis (Green tea)	Luteolin-6C-glucosyl-2''-O-glucoside	675µg/ml
	Gallocatechin gallate	225µg/ml
Messua ferrea (Essential oil)	Purified fraction (Containing linoleic acid and oleic acid)	78µg/ml

4.3 Cytotoxicity Assay of the Purified Compounds

The isolated purified compounds and the purified essential oil were investigated for their cytotoxicity using RAW 264.7 macrophage cell lines. The compounds were used in 5 different concentrations of 62.5µg/ml – 1mg/ml. The compounds were dissolved in sterile double distilled water and filtered through a 0.22 filter membrane. The essential oil was applied directly at different concentrations of 1,5,10, 20 and 40 µl into the 96 well plates in which the cell lines were seeded. The plates were incubated in a CO_2 incubator for 12h and then estimated for cytotoxicity using MTT. None of the compounds and the essential oil was found to be toxic.

4.4 Antioxidant Assay of the purified compounds

The antioxidant activity of both the purified compounds and the essential oil fraction containing linoleic acid and oleic acid were assayed using the DPPH free radical scavenging method. Five different concentrations of Luteolin-6C-glucosyl-2''-O-glucoside and gallocatechin gallate were taken from 62.5µg/ml – 1mg/ml, while for the essential oil fraction, the concentrations ranged from 5, 10, 20, 40 and 80µl. It was observed that at the highest concentration the compounds as well as the oil fraction showed more than 93% inhibition of DPPH free radical. Ascorbic acid taken as the positive control showed 97% inhibition, slightly more than the tested samples. The higher antioxidant property of the compounds and the oil indicate pharmacological significance.

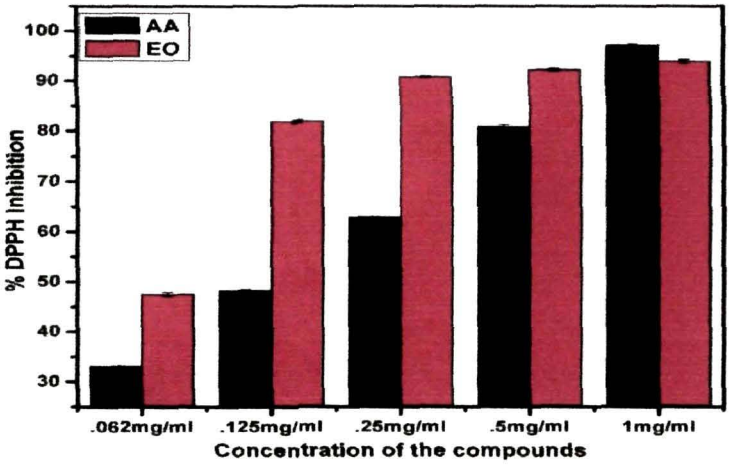


Fig 4.15: Antioxidant activity of Essential oil fraction from *Mesua ferrea*

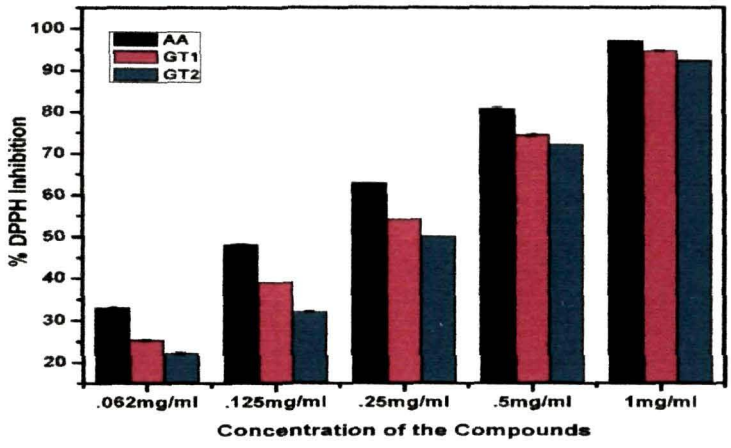


Fig 4.16: Antioxidant activity of *Camellia sinensis* (Green tea) compounds

4.5 *In silico* investigation of phytochemicals against selected *M. tuberculosis* target proteins

An effort was made to understand as to how the purified compounds from the plant extract, caused inhibition of the *Mycobacterium* species. In silico docking experiments were conducted to raise hypothesis as to the possible targets in the *Mycobacterium* species and about the target ligand binding.

4.5.1 Molecular Docking

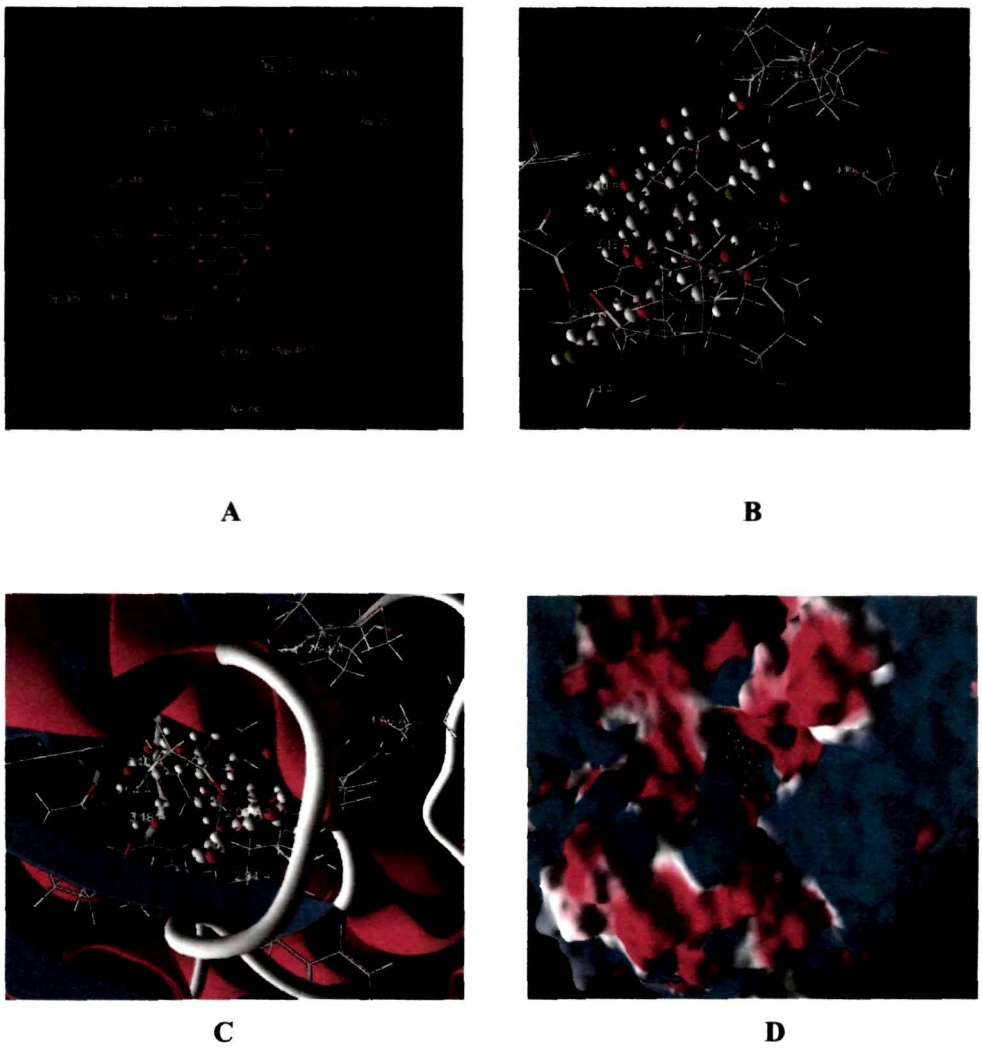
The purified and identified antimycobacterial molecules from the methanol extract of *Camellia sinensis* (Green tea) leaves and *Mesua ferrea* seed hexane extract were docked with the *Mycobacterium tuberculosis* cell wall proteins- **Glf T2 (PDB ID: 4 FIX), Inh A (PDB ID: 2B35) and mt Kas B (PDB ID: 2GP6).**

1. ***Camellia sinensis* (Green tea):**

The compounds Luteolin-6c-glucosyl-2"-O-glucoside and Gallocatechin gallate isolated and identified from green tea with activity against *M. smegmatis* ATCC 14468 Both the compounds were docked with the selected target proteins.

A.Glf T2

Luteolin-6c-glucosyl-2"-O-glucoside was found to dock with Glf T2 with better docking efficiency than Gallocatechin gallate (GCG). The Rerank score for Luteolin-6c-glucosyl-2"-O-glucoside was found to be -117.074 and a Moldock score of -130.174 while for GCG it was -107.074 and -125.74. The amino acids to which the ligands docked were Ala 398, Tyr 344, Asp 403, Trp 399, Met 397, His 413, Trp 309, Trp 370, Lys 369, Asp 372, Arg 171, Phe 169, Asp 257, pro 167. The various bond distances found to occur were 3.41 Å, 2.94 Å, 1.66 Å, 3.14 Å, 3.10 Å, 3.04 Å, 3.18 Å, 2.09 Å and 2.84 Å (Fig 4.17).



A **B**

C **D**

Fig 4.17: Docking study of Luteolin-6c-glucosyl-2"-O-glucoside and Gallocatechin gallate with Glf T2

A: Ligand mapping of the best docking pose

(Blue dash: H bonds, Red dash: Electrostatic bonds)

B: Orientation of the ligand w.r.t. the protein target

C: Protein target in 2° structure view

D: Ligand docking within the protein cavity

B. Inh A

GCG was found to be the best docked molecule among the two purified green tea molecules in case of the target protein Inh A. The Rerank score for GCG was found to be -59.3818 and the Moldock score -102.198. Luteolin-6c-glucosyl-2"-O-

glucoside was found to demonstrate a Rerank score of -22.3369 and a Moldock score of -85.0724. The amino acid residues interacted are Glu 218, Arg 185, Thr 188, Lys 186, Ile 184 and the distance of bonding was found to occur at 3.31 Å, 3.05 Å, 2.00 Å, 3.52 Å, 3.45 Å, 3.22 Å, 3.10 Å and 2.99 Å. (Fig 4.18).

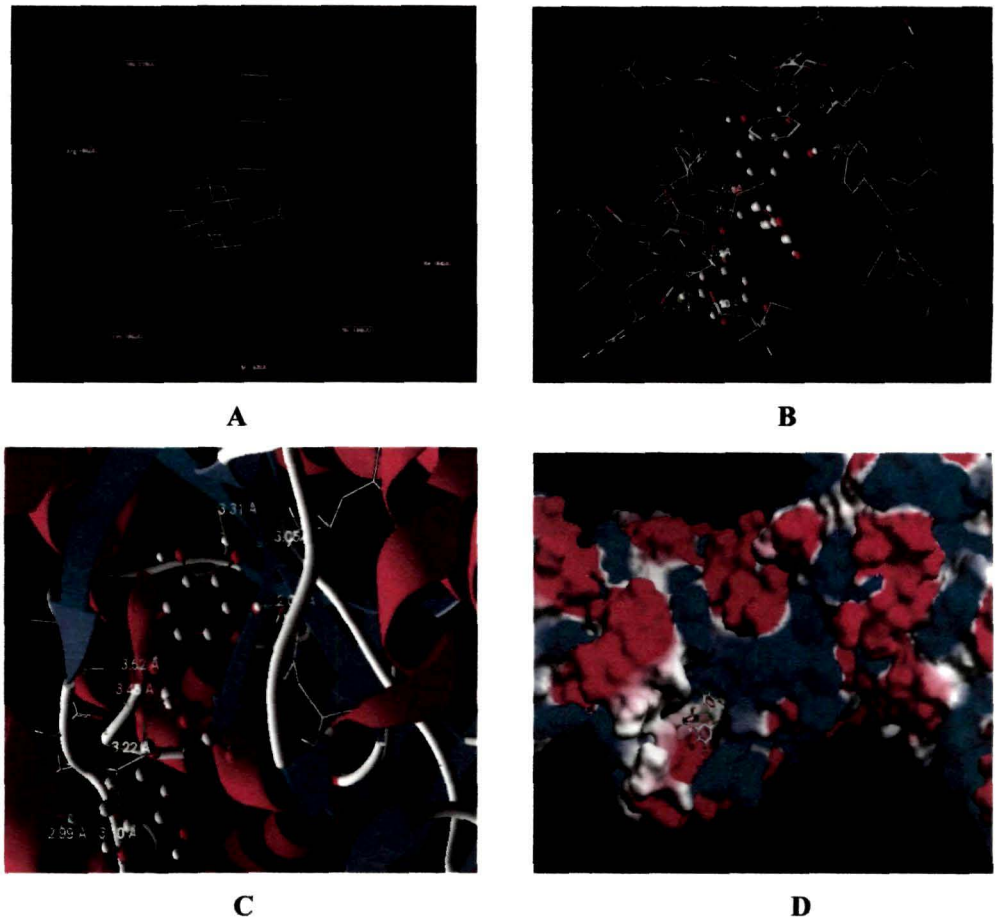


Fig 4.18: Docking study of Luteolin-6c-glucosyl-2"-O-glucoside and Gallocatechin gallate with Inh A

(A,B,C,D: same as above in Fig: 4.17)

C. mtKas B

In case of mt Kas B, GCG was found to dock with much more efficiency having a a Rerank score of -49.0554 and a Moldock score of -80.7944 than Luteolin-6c-glucosyl-2"-O-glucoside which exhibited a Rerank score of -47.5736 and a Moldock score of -83.918. The interacting amino acid residues were Ser 137, Asp 127,

Arg 131, Lys 143, Arg 84 and Phe 123. The bonding distance was found to be 3.10 Å, 2.89 Å, 3.06 Å, 3.10 Å, 3.18 Å, 2.62 Å, and 2.77Å. (Fig 4.19)

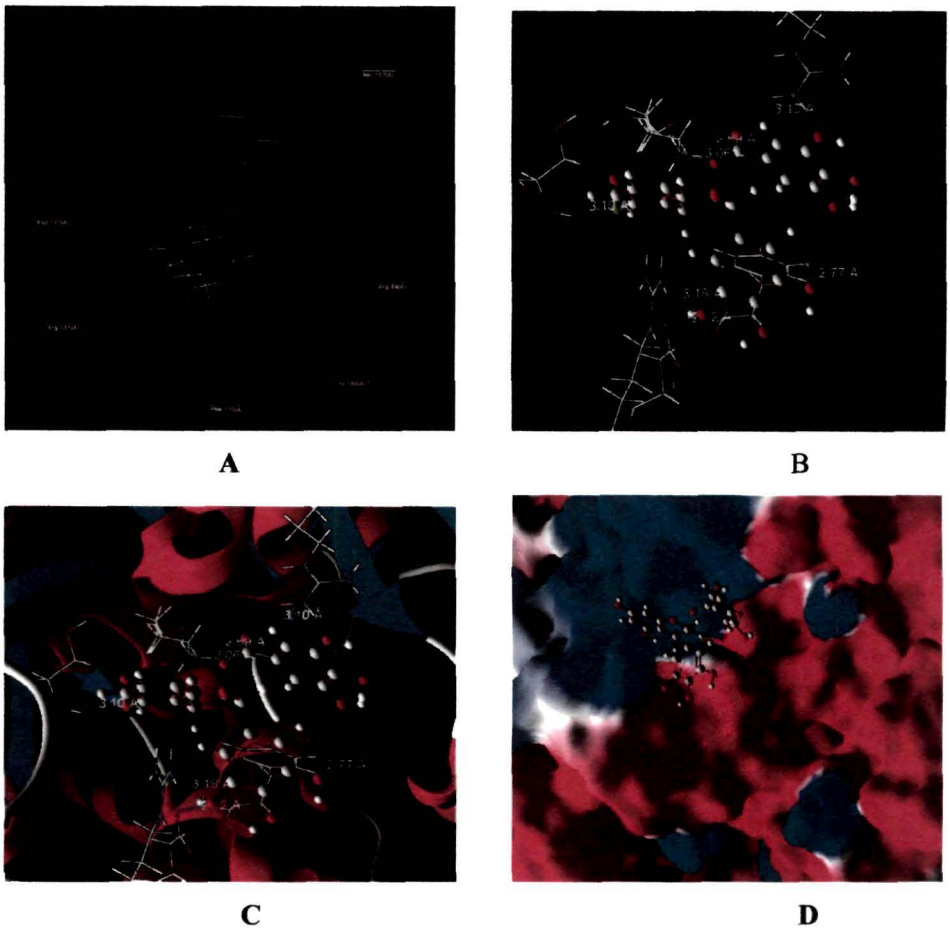


Fig 4.19: Docking study of Luteolin-6c-glucosyl-2"-O-glucoside and Gallocatechin gallate with mt Kas B

