1. INTRODUCTION

Tuberculosis (TB) is an infectious disease caused by the bacillus *M. tuberculosis*. It typically affects the lungs (pulmonary TB) but can affect other organs as well (extra-pulmonary TB). The transmission is mainly by aerosolic dispersion of the bacilli from patients infected with pulmonary tuberculosis due to coughing and sneezing.

1.1. History of Tuberculosis

Tuberculosis has claimed its victims throughout much of known human history. It reached epidemic proportions in Europe and North America during the 18th and 19th centuries, earning the sobriquet, "Captain Among these Men of Death." Understanding the pathogenesis of tuberculosis began with the work of Theophile Laennec at the beginning of the 19th century until the identification of the tubercle bacillus as the etiologic agent by Robert Koch in 1882. Modern techniques of molecular genetics and the sequencing of the genome of several strains of *M. tuberculosis* allow a more rigorous estimation of the time of origin of mycobacteria.

Early progenitor of *M. tuberculosis* was present in East Africa as early as 3 million years ago, and they suggest that it may have infected early hominids at that time (Gutierrez *et al.*, 2005). It is likely, however, that all modern members of the *M. tuberculosis* complex, including not only *M. tuberculosis*, but its African variants *M. africanum* and *M. canettii* as well as *M. bovis*, had a common African ancestor about 35,000–15,000 years ago.
(Brosch et al., 2002). There are written texts describing tuberculosis in India as early as 3300 years ago and in China 2300 years ago (Morse et al., 1967).

1.2. Causative organism

*Mycobacterium tuberculosis* is a slow growing, obligate aerobic and facultative intracellular organism primarily infecting lungs and the other parts of the body except the hair and nail. In the classic case of tuberculosis, organisms are always found in the well-aerated upper lobes of the lungs. Being a facultative intracellular parasite of macrophages, the bacterium has a slow generation time of 15-20 hours. Chains of cells in smears made from colonies grown in vitro often form distinctive serpentine cords.

*M. tuberculosis* is neither classified as Gram-positive nor as Gram-negative because it does not contain the chemical characteristics of either although the bacteria do contain peptidoglycan in their cell wall. The genus Mycobacteria and Nocardia are classified as acid-fast bacteria (AFB) due to their impermeability by certain dyes and stains. Despite this, once stained with heating, AFB will retain dyes even when treated with acidified organic decolorizing solvents.

The cell wall structure of *M. tuberculosis* is unique among prokaryotes and it is a major determinant of virulence for the bacterium. The cell wall complex contains peptidoglycan, but otherwise it is composed of complex lipids. Over 60% of the mycobacterial cell wall is lipid (Todar, 2008).
1.3. **Pathophysiology of TB**

Bacteria in the inhaled aerosols are engulfed by alveolar macrophages and tissue dendritic cells. The bacilli replicate inside the phagocytic cells and later cross the alveolar barrier to cause systemic infection (Bermudez *et al.*, 2002; Todar, 2008). This is observed during the development of adaptive immune response where by the bacilli establishes a protected niche and persists indefinitely (Chackerian *et al.*, 2002; Hinley-Wilson *et al.*, 2003). Activated T-lymphocytes, macrophages and other immune cells together form granuloma to curtail the necrotic tissue thereby restricting replication and spread of bacilli (Scanga *et al.*, 2001). However, the bacteria have evolved effective strategies to escape from the host immune response and survive in non-replicating latent state. Thus in such individuals, the pathogen is not completely eliminated from the system and the bacteria remain quiescent or dormant of latent state (Hingley-Wilson *et al.*, 2003; Frieden *et al.*, 2003; Tufariello *et al.*, 2003).

1.4. **TB and Human Immunodeficiency Virus (HIV)**

Tuberculosis is one of the earliest opportunistic diseases and a leading cause of death in HIV infected persons and the latter is the most powerful risk factor for the progression of active TB disease from a latent TB infection (Bruchfeld *et al.*, 2002). In most developing countries, although the disease has always been endemic, its severity has increased because of the global HIV pandemic. Of the 9.4 million incident cases in 2009, an estimated 1.1 million (12%) were HIV-positive. Of these HIV positive cases, 78% were in the African region and 13% were in the South-East Asia region (WHO, 2010). Around 30% of the AIDS related deaths are due to tuberculosis (Grange and
Zumla 2002). In the year 2011, among 23 states 6 lakh TB patients were ascertained for their HIV status (67% of TB patients registered) and about 44,000 HIV-infected TB patients were diagnosed (RNTCP annual report, 2012). Malnutrition is an important risk factor for the development of TB. Malnourished individuals have an increased likelihood of primary or latent infection progressing to active disease (Parasca et al., 2006).

1.5. Epidemiology

World Health Organization (WHO) declared a global emergency in the year 1993 because of the resurgence of tuberculosis (WHO 1993). With the introduction of newer strategies such as DOTS and PMDT, quite a high number of lives have been saved. Global burden of TB remains enormous. Burden of TB morbidity and mortality in children (≤15 years) and women (≥15 years) remain undefined. It was estimated in 2012 that there were 2.9 million cases of new TB and 410,000 deaths due to tuberculosis. Among the children the estimated infection was 530,000 with 72,000 deaths in the same year. The above estimates are slightly higher than the report by WHO in 2012, indicating the improvement in the new surveillance and case detection.