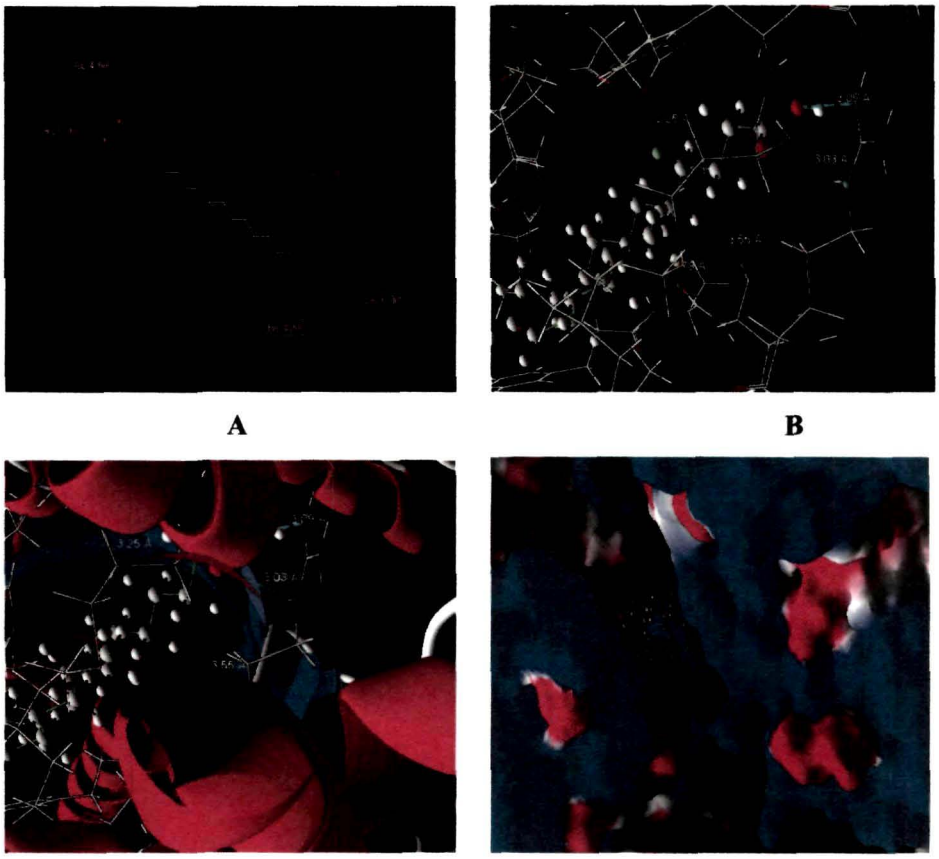
(A,B,C,D: same as above in Fig: 4.17)

2. ***Mesua ferrea***: Linoleic acid and Oleic acid identified as the antimycobacterial compounds from the hexane fraction of *M. ferrea* were docked with the target proteins with 2000 iterations.

A. Glf T2

Linoleic acid exhibited a better Rerank score of -54.0174 and a Moldock score of -88.5801 as against Oleic acid which showed scores of -33.5739 and -71.8612. The amino acid residues which interacted with the ligands were Arg 610, Asp 426, Pro

157, His 382 and Leu 613. The bond distances that occurred were 3.25 Å, 3.09 Å, 3.03 Å, 3.55 Å and 3.78Å.(Fig 4.20).



A **B**

C **D**

Fig 4.20: Docking study of Linoleic acid and Oleic acid with Glf T2

(A,B,C,D: same as above in Fig: 4.17)

B. **Inh A**

In case of Inh A, Linoleic acid demonstrated the best Rerank score of -79.1535 and a Moldock score of -107.516 while Oleic acid exhibited -74.884 and -98.1139 as Rerank and Moldock score. Ser 152, Ile 257, Tyr 259 and His 265 were the interacting amino acid residues while the bonding distance observed were 1.91 Å, 3.92 Å and 3.76Å. (Fig 4.21).

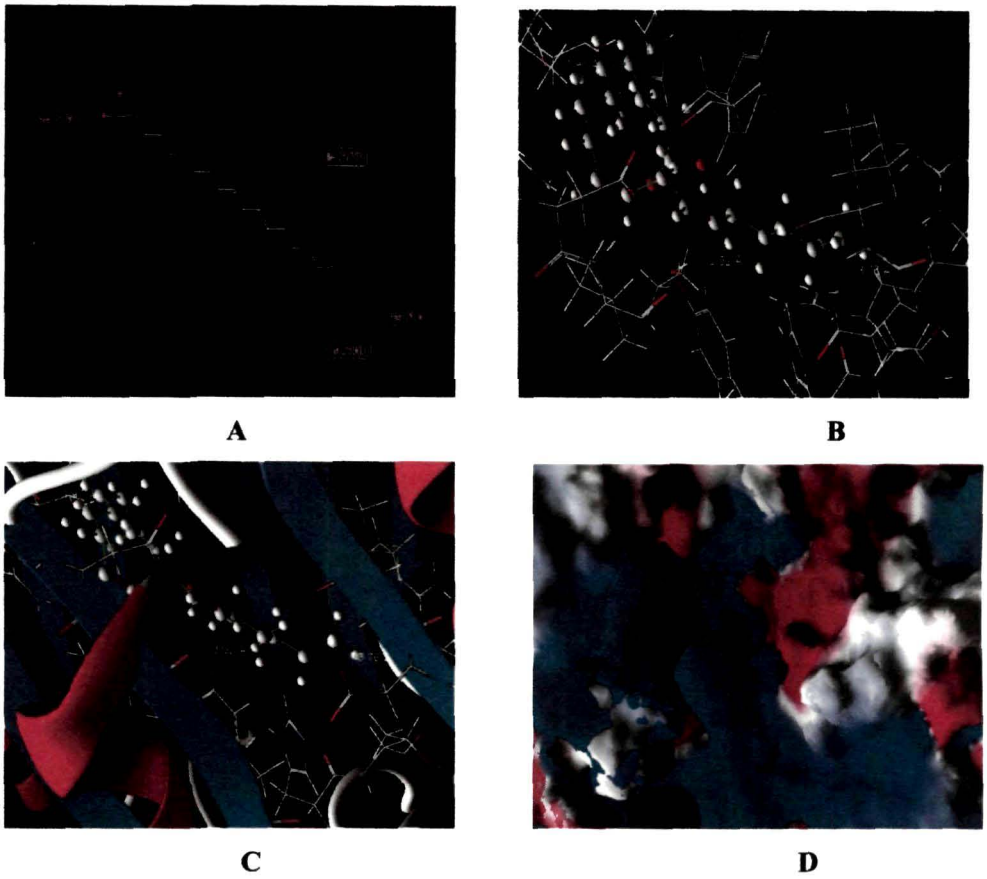


Fig 4.21: Docking study of Linoleic acid and Oleic acid with Inh A

(A,B,C,D: same as above in Fig: 4.17)

C. mt Kas B

Oleic acid was the best docked ligand in case of the target protein mtKas B with a Rerank score of 315.301 and a Moldock score of -45.948, while linoleic acid exhibited a score of 424.186 and -17.7098 as Rerank and Moldock score. Ser 347, Glu 241, Gly 242, Glu 198, Val 348, Thr 199, Glu 202, leu 115, Glu 119 were the amino acid residues interacting with the ligands. The bonding distance observed was found to be 2.36 Å, 1.35 Å, 3.08 Å, 3.05 Å. (Fig 4.22).

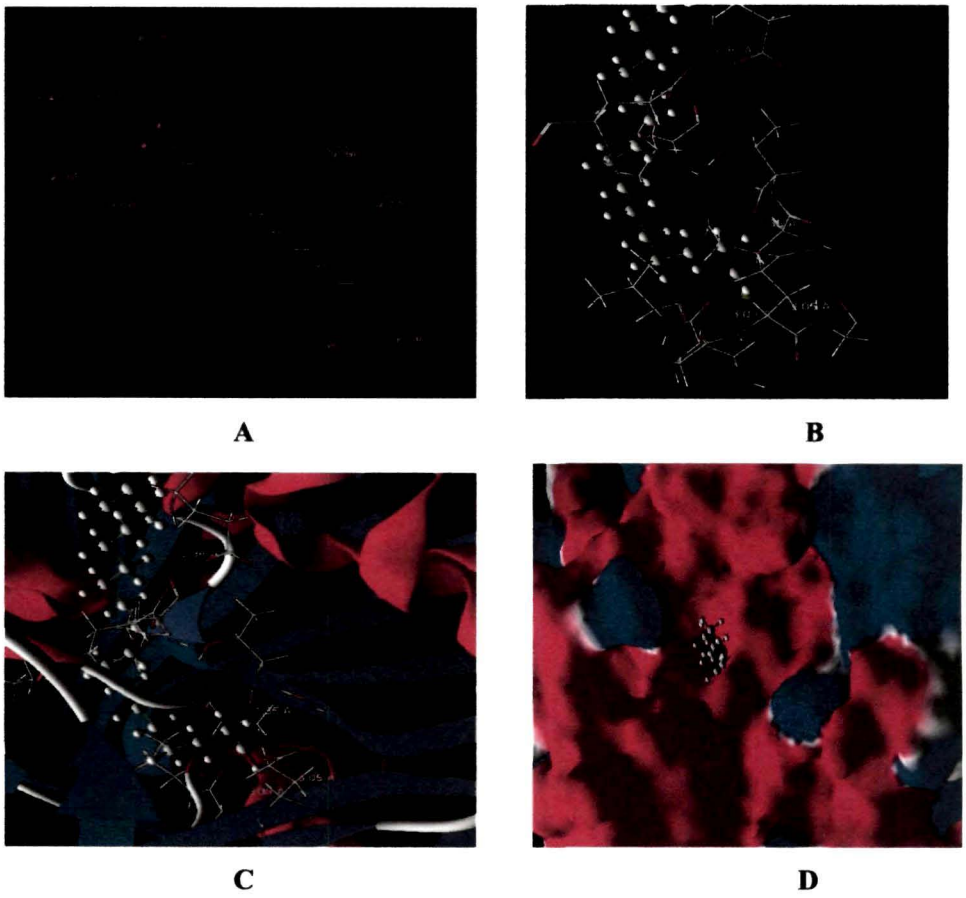


Fig 4.22: Docking study of Linoleic acid and Oleic acid with mt KasB

(A,B,C,D: same as above in Fig: 4.17)

The results of the docking studies reveal that A, B, C and D are the most likely target in the *M. smegmatis* / *Mycobacterium* for A,B,C and D ligands purified and identified from *C. sinensis* and *M.ferrea* .

4.5.2 ADME-TOX Studies

The ADME- Tox studies was carried out to hypothesize the various pharmacological properties of the purified and identified molecules and also to ascertain their toxicity against various model systems in silico. The Lipinskis Rule of Five recommends that for a drug to be orally absorbed, it should not have a molecular weight of more than 500, its Log P should be less than 5, it must have less than 5 hydrogen bond donors (HBD) and less than 10 hydrogen bond acceptors (HBA). The total polar surface area (TPSA) of a molecule has significant attributes to the properties of a drug molecule. Molecules with TPSA more than 140 have poor

intestinal absorption (Nuez and Rodriguez, 2008). In the present study as detailed in Table 4.7, it was found that both linoleic acid and oleic acid follow all the properties of Lipinskis Rule, except the xLogP value. In case of GCG and Luteolin-6c-glucosyl-2"-O-glucoside, many violations were observed with more than the required HBD and HBA as well as high molecular weight and with 610.52 Dalton. The TPSA values for linoleic and oleic acid were found within the stipulated range suggesting that they can be absorbed well in the intestine.

Table 4.7: Lipinski's Rule of 5 filtering of compounds

Sl No	Compound	HBA	HBD	MOL WT	XLogP	TPSA
1	Linoleic acid	1	2	280.44	7.86	37.3
2	Oleic acid	1	2	282.46	8.19	37.3
3	Gallocatechin gallate	8	11	458.37	2.98	197.37
4	Luteolin-6c-glucosyl-2"-O-glucoside	11	16	610.52	-1.9	276 52

The in silico toxicity studies, (Table 4.8) in mice and rats were carried out to determine the health effect predictions of the identified molecules. From the LD_{50} values, in mice, the linoleic acid had the lowest LD_{50} value of 260mg/Kg and 45mg/Kg when administered through IP and IV routes, while GCG and luteolin-6c-glucosyl-2"-O-glucoside had the lowest LD_{50} values of 2400mg/Kg and 200 mg/Kg when administered through the Oral and SC route. In case of rats, linoleic acid and luteolin-6c-glucosyl-2"-O-glucoside had the lowest LD_{50} value in case of IP and oral delivery.

Table 4.8: Toxicity parameters prediction of compounds

Sl No	Compound	LD_{50} mouse[a] (IP)	LD_{50} mouse[a] (oral)	LD_{50} mouse[a] (IV)	LD_{50} mouse[a] (SC)	LD_{50} rat[b] (IP)	LD_{50} rat[b] (Oral)
1	Linoleic acid (mg/kg)	260	3400	45	390	310	3600
2	Oleic acid (mg/kg)	310	2700	140	1500	370	22000
3	Gallocatechin gallate (mg/kg)	890	2400	240	490	330	5400
4	Luteolin-6c-glucosyl-2"-O-glucoside (mg/kg)	1100	2500	1700	200	1300	1800

[a] Estimates LD_{50} value in mg/kg after intraperitoneal (IP), oral, intravenous (IV) and subcutaneous (SC) administration to mice.
[b] Estimates LD_{50} value in mg/kg after intraperitoneal (IP), oral, intravenous (IV) and subcutaneous (SC) administration to rats.

The pharmacological properties, *viz* (Table 4.9), like permeability, solubility and absorption were investigated. The permeability through MDCK epithelial cell line and Caco cell line were studied. Both linoleic acid and oleic acid had better permeability in case of MDCK cell line while GCG and luteolin-6c-glucosyl-2"-O-glucoside exhibited poor permeability. Also in Caco cell line, both linoleic acid and oleic acid demonstrated better permeability than luteolin-6c-glucosyl-2"-O-glucoside and GCG. All the four compounds exhibited permissible range of IC_{50} values in case of HERG K^+ channel suggesting that the compounds do not cause any blockage of the HERG K^+ channel. The blood/ brain coefficient values were within the acceptable limits in case of all the compounds. Both GCG and luteolin-6c-glucosyl-2"-O-glucoside exhibited better aqueous solubility as compared to linoleic acid and oleic acid, however the % oral absorption in human was nil for both GCG and luteolin-6c-glucosyl-2"-O-glucoside while linoleic acid and oleic demonstrated good oral absorption.

Table 4.9: ADME and pharmacological parameters prediction

Sl No	Compound	QPP MDCK [a]	QPLog HERG[b]	QPP Caco[c]	QP LogBB[d]	QP logS[e]	%Human Oral Absorption[f]	Rule of 5[g]
1	Linoleic acid (mg/kg)	1042.9	-4.366	1993.682	-0.112	-2.169	92.778	0
2	Oleic acid (mg/kg)	1042.9	-4.338	1993.708	-0.11	-2.123	92.642	0
3	Gallocatechin gallate (mg/kg)	17.498	-3.162	45.42	-1.47	2	0	1
4	Luteolin-6c-*glucosyl*-2"-*O*-glucoside (mg/kg)	19.475	-3.452	50.148	-1.514	2	0	2

[a] Predicted value of MDCK cell permeability in nm/s (Acceptable Range: <25 is poor, >500 is great)

[b] Predicted value of IC_{50} for blockage of HERG K^+ channels (Concern below -7)

[c] Predicted value of Caco-2 cell permeability in nm/s (Acceptable Range: <25 is poor, >500 is great)

[d] Predicted value of blood/brain barrier partition coefficient (Concern value is -3.0 to -1.2)

[e] Predicted value of aqueous solubility (Concern value is -6.5 to -0.5)

[f] Predicted value of human oral absorption on 0-100% scale (acceptable range: <25% is poor, >80% is high)

[g] Number of violations of Lipinski's Rule of five (Lipinski et al, 1997)

Thus the results of the in silico ADME TOX studies indicate that A and B satisfy the requirements in terms of absorption, permeability, solubility and toxicity for forwarding as potential antimycobacterial agents after *in vitro* validation of these results.

4.5.3 Quantitative Structure Activity Relationships (QSAR)

Quantitative Structure Activity Relationships analysis involves the ability to predict various properties of a drug molecule, *viz.*, pharmacokinetic, physiochemical and toxicological, which are essential in the development of lead molecules. QSAR analysis helps in reducing expensive drug experimentation and late development failures (Winkler, 2002).

The list of studied compounds along with their IC_{50} values, are tabulated in Table 6.4. The chemical hardness (η), chemical potential (μ) and electrophilicity (ω) values computed at 6-311g** level for all compounds are given in Table 6.5, along with the physical parameters such as hydration energy, logP, surface area, refractivity and polarizability obtained from the MM+ computations with Hyperchem software.

4.5.3.1 QSAR analysis

Based on the DFT calculations, different descriptors were selected for QSAR modeling such as the energy of highest occupied molecular orbital (E_{HOMO}), energy of the lowest unoccupied molecular orbital (E_{LUMO}), energy of the next lowest unoccupied molecular orbital (E_{NL}), energy difference between LUMO and HOMO (E_{L-H}), electorphilicity (ω), hardness (η), etc. The HOMO and LUMO descriptors are frequently calculated as these orbitals can influence the chemical reactivity and hence the reaction mechanism. The HOMO features the susceptibility of a molecule towards attack by nucleophile, whereas LUMO characterizes the susceptibility of a molecule towards the attack by electrophiles in chemical reactions (Iczkowski and Margrave, 1961).

In addition, molecular mechanics (MM+) parameters such as hydration energy, molecular refractivity index, polarizibility, surface area (SA), hydrophobicity (logP) of the compounds were also selected. The biological activity data (IC_{50}) of compounds against various targets were taken from the results reported in Table 4.10.

At the first step, we performed the simple linear regression for twenty one compounds by considering the IC_{50} as dependent variable and other descriptors as single individual independent variable. However, the correlation of IC_{50} with all the individual descriptors was found to be very much insignificant except in case of polarizability and there exists auto correlation among the various parameters. So, we have perform multiple regression analysis keeping polarizibility as a fixed descriptor and then MLR equations were generated utilizing the pool of all calculated descriptors. The autocorrelation values of the parameters are shown in Table 4.11. The predictability of the models was determined using the "leave one out (LOO)" cross-validation method.

The QSAR equations having significant statistical parameters for all the compounds are represented in the Table 4.12. The equation was obtained by considering the IC_{50} as a dependent variable and energy of highest occupied molecular orbital (E_{HOMO}), polarizability, E_{HOMO-1}, hydration energy, surface area, logP, and molar refractivity as independent variables.

Here, R^2 is the square of correlation coefficient, SD is the standard deviations of regression, F is the overall F-statistics for the addition of each successive term, and

p is the p-values using the F statistics. In general, a regression model is significant at p-value < 0.05 using the F statistics (Cho, et al, 2001) and so these models are statistically significant. However, according to the generally statistical standards, a model with R^2 > 0.80 (Yao, et al, 2003) is acceptable. Therefore these QSAR equations should be further improved to become a statistically significant model.

To improve R^2, LOO (Leave One Out) method suggested by Dietrich et al. and Cornish-Bowden and Wang (Dietrich, et al, 1980, Bowden and Wong, 1978 and Barua et al, 2012) was applied in which a compound was considered as outlier if its corresponding R^2, called jackknife R^2 (Rj2) value obtained from the regression analysis after deleting the compound, was comparatively higher than the other Rj2 values. The calculated Rj2 values are presented in Table 6.7. It is clear that compounds 7, 11, 12, 15 and 16 have higher rj2 values. Therefore, these compounds may be considered as outliers. The obtained QSAR equations after deleting compounds are shown in the Table 4.13

The finally developed model reveals that decreasing values of E_{HOMO}, polarizability, E_{HOMO-1} and logP and increasing values of hydration energy, surface area and MR of the molecules enhance the activity of such class of compounds. A plot between the experimental and calculated logRA values (Figure 4.23) for the best fit model shows that the selected descriptors are capable of predicting the activity of such class of molecules.

Table 4.10: IC_{50} values of the selected phytochemicals

Sl No	Name of the Selected Molecules	IC50 value	References
1	+- Catechin (+-C)	25	Si, et al, 2006
2	-- Catechin (--C)	25	"
3	-- Epicatechin (--EC)	25	"
4	+- Epicatechin (+-EC)	25	"
5	Gallocatechin (GC)	6	"
6	-Epigalloacatechin (-EGC)	2.5	"
7	Epigallocatechin gallate (EGCG)	16	"
8	Linoleic acid (LA)	39	Ferrino, et al, 2012
9	Oleic acid (OA)	150	"
10	Stearic acid (SA)	200	Dantas et al, 2002
11	Palmitic acid (PA)	18	Raychowdhury, et al, (1985)
12	Arachidic acid (AA)	80	"
13	Myristic Acid (MA)	0.75	Batovska, et al, (2009)
14	Chlorogenic acid (Ch A)	177	Zaffarano (2003)
15	Caffeic acid (CA)	74.6	"
16	Gallic acid (GA)	76	Akiyama, et al, 2001
17	Myrecitin (Myr)	7.82	D'Souza, et al, 2010
18	Kaempferol (Kmp)	25.6	Alvarez et al, 2008
19	Quercitin (Qur)	59	"
20	Theobromine (Thb)	486.76	Hayallah, et al, 2011
21	Theophylline (Thp)	1000	"

Table 4.11: Table showing the values of the various descriptors for the selected phytochemicals

	E HOMO	E LUMO	E (HOMO-1)	ENL	E (LUMO - HOMO)	μ	η	ω	SA	logP	HE	MR	Pol
+-C	-0 227	-0 034	-0 23	-0 03	0 194	-0 131	0 097	0 088	367 72	2 18	-31 7	29 31	28 65
--C	-0 221	-0 033	-0 224	-0 027	0 188	-0 127	0 094	0 086	354 23	2 18	-30 52	29 31	28 65
--EC	-0 225	-0 037	-0 228	-0 034	0 188	-0 131	0 094	0 092	358 8	2 18	-30 8	29 31	28 65
+-EC	-0 226	-0 03	-0 229	-0 027	0 196	-0 128	0 098	0 084	355 1	2 18	-31 12	29 31	28 65
GC	-0 229	-0 037	-0 232	-0 025	0 192	-0 133	0 096	0 093	374 99	2	-36 98	30 7	29 28
-EGC	-0 225	-0 04	-0 227	-0 026	0 185	-0 132	0 092	0 095	365 36	2	-35 81	30 7	29 28
EGCG	-0 228	-0 069	-0 231	-0 048	0 159	-0 148	0 08	0 138	530 01	5 09	-54 38	40 32	42 78
LA	-0 248	-0 019	-0 25	-0 017	0 229	-0 134	0 115	0 078	715 78	8 14	-4 85	68 78	34 14
OA	-0 248	-0 029	-0 291	-0 019	0 219	-0 139	0 11	0 087	753 84	8 26	-3 68	76 19	34 33
SA	-0 294	-0 018	-0 297	-0 014	0 276	-0 156	0 138	0 088	782 42	8 39	-2 12	83 6	34 53
PA	-0 295	-0 018	-0 299	-0 014	0 277	-0 157	0 139	0 088	710 18	7 59	-2 87	74 4	30 86
MA	-0 296	-0 018	-0 301	-0 014	0 278	-0 157	0 139	0 089	637 94	6 8	-3 62	65 2	27 19
AA	-0 293	-0 018	-0 297	-0 014	0 275	-0 156	0 138	0 088	854 66	9 18	-1 37	92 81	38 2
Ch A	-0 242	-0 089	-0 267	-0 038	0 153	-0 165	0 076	0 179	463 71	3 93	-34 32	46 73	32 45
CA	-0 234	-0 081	-0 263	-0 034	0 154	-0 157	0 077	0 161	327 44	3 73	-23 78	13 25	17 74
GA	-0 254	-0 067	-0 263	-0 046	0 187	-0 161	0 093	0 138	267 76	2 89	-27 94	12 84	14 9
Kmp	-0 231	-0 083	-0 252	-0 048	0 148	-0 157	0 074	0 166	359 95	4 4	-29 14	18 04	27 9
Myr	-0 23	-0 085	-0 25	-0 043	0 145	-0 158	0 073	0 171	368 69	4 05	-39 74	20 81	29 18
Qur	-0 224	-0 091	-0 247	-0 043	0 133	-0 158	0 066	0 188	360 46	4 22	-33 38	19 42	28 54
Thb	-0 242	-0 055	-0 281	-0 022	0 186	-0 148	0 093	0 118	297 75	3 6	-7 9	25 21	17 04
Thp	-0 241	-0 056	-0 282	-0 019	0 185	-0 148	0 092	0 119	299 39	3 6	-7 61	25 21	17 04

Table 4.12: QSAR Model Equations with all 21 compounds

Sl No	QSAR equations	N	R^2	SE	P value		Dominating descriptors	F-Statistics
					X1	X2		
1	161 8697(±460 2312) - 1706 87(±1834 392)EHOMO -16 1262(±6 783673)POL	21	0 2476	210 00	0 364432	0 028738	EHOMO, POL	2 962
2	-298 617(±458 9122) - 3175 51(±1578 597) E(HOMO-1) -14 1429(±6 194507)POL	21	0 3562	194 26	0 059478	0 034795	E(HOMO-1), POL	4 979
3	641 9987(±184 6369) +5 8153(±2 721797)HE -13 6815(±6 13986)POL	21	0 3709	192 02	0 046617	0 038848	HE, POL	5 308
4	567 7488(±192 8863) +0 428667(±0 334457)SA -22 751(±8 898988)POL	21	0 2774	205 81	0 216219	0 019825	SA, POL	3 455
5	556 6707(±188 4215) +33 34416(±21 16554)logP -20 6536(±7 339133)POL	21	0 3070	201 55	0 117942	0 010297	logP, Pol	3 987
6	634 733(±194 4869) +3 785448(±2 305366)MR -23 453(±8 186419)POL	21	0 3141	200 50	0 13258	0 01148	MR, POL	4 123

Table 4.13: The acceptable QSAR Models with 16 compounds

Sl No	QSAR equations	N	R^2	SE	F-Statistics	P value		Dominating descriptors
						X1	X2	
1	-413 252(±350 1259) - 7826 5(±1638 385)EHOMO -45 5155(±6 603061)POL	16	0 8001	126 66	25 32	0 000361	1 1E-05	EHOMO, POL
2	-395 328(±333 3361) - 5945 72(±1191 451)E(HOMO-1) -33 6547(±5 70947)POL	16	0 8070	123 13	27 18	0 000247	5 28E-05	E(HOMO-1), POL
3	1367 629(±176 9006) +10 95794(±2 128728)HE -33 5928(±5 591274)POL	16	0 8148	120 62	28 59	0 000187	4 39E-05	HE, POL
4	1452 092(±171 9112) +1 353974(±0 241144)SA -66 5429(±8 241385)POL	16	0 8357	113 61	33 06	8 41E-05	2 02E-06	SA, POL
5	1526 302(±175 8156) +9 528266(±1 670847)MR -60 9025(±7 394307)POL	16	**0.8393**	**112. 36**	**33.94**	**7.26E-05**	**1.62E-06**	**MR, POL**

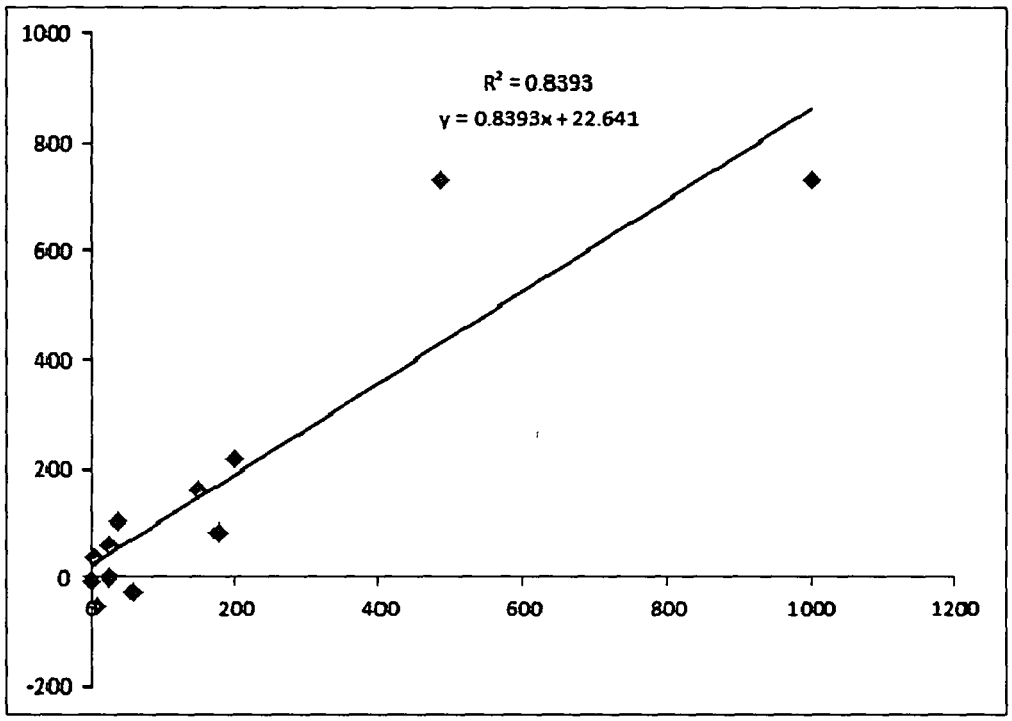


Fig: 4.23: Plot between the experimental and calculated logRA values

The *in silico* results relating to docking, ADME TOX and QSAR studies may be summarized as follows. In case of the docking efficiencies of the molecules with the three different target proteins all the targets were important drug targets, in the context of their importance in synthesis of the mycobacterial cell wall which is critical for the survival of the mycobacterium species. The cell wall synthesis mechanism in all the mycobacterium species is same. The *in silico* results obtained support the antibacterial activity results of the plant extracts in having antimycobacterial activity. Among all the compounds, linoleic acid was found to dock with better efficiency with both Inh A and Glf T2, while oleic acid demonstrated better docking efficiency with mt KasB. GCG from *C. sinensis* (green tea) docked with both Inh A and mt KasB while Luteolin-6c-glucosyl-2"-O-glucoside docked with Glf T2.

Overall, in case of Inh A, linoleic acid demonstrated the best docking score of all the four compounds with the best Rerank score of -79.1535. GCG was found to be the best docked molecule with a Rerank score of -49.0554 with mt Kas B and Luteolin-6c-glucosyl-2"-O-glucoside was found to be the best docked molecule with respect to GlfT2 having a Rerank score of -117.074.

The ADME-Tox study of the four molecules, demonstrated that the molecules with a higher LD_{50} value have less harmful effects on the human body than the molecules showing the least value. Therefore from the results it is evident that linoleic acid is toxic to both mice and rat when administered through IP and IV routes at a much lower value than the other molecules as revealed in the *in silico* studies. GCG was found to be toxic to mice through the oral route while in rats Luteolin-6c-glucosyl-2"-O-glucoside is toxic to mice through the oral route and in mice through SC route of administration. The fatty acid molecules linoleic acid and oleic acid showed better permeability in both MDCK and Caco cell line while GCG and Luteolin-6c-glucosyl-2"-O-glucoside were very poor in permeability. All the molecules exhibited scores within the permissible limits in terms of HERG potassium channel. GCG and Luteolin-6c-glucosyl-2"-O-glucoside were soluble in aqueous solvent while linoleic acid and oleic acid showed poor aqueous solubility due to their lipophilic nature. However, linoleic acid and oleic acid were better absorbed orally as compared to GCG and Luteolin-6c-glucosyl-2"-O-glucoside.

The QSAR studies enabled us to obtain some equations which enable in prediction of the drug like properties of the molecules based on calculations using various molecular descriptors. For this study, 21 molecules were taken which have been selected from *C. sinensis* and *M. ferrea.* The initial equations obtained had a R^2 value of <0.37. However for a equation to be significant the R^2 value should be >0.80. Therefore to obtain better equations, the molecules had to be eliminated one by one to observe in the absence of which molecules a better equation with R^2 value of >0.80 is obtained. Therefore five molecules were eliminated. The final QSAR equations were calculated on the basis of the 16 molecules that were left in the study. The final equations had a R^2 value of >0.80 and were statistically significant.

4.6 Extraction and Characterization of MCC from *Setaria glauca* (P) beauv L and its drug release studies

4.6.1 Flowchart Diagrammatic representation of MCC preparation

The alpha cellulose obtained as a result of alkaline degradation of the raw material was white in colour and fibrous. The alpha cellulose was then acid hydrolysed to obtain the microcrystalline cellulose (MCC). The MCC obtained was also white in colour and crystalline in nature. All the obtained alpha cellulose and MCC were stored in air tight sample containers and stored at room temperature until further use.

Fig 4.24: Flowchart Presentation of preparation of Microcrystalline Cellulose

4.6.2 Scanning Electron Microscopy (SEM)

MCC was extracted from *S. glauca* (L) P Beauv by initial treatment of the dried grass with sodium hydroxide which removed lignin and hemi-cellulose. Subsequent treatment with the bleaching agents helped in further removal of any residual traces of lignin and hemi-cellulose. The α-cellulose obtained was fibrous and white in color. The MCC finally obtained through acid treatment of the α-cellulose was powdery and white. The electron micrograph of the SEM study presented in Figure 4.26, revealed the MCC as individually arranged rods with variable length in the range of 5-30 μm. The thickness of the rods was between 5μm-7μm. Such rod like shapes of the MCCs from different lignocellulosic materials have been reported earlier (Adel, et al, 2011).

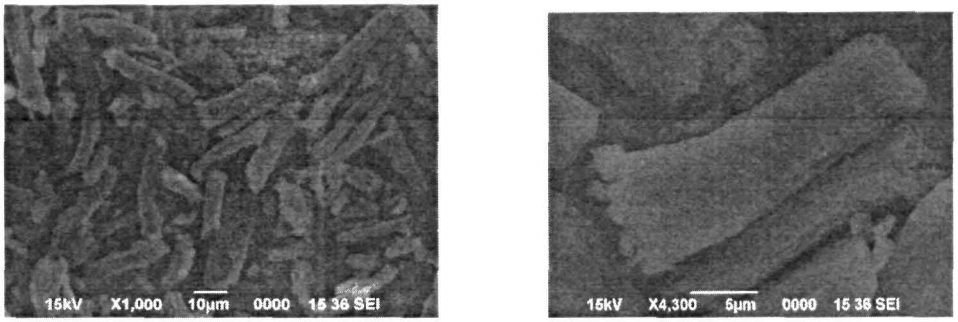


Fig 4.25: Scanning Electron Micrograph of Microcrystalline Cellulose

4.6.3 Thermo Gravimetric Analysis (TGA)

The thermal property of MCC has been investigated as they have wide variety of applications in different industries. Figure 4.27, presents the thermal curves of both the α-cellulose and the MCC showing a two step degradation pattern. The initial loss in weight due to moisture was 15 % in the case of α-cellulose whereas it was 10% in the case of MCC. The percentage of weight loss for α- cellulose in the two step degradation process was 50 % and 25 % while for MCC it was 70 % and 20%. The weight loss in the first step degradation process for α- cellulose began at 270° C while for MCC it was from 286° C. Thus in α- cellulose the peak temperature difference was 16°C for weight loss with reference to that in MCC. The weight loss in the first step degradation was mainly due to the decomposition of the cellulose content (Keshk, et al, 2011). Similarly, the weight loss in the second step began at 353° C for of α - cellulose and at 377.5 °C for MCC. Thus the difference in the peak weight loss temperature was 24.5° C. Since material with higher crystallinity requires higher degradation temperature. The degradation temperature for MCC indicated higher crystallinity in comparison to α-cellulose (Yang, et al, 1996). During industrial processing the temperature rises abruptly, thermal stability of the composite is highly desirable. In the present study, the MCC obtained was found to be highly stable till 286° C and therefore it can be expected that MCC prepared from *Setaria glauca*, (L) P Beauv would remain stable during industrial processing for varied applications. The final ash content of α-cellulose was found to be 7.9% while that of MCC from fodder grass was 0.35% at 876 °C.

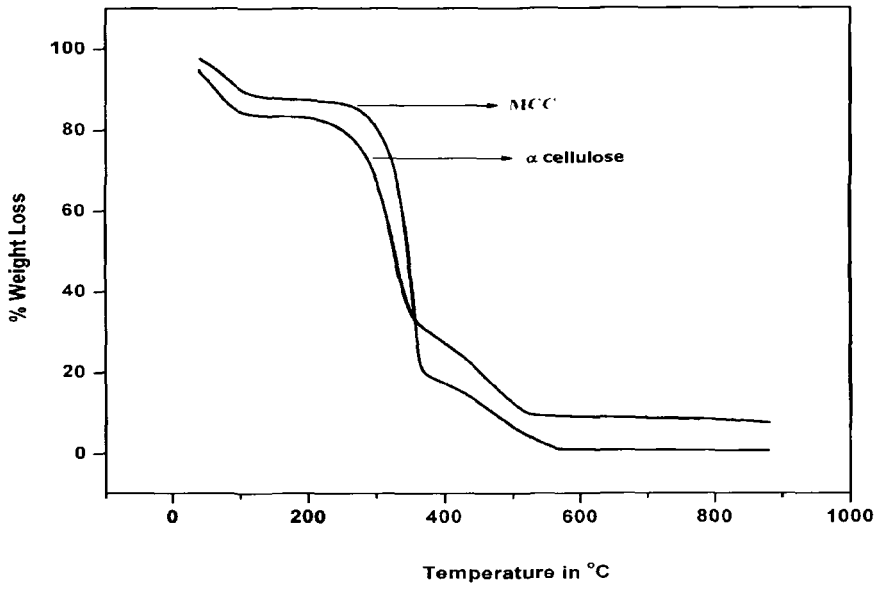


Fig 4.26: TGA thermogram of Microcrystalline Cellulose

4.6.4 Density Scanning Calorimetry (DSC)

The DSC thermogram (Fig 4.28) of the MCC revealed two different endothermic peaks. The two peaks demonstrate the enthalpy of dehydration and decomposition of cellulose. The first endothermic peak from 27°C TO 102°C is due to the evaporation of moisture from the MCC while the second endothermic peak from 230°C to 256°C is associated with the decomposition of the MCC corresponding and further complementing the results already obtained in the TGA thermogram.

The thermal degradation of the cellulose is due to its supramolecular structure. The 1st endothermic peak corresponding to moisture degradation is characteristic of cellulose. The shift of maximum temperature in case of the dehydration process to a higher temperature indicates lesser degree of crystallinity.

The second endothermic peak indicated the breakage of the glucosidic bond which further leads to the decomposition of the material. This degradation corresponds to the thermal degradation of the amorphous domain of the cellulose.

Thus the results obtained helps in establishing the relation between both the TGA and DSC thermograms. Also the crystallinity of the samples can be ascertained

with the first step endothermic reaction.

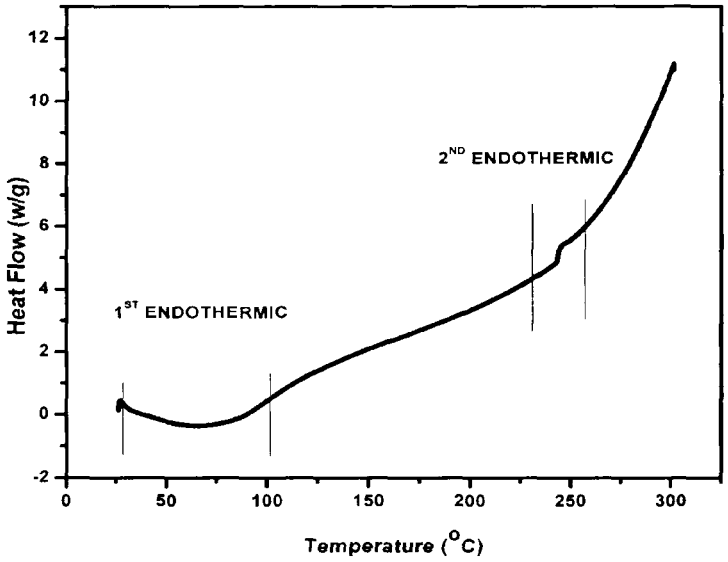


Fig 4.27: DSC thermogram of Microcrystalline Cellulose

4.6.5 FT-IR

The FTIR spectroscopy studies have shown that the different absorption bands obtained from the spectrum can be used to study the ordering pattern of the cellulose polymers (Fig: 4.29). The *ratio of crystallinity* determined by FTIR was calculated by two methods taking into account the following absorbance ratios,

i. Absorbance ratio from 1370 cm^{-1} (A_{1370}) and 2904 cm^{-1} (A_{2904}) bands:

$Cr.R. = A_{1370} / A_{2904}$

ii. Absorbance ratio from 1428 cm^{-1} (A_{1428}) and 900 cm^{-1} (A_{900}) bands:

$Cr.R. = A_{1428} / A_{900}$

The results obtained demonstrate the alteration of crystalline organization through the conversion of ά cellulose to MCC. The spectrum of the ά cellulose was observed with reduction in intensity of the bands which are characteristics of the crystalline domains. The broad band obtained in the 3100-3600 cm^{-1} region due to OH- stretching vibration gives considerable information on the hydrogen bonds. The peaks characteristics of the hydrogen bonds from the MCC spectra are sharper as compared to that of ά cellulose which can be correlated with the simplification of the intra and intermolecular hydrogen bonds. In the case of ά cellulose, the shifting of the

peak to a lower wavenumber value was observed. The presence of a more crystalline order in MCC can be further confirmed by the shift of the spectra from 2901 cm^{-1}(C–H stretching vibration), to higher wavenumber value (2904 cm^{-1}) and by the strong increase in the intensity of this band. The absorption bands from the 1500-447 cm^{-1} region in ά cellulose are reduced in intensity. Also the FTIR absorption band assigned to a symmetric CH2 bending vibration, at 1428 cm^{-1}, increased in MCC. This band is also known as the "crystallinity band". Thus an increase in its intensity demonstrates higher degree of crystallinity. Also the peaks at 1062, 1370 and 1645 corresponding to the C-O-C pyrannose ring skeletal vibration, OH in bending vibration and bending of absorbed water was found to be very strong and with a higher intensity than in the case of ά cellulose. These peaks are important characteristics of MCC. The ratios of crystallinity obtained from FTIR spectra using the two methods above were 87.88 and 93.64 % agreed with the crystallinity indices as established by X-ray diffraction (about 80 %).Thus the FTIR spectra revealed the alteration of the crystalline organization of the MCC through the increase in intensity and even appearance of the crystalline bands.

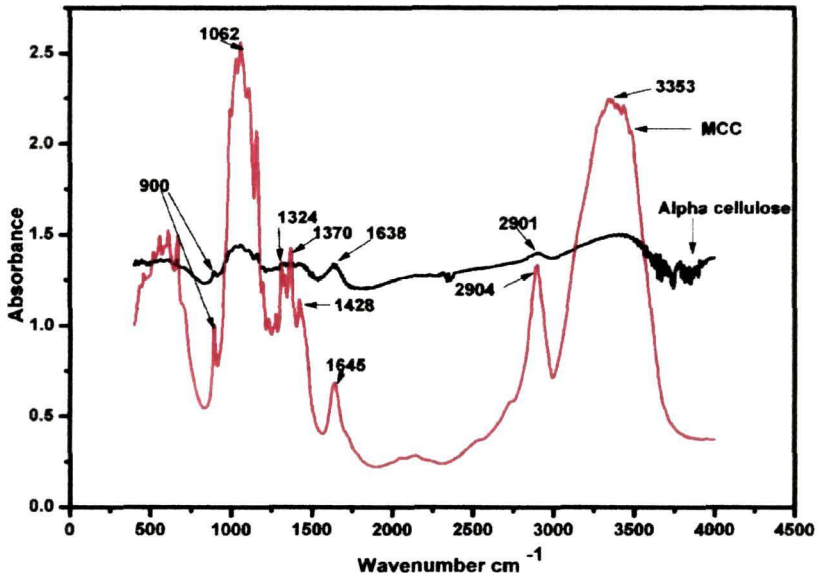


Fig 4.28: FTIR spectrum of Microcrystalline Cellulose

4.6.6 X-Ray Diffractogram (XRD) Analysis

The XRD diffractogram studies of the cellulose samples were carried out to investigate the crystallinity of the MCC obtained from *S. glauca* (L) P Beauv. The diffractograms for MCC in Figure 4.30 showed peaks at 2θ = 22.30° and 20.35° while for ά cellulose a peak was observed at 2θ = 23.00°, which represents a Cellulose I structure. The strong reduction or absence of peaks corresponding to the planes (101), (10$\bar{1}$) and (002) indicates an amorphous structure for the ά cellulose. The diffractogram of the ά cellulose corresponds to the values of the Bragg angle which is a characteristic of α-cellulose. An increase of the crystallinity degree to values of around 80% was found in MCC. This increase in the crystallinity may be attributed to an increase in the intra and intermolecular hydrogen bonding which occurs during the continuous transformation of ά cellulose to MCC. It has been reported that the higher crystallinity in case of MCC was due to its tensile strength and also due to the removal of the hemicellulose and the lignin otherwise present in cellulose. It is pertinent to point out that MCC with high crystallinity can be useful as a better reinforcement agent for preparation of composite materials.

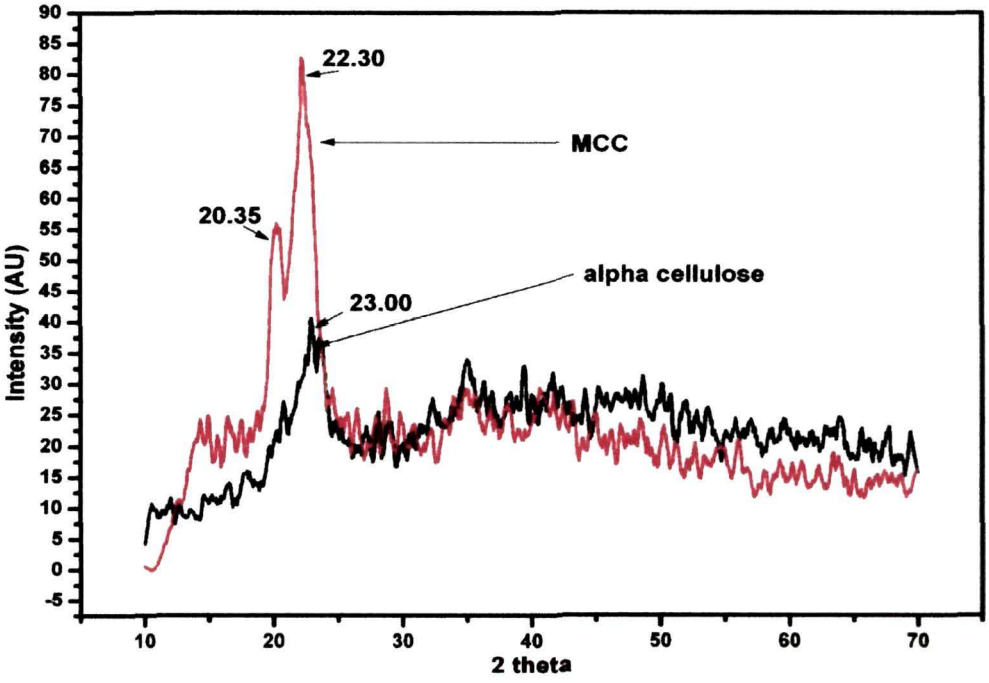


Fig 4.29: XRD diffractogram of Microcrystalline Cellulose (MCC) and α-cellulose

4.6.7 Drug release studies

The drug release studies were performed using the SIF (pH 7.4) in a cell free system which is presented in figure 4.31. Isoniazid was used as the drug of choice. The drug release from the beads has been calculated in terms of isoniazid equivalent. The total time period for the study was 24 hours.

In SIF, the drug release of the beads prepared with only sodium alginate and isoniazid was observed after the 10^{th} minute with release of 0.063 μg of isoniazid equivalent. The rate of drug release gradually increased and the maximum release of 0.11 μg, was observed after 40 mins. The amount of released drug remained the same till the first 4 hours. The release gradually decreased and after 24 hours the release was lowest at 0.024 μg of isoniazid equivalent. When MCC (250 mg) was added to sodium alginate and isoniazid, the drug release was observed after 10 minutes with 0.035 μg of isoniazid equivalent. The release gradually increased and after 3 hours it was found to be 0.521 μg of isoniazid equivalent which was much higher in comparison to the amount released from the beads without the MCC. The release of the drug gradually decreased after 4 hours and only 0.382 μg was released after 24 hours. Thus it was found that with the presence of MCC, the release of the drug increased in the intestinal fluid medium. In order to examine whether the relative proportion of MCC incorporated to the alginate beads have any influence in the release of drug, beads containing 500 mg of MCC were prepared. The release of 0.030 μg of isoniazid equivalent was observed after 10 minutes. Thereafter the rate of release of the drug increased and a maximum of 0.326 μg was found to be released after 3 hours. However the release rate declined and after 24 hours only 0.231 μg of isoniazid equivalent was released. It was found that the release of the drug was lower from the beads containing MCC at 500 mg than from the beads with MCC at 250 mg concentration.

As observed, a high concentration of MCC inhibited the release rate of the drug. However, in both the cases the release of the drug were sustained for a longer period (upto 24 hours) and the release was more than that of the release from the beads without MCC.

The above observation shows that incorporation of MCC in the alginate micro beads enhanced the drug binding property which in turn resulted in greater release of the drug from the beads in comparison from the ones without MCC.

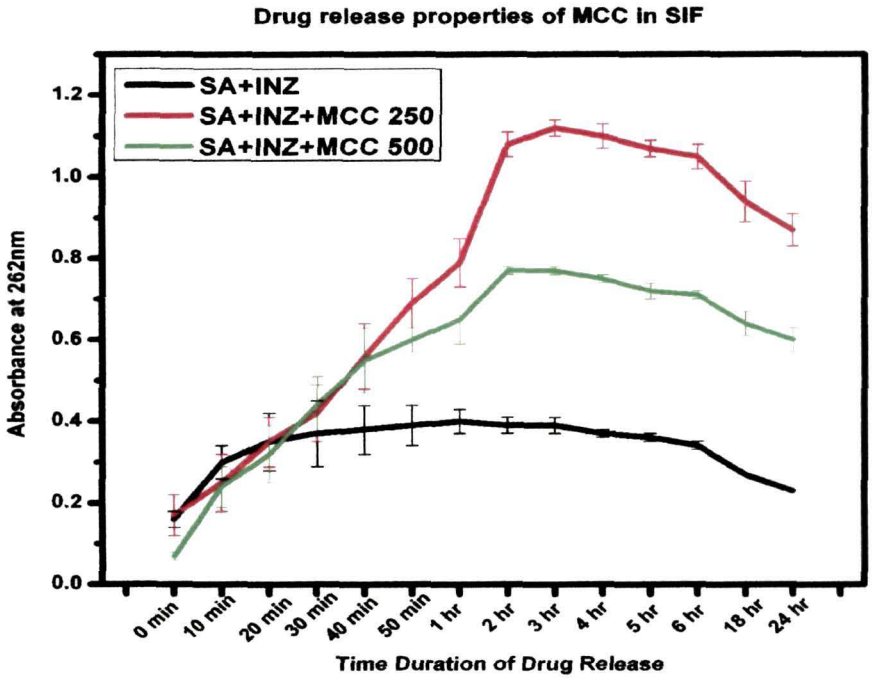


Fig 4.30: Drug release studies of Isoniazid from Alginate and Microcrystalline Cellulose beads

4.6.8 Characterization of the beads

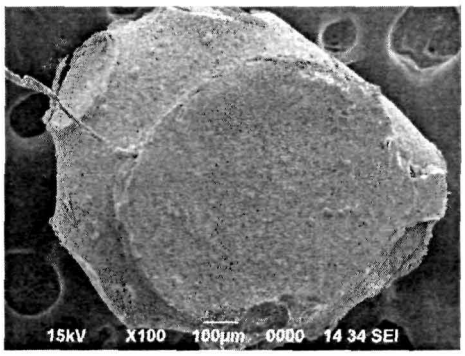
4.6.8.1 SEM

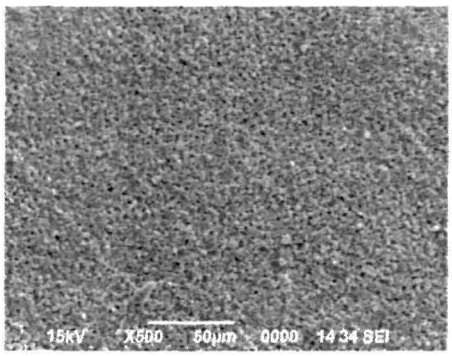
The SEM micrographs of the beads are given in Fig 4.32. It has been observed that the beads are of approximately 1mm in size and are generally round in structure. The sodium alginate beads at a magnification of 500x seemed to be of rectangular crystalline rod of about 3-5um in size. Isoniazid when added to seem to be like crystalline rod like but a little bigger in size. When MCC was added at a concentration of 250 mg, the isoniazid and alginate crystals was observed to be embedded in between the MCC. However when the concentration of the MCC was increased to 500 mg, the isoniazid as well as the alginate crystals were deeply embedded and could not be observed very clearly.

The SEM images explain the phenomenon, of sustained release of isoniazid from the beads. In case of the beads with only alginate, Isoniazid was released within the first 40 minutes, and after 24 hours the release was minimal, however when MCC was incorporated the release rate was enhanced till the 24^{th} hour. Sustained release of

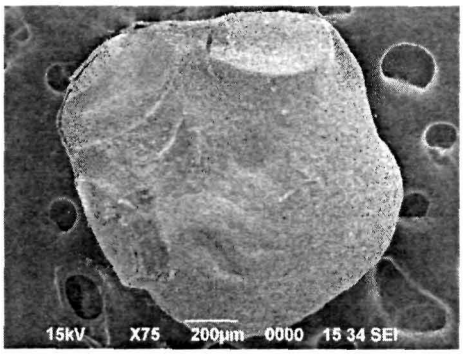
the drug was also observed in case of the beads with 500 mg of MCC, but the release of the drug was less than in case of the beads where MCC was in a a concentration of 250mg. This might be due to the fact that with increase in the concentration of MCC the isoniazid is deeply lodged within the MCC, thus releasing in lesser amount.

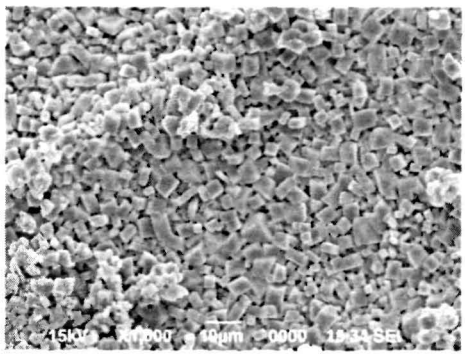
A.



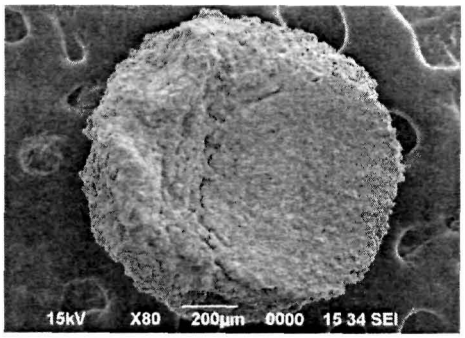


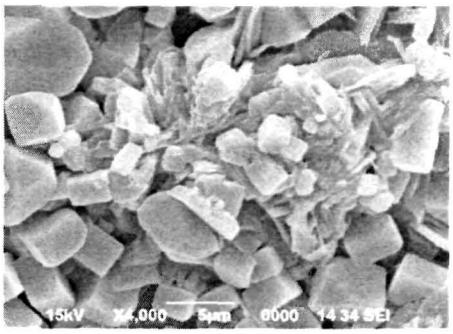
B.





C.





D.

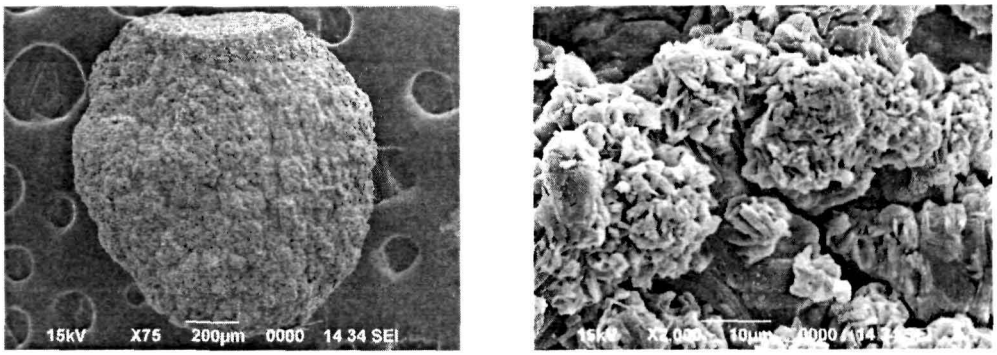


Fig 4.31: Scanning Electron Micrographs of
A) Sodium alginate beads
B) Sodium alginate + Isoniazid beads
C) Sodium alginate + Isoniazid + MCC (250 mg) beads and
D) Sodium alginate + Isoniazid + MCC (500 mg) beads

4.6.8.2 TGA

The thermo gravimetric analysis of the beads was carried out to find out the stability of the beads (Fig: 4.33). In all the four types of different composition of beads, a two step degradation pattern was observed. In the first step degradation pattern the initial weight loss in the sodium alginate beads was observed at 190° C, for the SA+INZ beads it was 202° C and for the beads with MCC (250mg) was 208° C while for MCC (500mg) was 210° C. The initial weight losses for moisture in all the beads are 24.4%, 20.37% and 13.86 % (for both types of beads containing MCC). Thus it is observed that when different materials were added to sodium alginate the weight loss was reduced and the stability of the material increased as can be observed from the peak temperature difference which is 20°C (210° C- 190°C). The second step degradation started from 311°C, 313°C, 338°C and 339°C for the Sodium alginate, Sodium alginate and Isoniazid, Sodium alginate, Isoniazid and Microcrystalline cellulose (250mg) and Sodium alginate, Isoniazid and Microcrystalline cellulose (500mg) beads. The weight loss observed in the second step degradation process was found to be 38.5%, 38.08%, 43.86% and 44.72%.

Thus from the TGA studies, it has been observed that the stability of the beads increased when microcrystalline cellulose was added. Also it is reported that materials with higher crystallinity requires a higher temperature for degradation and also are better as drug release source.

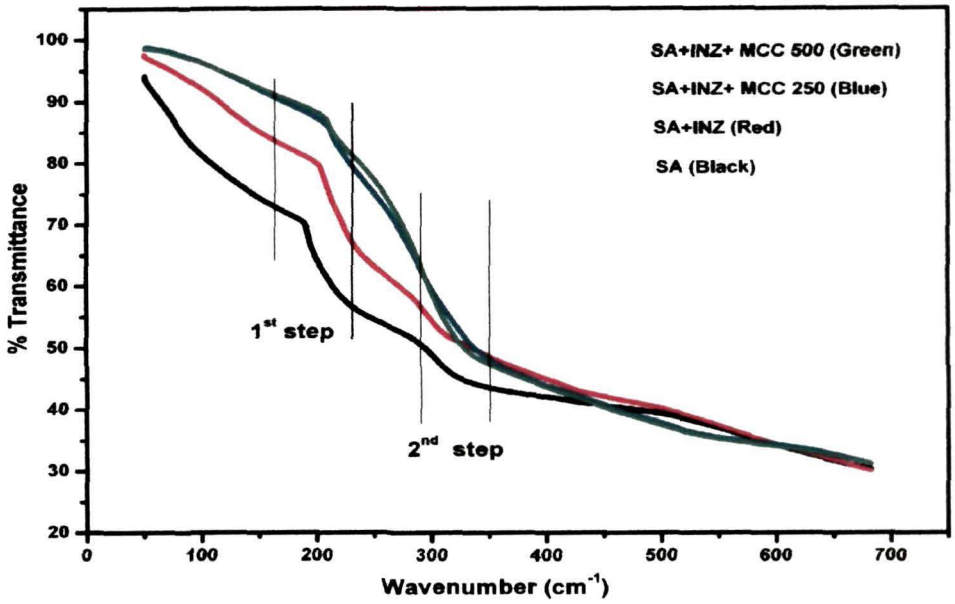


Fig 4.32: TGA of the beads

a) Black colour represents only sodium alginate beads (for reference).
b) Red colour represents Sodium alginate + Isoniazid beads
c) Blue Colour represents Sodium alginate + Isoniazid + MCC (250 mg) beads and d) Green colour represents Sodium alginate + Isoniazid + MCC (500 mg) beads

4.6.8.3 DSC

The DSC thermograms of the three beads are presented in fig 4.34. All the thermogram presents two endothermic peaks. As previously mentioned, the first peak represents the enthalpy of dehydration and the second peak the enthalpy of decomposition. In case of the sodium alginate and isoniazid beads the dehydration rate starts from 85° C to 135° C. The dehydration temperature for the beads in which MCC is present starts from 35° C to 120° C. The peak for decomposition of all the beads starts from 192 °C onwards. The increase of temperature for the first endothermic peak incases of the sodium alginate and isoniazid bead suggest that the beads are amorphous in nature, while the others are crystalline. This also further proves the crystalline nature of the MCC as well as the better drug release efficiency of the beads in which MCC has been incorporated. The decomposition of the beads at 192° C is due to the starting of the degradation of the sodium alginate present in the beads as has been already observed through the TGA analysis of the beads.

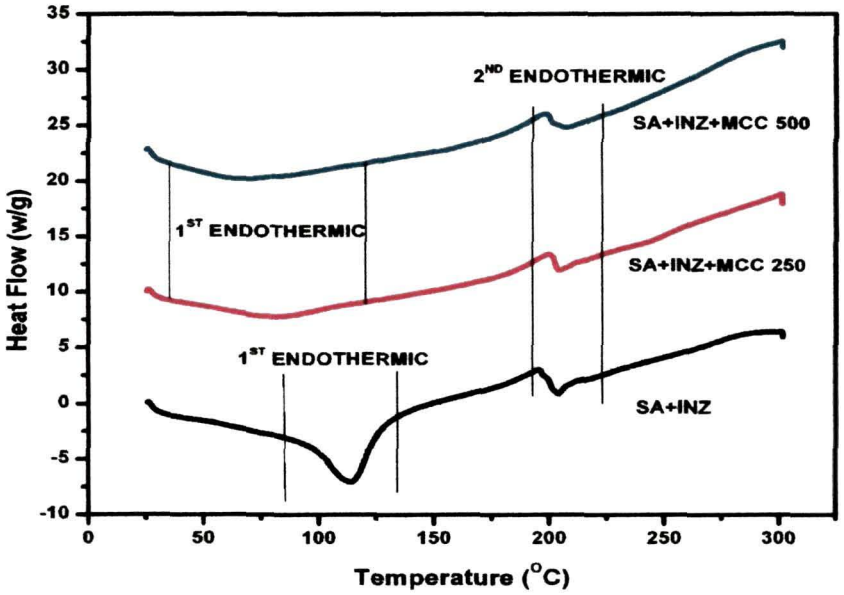


Fig 4.33: DSC thermogram of the beads

a) Black colour represents Sodium alginate + Isoniazid beads
b) Red Colour represents Sodium alginate + Isoniazid + MCC (250 mg) beads and
c) Blue colour represents Sodium alginate + Isoniazid + MCC (500 mg) beads

4.6.8.4 FTIR

The FTIR of the beads was carried out to investigate the changes occurring in the beads when mixed with isoniazid and MCC (Fig: 4.35). In case of only sodium alginate beads a peak was observed at about 1650cm^{-1}, which was subsequently displaced to 1655 cm^{-1} when isoniazid and MCC was added. Similarly the band peak at 1328 in case of sodium alginate and isoniazid beads to be displaced to 1328 cm^{-1} when MCC was added. Finally the broad peak at 1032 cm^{-1} was more prominent when MCC was added to the beads. This peak is not observed in the sodium alginate beads and is very low in the beads with combination of both sodium alginate and isoniazid. The study therefore demonstrates the binding of sodium alginate, isoniazid and MCC in the beads.

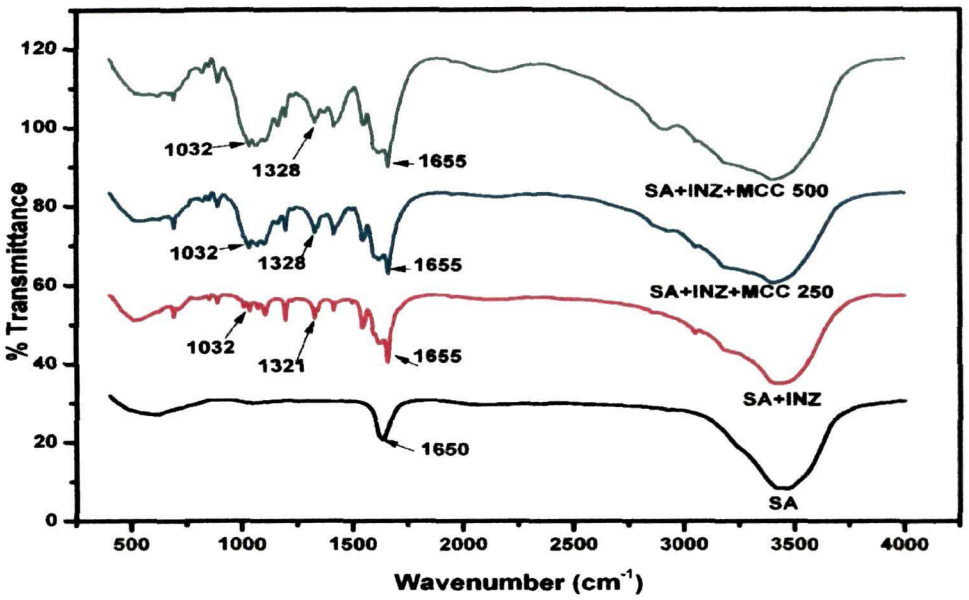


Fig 4.34: FTIR spectra of the beads

4.6.9 Antioxidant activity

The assay for antioxidant activity was carried out to find the radical scavenging properties of MCC. It was found that MCC at a concentration of 50 mg could scavenge DPPH free radicals up to 78.29% while at 10 mg the percentage scavenging was only 62.07%. Sodium alginate beads demonstrated very weak scavenging activities at both concentrations. However the sodium alginate and isoniazid combined beads at concentrations of 10 mg and 50 mg possessed very high DPPH scavenging activity of 94.32 and 94.64%. The sodium alginate, isoniazid and MCC (250 mg) beads presented a slightly higher activity at 94.34 and 94.56 % while the sodium alginate, isoniazid and MCC (500 mg) beads demonstrated 94.34 and 95.16% DPPH free radical scavenging activity. Sodium alginate had the least radical scavenging activity at less than 45% for both the concentrations. Ascorbic acid taken as the positive control demonstrated a scavenging activity of 97.51%. Thus the antioxidant property of MCC could be of advantage when used as a drug delivery vehicle.

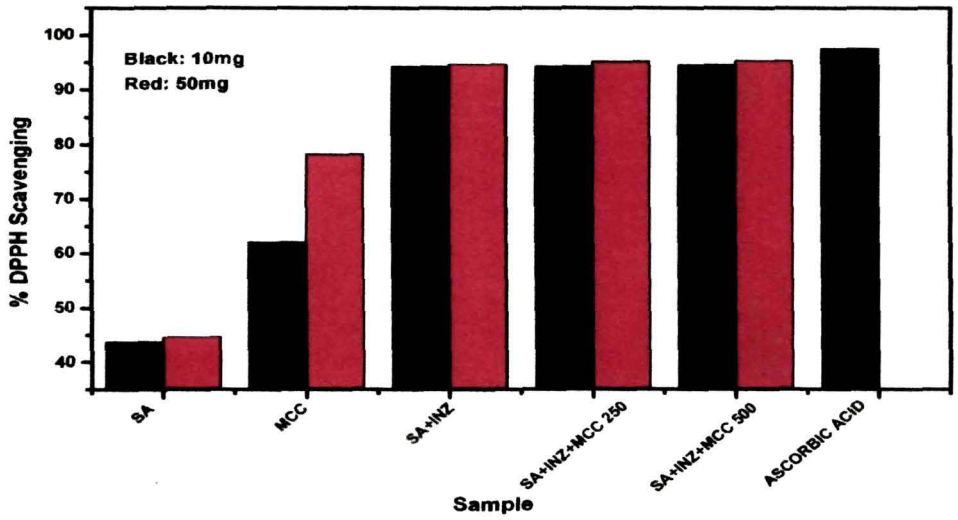


Fig 4.35: DPPH free radical scavenging of the prepared beads and MCC.

a) SA+INZ: Sodium alginate + Isoniazid,
b) SA+INZ+MCC 250: Sodium alginate + Isoniazid+ Microcrystalline cellulose 250 mg,
c) SA+INZ+MCC 500: Sodium alginate + Isoniazid+ Microcrystalline cellulose 500 mg

4.6.10 Haemolytic activity

Whether MCC had any cell damaging affect was investigated in terms of its hemolytic activity in erythrocytes. It was found that MCC did not cause any damage to the blood cells. Its activity was the same as that of the PBS buffer (pH 7.4) even after two hours of incubation which indicated no cytotoxic affect in the cells. This suggests that the MCC has no cell damaging properties and thus is non toxic to the erythrocytes.

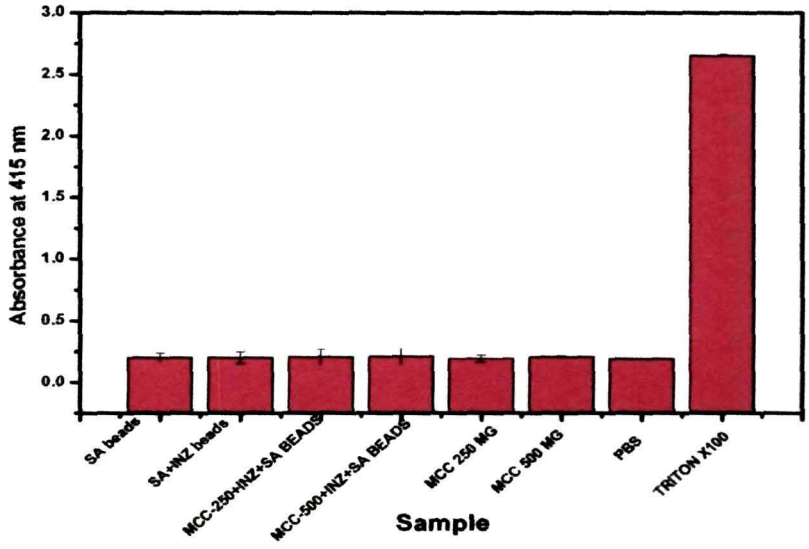


Fig 4.36: Haemolytic assay of Different beads

MCC based drug release studies have been gaining importance in the pharmaceutical industry because of its sustained release properties. In the present work the MCC isolated from *Setaria glauca* L (P) Beauv was obtained by acid hydrolysis method. The MCC was found to be thermally stable and having a higher crystallinity as estimated from the TGA, XRD and FT-IR studies. Also the MCC demonstrated antioxidant property. The MCC also did not have any cell damaging properties against erythrocytes as was evident from the haemolytic activity. The sodium alginate based beads containing MCC and Isoniazid was found to release the drug in a sustained manner. Thus it has been shown that the MCC obtained from a low cost source, *S. glauca,* a grass species can be a valuable addition to the pharma industry on the basis of its thermal, crystalline, antioxidant, non-haemolytic and sustained drug releasing properties.

Thus the promising findings of sustained drug release from MCC-alginate composites without any cytotoxicity could be forwarded as an attractive candidate for further exploration as a drug delivery vehicle.

Chapter V

Conclusion and Future Works

Medicinal plants have been the forefront in the treatment of various diseases among the rural people. Nearly 2/3rd of the world's populations still use medicinal plants for their basic needs. New drugs are still being discovered from natural products. With the emergence of new diseases and of resistant varieties of several diseases the need for discovering new drugs is on the rise. Medicinal plants contains a diversity of phytochemicals belonging to many different classes of compounds and thus offers themselves as a platform for the discovery of new drug molecules. Resistant forms of various diseases like tuberculosis and malaria along with cancer and AIDS require newer drugs to be treated with. Presently all the known drugs for TB have a resistant strain against it, thus making the eradication of TB a far cry. Similar is the case with malaria, in which various resistant strains have emerged to the first line drug artemisinin.

Therefore the need of the hour is renewed research in newer areas of drug discovery of which screening of plants against such diseases is a major thrust area.

One of the general objectives of the work was to explore local traditional knowledge pertaining to treatment of various respiratory diseases prevalent in the region with a specific interest in tuberculosis. Accordingly field visits were undertaken in Tinsukia (Upper Assam) and Sonitpur (Middle Assam) for identifying local plant species which are generally prescribed by traditional healers for various respiratory ailments. Sixty-four plant extracts from twenty one different plant parts of ten different angiosperm plants were screened for their antimycobacterial activity against the model system *M. smegmatis* ATCC 14468. Of the sixty four extracts, 12 of the extracts belonging to five different plant species were found to exhibit antimycobacterial activity. The five plants species are *Camellia sinensis* var assamica (Green tea and Assam orthodox tea variety), *Xanthium strumarium, Messua ferrea, Aegle marmelos* and *Jatropha curcus.* The screening of these medicinal plants was in line with one of the specific objectives of the research work. All the positive plant extracts were assayed for their phenolics and flavonoid contents to ascertain the type of compounds present. Among the phenolic content assayed, *C. sinensis* exhibited

both the highest phenolic and flavonoid contents while *X. strumarium* showed lowest amount of phenolics and *J. curcus* demonstrated lowest amount of flavonoid content. The plant extracts also showed varied antioxidant potential with *M. ferrea* and *C. sinensis* showing the highest antioxidant activity and *X. strumarium* the lowest. None of the plant extract had any cell damaging against erythrocytes as evident from the haemolytic activity test.

Isolation and characterization of the active compounds from the screened plant species was a major objective of the research. Therefore two of the plant extracts, the methanol leaf extract of *C. sinensis* and the hexane seed extract of *M. ferrea* were further selected for finding out the active compounds. Isolation and purification of the *C. sinensis* extracts were carried out using column chromatography and HPLC which led to the isolation of seven different fractions. Two of the fractions were found to demonstrate antimycobacterial activity at MIC of 675 µg/ml and 225 µg/ml. The compounds were identified as Luteolin-6C-glucosyl-2′′-O- glucoside and Gallocatechin gallate. This is the first report of both the compounds showing antimycobacterial activity. From the hexane extract of *M. ferrea*, FAMEs were isolated which were further characterized using GC-MS. This led to the identification of linoleic acid and oleic acid which has also been reported to possess antimycobacterial activity.

Another major objective of the work was to undertake *in silico* approaches for raising hypothesis towards understanding the probable mechanism of drug target interaction relating to the resolved and identified molecules from the selected plant species. Molecular docking studies of the identified compounds confirmed the *in vitro* antimycobacterial activity of the plant extracts. The cell wall synthesizing proteins, Inh A, Glf T2 and mt Kas B were taken as the targets receptors for the ligand compounds. All the molecules docked with efficiency with the above targets. Linoleic acid, Gallocatechin gallate and Luteolin-6c-glucosyl-2"-O-glucoside were found to be the best docked among all the four molecules with respect to Inh A, mt Kas B and Glf T2. ADME-TOX studies revealed the pharmacological and toxicity properties of the molecules. It was found that the compounds were tolerable to higher doses without any toxic activity. Though permeability in MDCK and Caco cell lines were observed only in linoleic and oleic acid while the rate of permeability was poor in case of Gallocatechin gallate and Luteolin-6c-glucosyl-2"-O-glucoside. However the

aqueous solubility in Gallocatechin gallate and Luteolin-6c-glucosyl-2"-O-glucoside was much better than linoleic and oleic acid. The QSAR studies led to the development of five different equations of which the best equation was observed in the case of the descriptors Molecular Refractivity and Polarizability with a R^2 value of >0.83.

The efficacy of a drug depends on its effective release without which the disease may aggravate further with serious biomedical implications. For example the first line drugs of tuberculosis are prescribed as multiple oral administrations to the TB patients daily for atleast 6-8 months. This makes patient compliance of the prescribed drugs difficult. Therefore in line with a major objective of the thesis investigations were made for forwarding effective delivery mechanism of isoniazid which is one of the major first line drugs. The exploration centered around developing a microcrystalline cellulose based system for which a commonly available fast growing grass species *Setaria glauca* L (P) Beauv was explored. Microcrystalline cellulose (MCC) was extracted from *Setaria glauca* L (P) Beauv, an important fodder plant species. The MCC was further characterized using SEM, FTIR, TGA, DSC and XRD. The MCC was combined with isoniazid and sodium alginate to prepare beads of approximately 1μ in size. The beads were found to release the drug isoniazid in a sustained release manner over a period of twenty four hours. The microcrystalline cellulose was found to be highly stable and having a high crystallinity. Also the MCC was found to possess antioxidant activity. No cell toxicity towards erythrocytes was observed as seen from the haemolytic activity assay. Thus the MCC can be put forward as a potential excipient in the pharma industry for treatment of tuberculosis and other diseases.

Future Works

- Screening of more plants for antimycobacterial activity has to be carried out.
- Identification of molecules from the other active plants also needs to be carried out.
- Antimycobacterial activity screening to be carried out in TB strains and infected animal models to validate the potential of the plant molecules.
- Computational studies including ADME-TOX, Molecular Docking and QSAR studies to be carried out to identify more phytochemicals to find out hit-to-lead molecules.
- Different species of plants may be screened for extraction of microcrystalline cellulose.

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Appendices

Middlebrook 7H9 Broth Base

Composition**

Ingredients	Gms / Litre
Ammonium sulphate	0.500
Disodium phosphate	2.500
Monopotassium phosphate	1.000
Sodium citrate	0.100
Magnesium sulphate	0.050
Calcium chloride	0.0005
Zinc sulphate	0.001
Copper sulphate	0.001
Ferric ammonium citrate	0.040
L-Glutamic acid	0.500
Pyridoxine	0.001
Biotin	0.0005
Final pH (at 25°C)	6.6±0.2

**Formula adjusted, standardized to suit performance parameters

MHA media

Composition**

Ingredients	Gms / Litre
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Agar	17.000
Final pH (at 25°C)	7.3±0.1

**Formula adjusted, standardized to suit performance parameters.

MHB

Composition**

Ingredients	Gms / Litre
Beef, infusion from	300.000
Casein acid hydrolysate	17.500
Starch	1.500
Final pH (at 25°C)	7.4±0.2

**Formula adjusted, standardized to suit performance parameters.

PBS (1X solution)

Ingredients	Gms/Litre
NaCl	0.8
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

Publications

Original Research Publications

1. **Ranjan D Kalita**, Yutika Nath, Martins E Ochubiojo, Alak Kumar Buragohain, 2013, Extraction and Characterization of Microcrystalline Cellulose from fodder grass; Setaria glauca (L) P. Beauv, and its potential as a drug delivery vehicle for isoniazid, a first line antituberculosis drug, Colloids and Surfaces B: Biointerfaces, 2013, 108, 85-89. (I.F. 3.456)
2. Emeje M., **Kalita R.**, Isimi C., Buragohain A., Kunle O., Ofoefule S., Synthesis, physicochemical characterization, and functional properties of an esterified starch from an underutilized source in Nigeria, African Journal of Food, Agriculture, Nutrition and Development (AJFAND Online), 2012, Volume 12(7).
3. Harekrishna Deka, Niranjan Karak, **Ranjan Dutta Kalita**, Alak Kumar Buragohain, Biocompatible hyperbranched polyurethane/multi-walled carbon nanotube composites as shape memory materials, Carbon, 2010, Volume 48(7) :2013-2022. (I.F. 5.378)
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1. Gazi A Ahmed, Alak K Buragohain, Partha P Nath, Ankur Gogoi, Nirmal Mazumdar, **Ranjan D Kalita,** Amarjyoti Choudhury. FTIR and luminescence studies of nanoporous diatom frustules Photonics and Nanostructures, Narosa Publications, New Delhi, 2010, 83-89 (In Press)

Presentations in Conference/ Seminars

1. **Ranjan Dutta Kalita** and Alak Kumar Buragohain, Characterization and *in silico* studies of a potent antimycobacterial agent from *Messua ferrea* (L) seed, accepted for presentation at the The 5th Congress of European Microbiologists (FEMS), Leipzig, Germany, July 21-25, 2013.

2. **Ranjan Dutta Kalita**, Nilakshi Barua, Lipika Aidew and Alak Kumar Buragohain, Evaluation of the antimycobacterial potential of *Xanthium strumarium*, **National** Seminar on Medicinal Plant and Microbe Diversity and their Pharmaceuticals, Tezpur University, Tezpur, Assam, India, December 19- 21, 2010.

3. **Ranjan Dutta Kalita**, Nilakshi Barua, Lipika Aidew and Alak Kumar Buragohain, "Antimycobacterial activity of the methanol extract of latex from *Jatropha curcus*", Indian National Science Academy Platinum Jubilee **International** Symposium on Research in Molecular Medicine Based on Natural Resources and Traditional Knowledge, National Chemical Laboratory, Pune, India, November 21-23, 2009.

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Extraction and characterization of microcrystalline cellulose from fodder grass; *Setaria glauca* (L) P. Beauv, and its potential as a drug delivery vehicle for isoniazid, a first line antituberculosis drug

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ABSTRACT

Microcrystalline cellulose (MCC) is generally produced through acid hydrolysis of woody plants and agro sources. MCC synthesized from a common wild grass *Setaria glauca* (L) P. Beauv was characterized to explore the possibility of application in pharmaceutical industry especially as a drug delivery vehicle. The SEM, TGA, XRD and FTIR investigations of the prepared MCC reveal that the 5–30 μm long, non aggregated MCC rods have high crystallinity index of 80% and were stable at 286 °C. The preliminary investigation of the MCC incorporated micro beads containing isoniazid, one of the first line drugs for treatment of tuberculosis was carried out in the simulated intestinal fluid (SIF). The MCC incorporated micro beads with isoniazid drug load showed sustained release upto 24 h with release of 0.521 μg of isoniazid equivalent drug in the SIF system. No cytotoxicity of the MCC was observed in the haemolytic assay. The MCC also showed good antioxidant activity. Thus, the study reveals that the MCC can be prepared from an inexpensive and abundant grass species. The MCC have properties advantageous for application in the pharmaceutical industry and may be explored further in drug delivery research.

1. Introduction

Microcrystalline cellulose (MCC) is increasingly considered as an important derivative from cellulose in the context of its potential applications in pharmaceutical, food and cosmetic industries. Attributes like strength, fibrous nature, stiffness, crystallinity, lightness, biodegradability, water insolubility and renewalibility make MCC attractive for applications in diverse industrial fields [1]. Generally, MCC is obtained from wood pulp and purified cotton linters. There are however reports of preparation of MCC from various other plant sources like groundnut shell, groundnut husk and rice husk [2–4], cereal straw [5], corncob and baggase [6], Indian bamboo [7], *Lufa cylindrica* [8], rice straw [9] and jute [10]. The properties of MCC obtained from different sources vary with respect to porosity, crystallinity, surface area, morphology *etc.* [11].

The importance of MCC as a versatile excipient in the drug research has been highlighted [12]. MCC extends advantages as a water retainer, suspension stabilizer, thickening agent and in the reinforcement of drug tablets. It is used for the direct compression of tablets because of its tablet binding, low chemical reactivity and high compactability [11]. Application of MCCs for slow release of drugs from capsules and tablets is opening up new trends of research in exploring MCCs as drug delivery vehicles. They are also used as a diluent and disintegrating agent for release of oral solid dosages. MCCs possess the characteristics and advantages for use as a constituent of oral dosage forms [12,13]. MCC is considered as one of the best and most useful fillers because of its excellent compactibility at low pressures, superior disintegration properties and also high dilution potential. The chemical inertness of MCC and its compatibility with most drugs make MCC a highly sought after pharmaceutical agent [14].

The efficacy of a drug depends to a great extent on its effective release without which the disease may aggravate further with serious biomedical implications. An example of this is witnessed in the case of the first line drugs of tuberculosis. Tuberculosis (TB) patient is prescribed multiple oral administrations of the first line drugs (*e.g.*, isoniazid) daily for at least six to eight months. This makes patient compliance of the prescribed drugs difficult and therefore precludes effective treatment of the disease. There is urgency therefore in not only discovering new drug molecules but also for developing an effective drug delivery system. Such a system should have a longer release time to facilitate the release of the drug over

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a longer period of time so that multiple daily oral administration of the drug is not required [15]. MCC based drug delivery system has been proposed with its implicit advantages of targeted delivery in the pathogen infected cells enhancing its therapeutic value and sustained release minimizing frequent drug dosing issue [16].

Setaria glauca (L) P. Beauv is a naturally growing abundant annual grass species which is abundantly found in Assam, India. The grass is fast growing, attaining a height of about 1 m and is extensively used as fodder. There is no report of the cellulose derived from this species being subjected to value addition for diverse applications especially in the pharmaceutical industry.

We report the preparation of MCC from the fodder grass, *S. glauca* (L) P. Beauv, a common grass species. The MCC so derived has been characterized to determine its thermal, crystalline, morphological, antioxidant and cytotoxic properties. The release of isoniazid as a first line drug used for the treatment of tuberculosis from MCC incorporated alginate micro beads was also studied.

2. Materials and methods

2.1. Preparation of microcrystalline cellulose (MCC)

The protocol for extraction of MCC was that of Bhattacharya et al. [17] with some modifications. The grass samples were first cut into small pieces and then washed thoroughly with water before drying them at room temperature. The dried samples were treated with NaOH (4%) and boiled at 80 °C in water bath for 4 h. The samples were washed with water until neutral pH and then bleached with a solution of sodium hypochlorite and hydrogen peroxide in 1:1 ratio for 40 min at 100 °C. The samples were rewashed with water until neutral pH yielding the α-cellulose fibers. For extraction of MCC, 10 g of the α-cellulose was acid hydrolyzed using HCl (2.5 N) at 100 °C for 30 min. The extracted MCC was washed thoroughly with water for complete removal of HCl.

2.2. Characterization of the MCC

2.2.1. Scanning electron microscopy (SEM)

MCC was submitted to SEM for morphological study. A small quantity of the MCC was fixed to a carbon stud. A platinum coating was laid on the stud with a JEOL JFC 1600 auto fine coater. The SEM study was carried out in a JEOL 6390 instrument.

2.2.2. Thermal gravimetric analysis (TGA)

TGA studies were carried out using a Perkin Elmer STA 6000 instrument. In this study a small amount of the sample was placed in a pan inside the instrument in a controlled environment. The TGA spectra were recorded in an ambient nitrogen atmosphere at a heating rate of 20 °C/min. The TGA was carried out to determine thermal stability of the MCC and also to compare the degradation pattern of the α-cellulose and the MCC.

2.2.3. Fourier transform infra red (FTIR)

FT-IR study was conducted to determine the functional groups present in the MCC. The MCC was mixed with potassium bromide (KBr) and prepared as tablet discs using a hydraulic press with 10 tons pressure. The discs were then scanned in the spectral range of 400–4000 cm^{-1} to find out the percentage transmittance (%T) of the MCC. The study was done in a Perkin Elmer spectrum 100 series instrument.

2.2.4. X-ray diffraction (XRD)

XRD study was carried out to find out the crystallinity of the compound. RIGAKU miniflex instrument using Ni filtered Cu Kλ radiation (λ = 1.5406 Å) was employed for the study. The operating voltage and current was 30 kV and 15 mA respectively. Crystallinity index has been calculated using various methods as described previously [22].

2.3. In vitro drug release studies

The drug release profiles of MCC in two different concentrations from sodium alginate beads with incorporated isoniazid, a first line anti-tuberculosis drug were studied. Beads containing only sodium alginate and isoniazid were taken as control. The *in vitro* assay was carried out in simulated intestinal fluid (SIF) by modifying the protocol of Pandey and Khuller [18]. MCC (250 mg, 500 mg) and isoniazid (250 mg) were added separately to 5% of sodium alginate prepared in a beaker and stirred with a magnetic stirrer until complete mixing took place. The resulting gelatinous solution was taken in a syringe and was dropped down in a 0.2 M calcium chloride solution leading to formation of small beads. The beads were filtered through Whatman filter paper No. 1 and allowed to dry in a hot air oven at 37 °C. The average size of the beads was 1 mm approximately. SIF was prepared using the following procedure. In 400 ml of distilled water 4 g of sodium chloride (NaCl), 100 mg potassium chloride (KCl), 720 mg disodium hydrogen phosphate (Na_2HPO_4) and 120 mg of potassium dihydrogen phosphate (KH_2PO_4) were added. The final volume was made upto 500 ml and the pH was maintained at 7.4. The absorbance was taken at 262 nm for SIF. The release study was performed by placing the beads in dialysis tube (Sigma) in a beaker containing the buffer solution. Constant stirring of 100 rpm was maintained for the release studies for 24 h.

2.4. Antioxidant assay

Antioxidant assay was performed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with slight modification of the protocol of Sharififar et al. [19]. DPPH solution was prepared by adding 4 mg of DPPH in 100 ml of methanol. MCC at two different concentrations of 10 and 50 mg were considered for the assay. The tubes were incubated at room temperature in the dark for 40 min after adding 3 ml of the DPPH solution to each tube. The absorbance was taken at 517 nm. The test was done in triplicates with methanol as blank. The percentage of radical scavenging (*I*%) was measured following the relation, $I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$, where, A_{blank} is the absorbance of the control reaction containing all the reagents except the beads. A_{sample} is the absorbance of the samples. The concentration of the MCC that caused 50% inhibition was calculated from the graph plotting inhibition percentage against the sample concentration. Ascorbic acid was taken as the positive control. All the tests were carried out in triplicate.

2.5. Haemolysis test

Haemolysis test was carried out according to Nair et al. [20]. Mammalian blood sample from goat was collected in a vial containing 4% trisodium citrate. It was centrifuged at 750 × *g*. The supernatant was discarded and the precipitate containing the erythrocytes was washed with PBS (pH 7.4) twice at 750 × *g* for 10 min. 48.5 ml of PBS was added to 1.5 ml of the erythrocyte suspension and was equally divided in 2 ml tubes. Beads containing two different composition of MCC and weighing 10 mg each were added to tubes containing 2 ml of the erythrocyte suspension and incubated for 2 h at 37 °C. After the incubation, the tubes were centrifuged at 750 × *g* for 10 min. Now, 200 μl of the supernatant was collected in a fresh tube and to it 2.8 ml of PBS was added. The absorbance was taken at 415 nm. PBS was taken as the negative control and TritonX 100 was taken as the positive control.

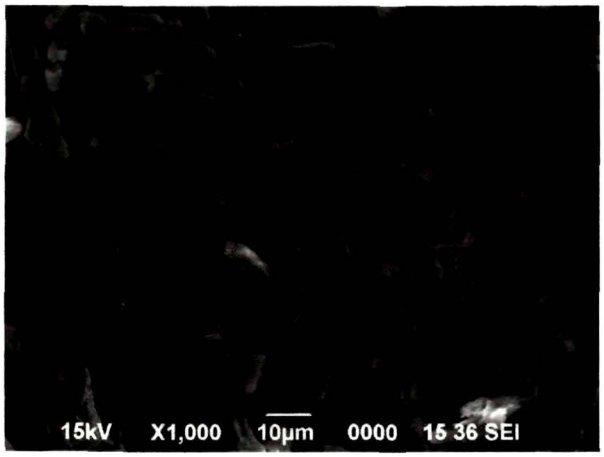

Fig. 1. SEM micrograph of MCC.

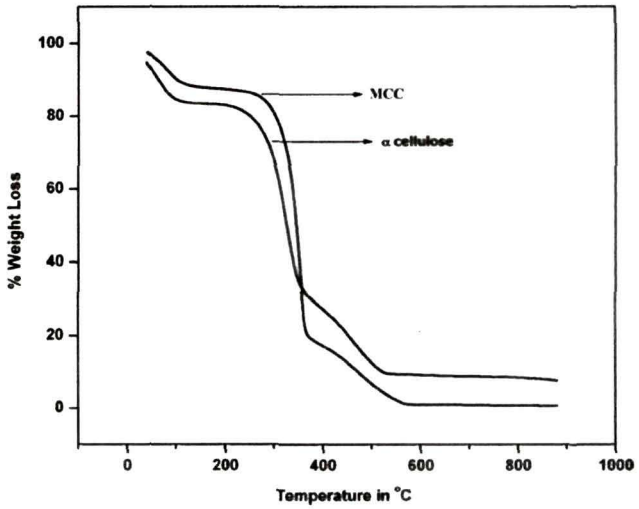

Fig. 2. TGA thermogram of MCC and α cellulose.

2.6. Chemicals and reagents

All the chemicals were purchased from Merck (Germany and India). Dialysis tubes were purchased from Sigma.

2.7. Statistical analysis

Statistical analysis were carried out with Origin Pro 8 Software and Microsoft Excel Software (1997–2003)

3. Results and discussions

MCC was extracted from *S. glauca* (L) P. Beauv by initial treatment of the dried grass with sodium hydroxide which removed lignin and hemi-cellulose. Subsequent treatment with the bleaching agents helped in further removal of any residual traces of lignin and hemi-cellulose [17]. The α-cellulose obtained was fibrous and white in color. The MCC finally obtained through acid treatment of the α-cellulose was powdery and white. The electron micrograph of the SEM study presented in Fig. 1 revealed the MCC as individually arranged rods with variable length in the range of 5–30 μm. The thickness of the rods was between 5 μm and 7 μm. Such rod like shapes of the MCCs from different lignocellulosic materials have been reported earlier [21].

The thermal property of MCC has been investigated as they have wide variety of applications in different industries. Fig. 2 presents the thermal curves of both the α-cellulose and the MCC showing a two step degradation pattern. The initial loss in weight due to moisture was 15% in the case of α-cellulose whereas it was 10% in the case of MCC. The percentage of weight loss for α-cellulose in the two step degradation process was 50% and 25% while for MCC it was 70% and 20%. The weight loss in the one step degradation process for α-cellulose began at 270 °C while for MCC it was from 286 °C. Thus in α-cellulose the peak temperature difference was 16 °C for weight loss with reference to that in MCC. The weight loss in the first step degradation was mainly due to the decomposition of the cellulose content [22]. Similarly, the weight loss in the second step began at 353 °C for of α-cellulose and at 377.5 °C for MCC. Thus the difference in the peak weight loss temperature was 24.5 °C. Since material with higher crystallinity requires higher degradation temperature [22,23]. The degradation temperature for MCC indicated higher crystallinity in comparison to α-cellulose. Since during industrial processing the temperature rises abruptly, thermal stability of the composite is highly desirable [10]. In the present study, the MCC obtained was found to be highly stable till 286 °C and therefore it can be expected that MCC prepared from *S. glauca*, (L) P. Beauv would remain stable during industrial processing for varied applications. The final ash content of α-cellulose was found to be 7.9% while that of MCC from fodder grass was 0.35% at 876 °C (Fig. 3).

The FTIR spectroscopy studies have shown that the different absorption bands obtained from the spectrum can be used to study the ordering pattern of the cellulose polymers. The *ratio of crystallinity* determined by FTIR was calculated by two methods taking into account the following absorbance ratios,

i. Absorbance ratio from 1370 cm^{-1} (A_{1370}) and 2904 cm^{-1} (A_{2904}) bands:
   $Cr.R. = A_{1370}/A_{2904}$
ii. Absorbance ratio from 1428 cm^{-1} (A_{1428}) and 900 cm^{-1} (A_{900}) bands:
   $Cr.R. = A_{1428}/A_{900}$

The results obtained demonstrate the alteration of crystalline organization through the conversion of α-cellulose to MCC. The spectrum of the α-cellulose was observed with reduction in intensity of the bands which are characteristics of the crystalline domains. The broad band obtained in the 3100–3600 cm^{-1} region due to OH– stretching vibration gives considerable information on the hydrogen bonds. The peaks characteristics of the hydrogen

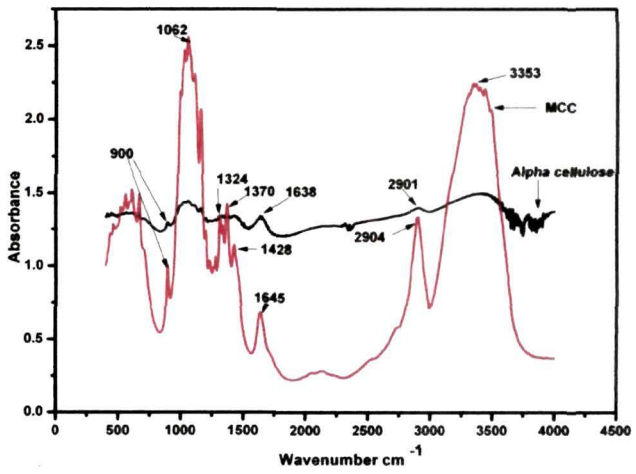

Fig. 3. FTIR spectra of MCC and α cellulose.

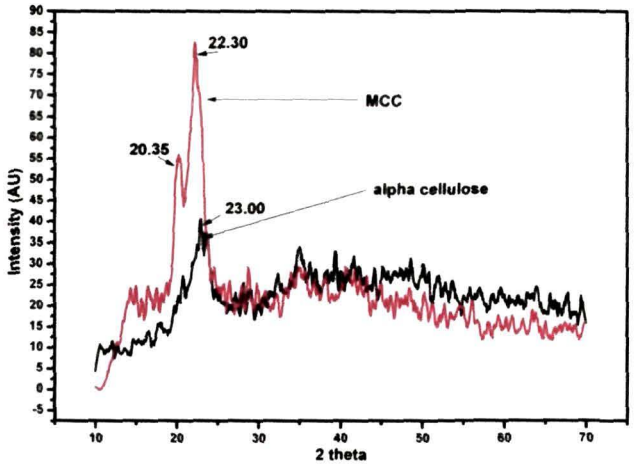

Fig. 4. X-Ray Diffractogram of MCC and α cellulose.

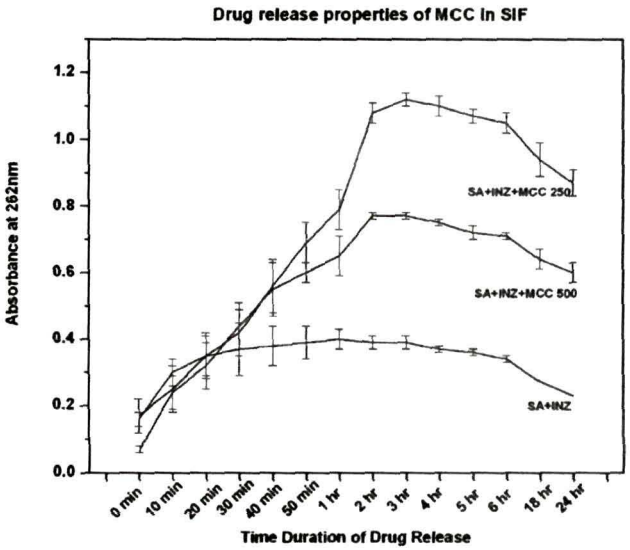

Fig. 5. Drug release property of MCC.

bonds from the MCC spectra are sharper as compared to that of α-cellulose which can be correlated with the simplification of the intra and intermolecular hydrogen bonds. In the case of α-cellulose, the shifting of the peak to a lower wavenumber value was observed. The presence of a more crystalline order in MCC can be further confirmed by the shift of the spectra from 2901 cm^{-1}(C—H stretching vibration), to higher wavenumber value (2904 cm^{-1}) and by the strong increase in the intensity of this band. The absorption bands from the 1500 cm^{-1} to 447 cm^{-1} region in α-cellulose are reduced in intensity. Also the FTIR absorption band assigned to a symmetric CH2 bending vibration, at 1428 cm^{-1}, increased in MCC. This band is also known as the "crystallinity band". Thus an increase in its intensity demonstrates higher degree of crystallinity. Also the peaks at 1062, 1370 and 1645 corresponding to the C—O—C pyrannose ring skeletal vibration, OH in bending vibration and bending of absorbed water was found to be very strong and with a higher intensity than in the case of α-cellulose. These peaks are important characteristics of MCC [22,24,25]. The ratios of crystallinity obtained from FTIR spectra using the two methods above were 87.88 and 93.64% agreed with the crystallinity indices as established by X-ray diffraction (about 80%) [27,28]. Thus the FTIR spectra revealed the alteration of the crystalline organization of the MCC through the increase in intensity and even appearance of the crystalline bands.

The XRD diffractogram studies of the cellulose samples were carried out to investigate the crystallinity of the MCC obtained from *S. glauca* (L) P. Beauv. The diffractograms for MCC in Fig. 4 showed peaks at $2\theta = 22.30°$ and 20.35° while for α-cellulose a peak was observed at $2\theta = 23.00°$, which represents a Cellulose I structure [10]. The strong reduction or absence of peaks corresponding to the planes (1 0 1), (1 0 −1) and (0 0 2) indicates an amorphous structure for the α-cellulose. The diffractogram of the α-cellulose corresponds to the values of the Bragg angle which is a characteristic of α-cellulose. An increase of the crystallinity degree to values of around 80% was found in MCC. This increase in the crystallinity may be attributed to an increase in the intra and intermolecular hydrogen bonding which occurs during the continuous transformation of α-cellulose to MCC [27]. It has been reported that the higher crystallinity in case of MCC was due to its tensile strength [10] and also due to the removal of the hemicellulose and the lignin otherwise present in cellulose. It is pertinent to point out that MCC with high crystallinity can be useful as a better reinforcement agent for preparation of composite materials [26].

The drug release studies were performed using the SIF (pH 7.4) in a cell free system which is presented in Fig. 5. Isoniazid was used as the drug of choice. The drug release from the beads has been calculated in terms of isoniazid equivalent. The total time period for the study was 24 h (Fig. 6).

In SIF, the drug release of the beads prepared with only sodium alginate and isoniazid was observed after the 10th minute with release of 0.063 μg of isoniazid equivalent. The rate of drug release gradually increased and the maximum release of 0.11 μg, was observed after 40 min. The amount of released drug remained the same till the first 4 h. The release gradually decreased and after 24 h the release was lowest at 0.024 μg of isoniazid equivalent. When MCC (250 mg) was added to sodium alginate and isoniazid (Figs. 7 and 8), the drug release was observed after 10 min with 0.035 μg of isoniazid equivalent. The release gradually increased and after 3 h it was found to be 0.521 μg of isoniazid equivalent which was much higher in comparison to the amount released from the beads without the MCC. The release of the drug gradually decreased after 4 h and only 0.382 μg was released after 24 h. Thus it was found that with the presence of MCC, the release of the drug increased in the intestinal fluid medium. In order to examine whether the relative proportion of MCC incorporated to the alginate beads have any influence in the release of drug, beads containing 500 mg of MCC were prepared. The release of 0.030 μg of

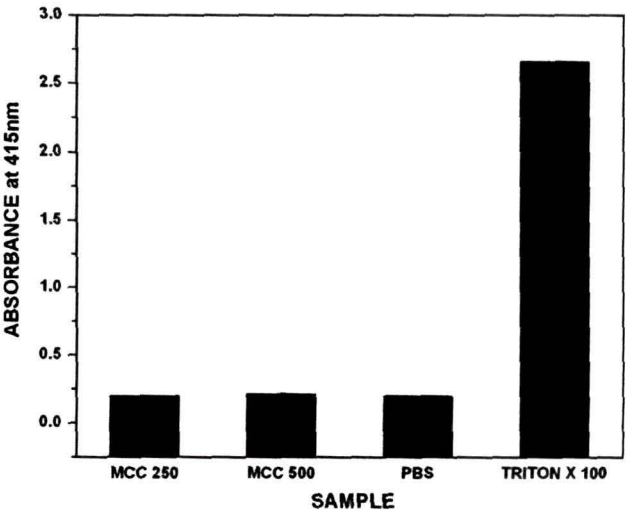

Fig. 6. Haemolytic activity of MCC.

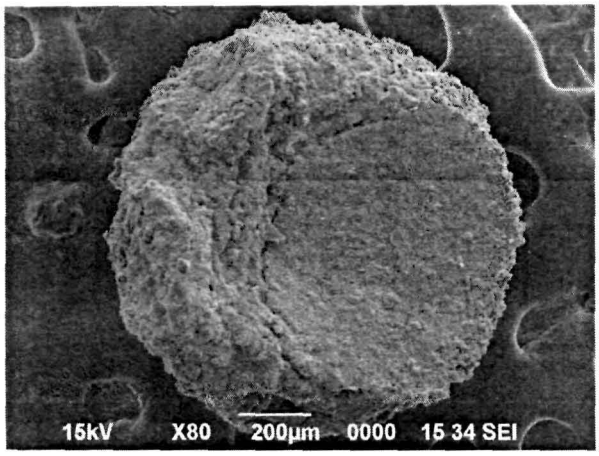

Fig. 7. SEM image of sodium alginate, isoniazid and MCC (250 mg) bead (80×).

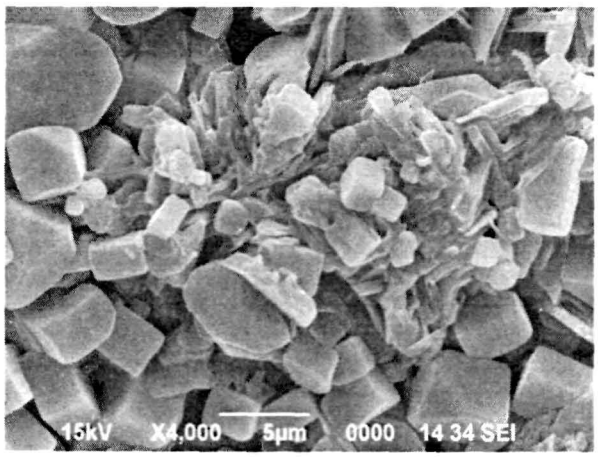

Fig. 8. SEM image of sodium alginate, isoniazid and MCC (250 mg) bead (4000×).

isoniazid equivalent was observed after 10 min. Thereafter the rate of release of the drug increased and a maximum of 0.326 µg was found to be released after 3 h. However the release rate declined and after 24 h only 0.231 µg of isoniazid equivalent was released. It was found that the release of the drug was lower from the beads containing MCC at 500 mg than from the beads with MCC at 250 mg concentration.

As observed, a high concentration of MCC inhibited the release rate of the drug. However, in both the cases the release of the drug were sustained for a longer period (upto 24 h) and the release was more than that of the release from the beads without MCC.

The above observation shows that incorporation of MCC in the alginate micro beads enhanced the drug binding property which in turn resulted in greater release of the drug from the beads in comparison from the ones without MCC.

The assay for antioxidant activity was carried out to find the radical scavenging properties of MCC. It was found that MCC at a concentration of 50 mg could scavenge DPPH free radicals up to 78.29% while at 10 mg the percentage scavenging was only 62.07%. Ascorbic acid taken as the positive control demonstrated a scavenging activity of 97.51%. The antioxidant property of MCC could be of advantage when used as a drug delivery vehicle.

Whether MCC had any cell damaging affect was investigated in terms of its hemolytic activity in erythrocytes. It was found that MCC did not cause any damage to the blood cells (Fig. 6). Its activity was the same as that of the PBS buffer (pH 7.4) even after 2 h of incubation which indicated no cytotoxic affect in the cells.

4. Conclusion

Microcrystalline cellulose prepared from a common fast growing fodder grass species *S. glauca* (L) P. Beauv was found to be with higher crystallinity and thermally stable at high temperature. It was found to possess antioxidant property and was without cytotoxic effect as evident from the hemolytic assay. The investigation also revealed that MCC supported sustained release of isoniazid, an antituberculosis drug over a considerable period of time. Thus, the MCC prepared from an inexpensive grass species may be further explored for application in the pharma industry. The promising findings of sustained drug release from MCC–alginate composites without any cytotoxicity could be forwarded as an attractive candidate for further exploration as a drug delivery vehicle.

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