In the year 2009, it was estimated that globally there were 9.4 million incident TB cases and there were 1.3 million TB deaths. India is the second-most populous country in the world, but it has more new TB cases annually than any other country. Out of the estimated global annual incidence 2 million were estimated to have occurred in India, thus contributing to a fifth of the global burden of TB. It is estimated that about 40% of Indian population is infected with TB bacillus (RNTCP annual report, 2011). Two of every five persons are infected with the tuberculosis bacillus. Of them, 10% will develop
the disease during their lifetime. Every day about 5000 people develop the disease and around 1000 die. TB kills more adults in the most productive age group of 15-54 (RNTCP -http://www.tbcindia.org). The risk for developing TB is also higher in persons with diabetes and in other chronic debilitating diseases leading to immune compromise, poor living conditions, tobacco smoking etc.

1.6. Diagnosis

Generalized symptoms of pulmonary TB includes chronic cough for three weeks or more, pain in the chest, haemoptysis, weakness or fatigue, weight loss, fever and night-sweats. The symptom of extra pulmonary TB depends on the site where tubercle bacilli reside and reproduce.

Classical evaluation for TB must include past medical history, physical examination, chest X-ray, tuberculin skin test and demonstration of bacilli by microbiology procedures. Microbiology investigations include smear microscopy and culture on solid or liquid based media.

World Health Organization has strongly recommended sputum smear examination as the preferred screening test. It is one of the simplest methods but requires at least $10^4$ AFB/ml of sputum for detection from concentrated specimens (Agarwal and Chauhan, 2005). Conventional TB diagnostics include sputum microscopy and culture of *M. tuberculosis*. Microscopy is simple, specific and rapid, it suffers from low sensitivity (30–70%) (Maher and Raviglione, 1995). In HIV infected populations microscopy is particularly insensitive and culture of *M. tuberculosis* is the gold standard for diagnosis of TB (Padmapriyadarsini *et al* 2011). Conventional culture of mycobacteria detects $10^3$ viable mycobacteria per ml of sample and in case of active
disease it is found to be 81% sensitive and 98.5% specific. The laboratory turnaround time for *M. tuberculosis* growth on solid culture media is around 3-4 weeks.

Culturing procedures on liquid media are more rapid compared to the conventional method (solid media). They include radiometric BACTEC 460 system and Mycobacteria Growth Indicator Tube (MGIT) system (manual and automated) from Becton and Dickinson, USA, BacT/Alert by Bio Merieux Inc., Durham., Septi-Check AFB manufactured by Roche and ESP II Culture system from Trek Diagnostic Systems, Ohio. Multi centric evaluation of diagnosis of TB employing liquid cultures has been reported. They are particularly useful in smear negative and paucibacillary cases. Although the cost of these tests has recently been reduced, most hospitals in developing countries may not find the test affordable. Resource limited countries need diagnostic tools that are sensitive, specific, cost effective, easy to perform, and easy to implement within the existing infrastructure (Trollip *et al*., 2001). Cost negotiations by WHO has lead to the wide spread accessibility of these newer technologies in resource limited settings.

Development of improved tools for the diagnosis of tuberculosis, including smear negative TB has been considered as a top priority (WHO, 2006; Albert *et al*., 2001). There are various non-conventional colorimetric; growth based; reporter based assays that have been extensively validated in research as well as in programmatic settings. These assays include nitrate reductase assay, thin layer agar method, microscopic observation and drug susceptibility (MODS), redox dye based assay, and reporter phage based assays. TB control program in Peru had previously demonstrated the
feasibility of MODS in programme settings. All these assays play a dual role as diagnostic and susceptibility test and can be used on direct and indirect specimens. With one time investment for minor instruments, the running cost per specimen become minimal.

Tests based on mycobacteriophages show promising results. Phage amplified biologically (PhaB) assay is relatively easy to perform, but requires the type of laboratory infrastructure that is needed for routine mycobacterial cultures. The turnaround time of phage-based tests is 2 days compared to about 2 hours (microscopy) or up to 2 months (culture) (Albert et al., 2001).

The luciferase gene from firefly has been widely used in biological reactions. It is one of the methods for measurement of adenosine triphosphate (ATP). The enzyme catalyzes the oxidation of luciferin to oxy-luciferin with ATP which is an indicator of growth and magnesium ions as catalysts. Light produced as a by-product of this reaction is measured using luminometer at 560 nanometer (Billar and DuBow 1998; Hastings et al., 1978). This principle is used in the LRP where the gene is inserted into mycobacteriophages. The assay format can be not only used for both diagnosis and drug susceptibility test (DST) of M. tuberculosis but also for screening extracts and compounds to assess their antitubercular activity. Though several reports are available regarding the use of LRP assay for diagnosis, further research is required for addressing certain critical areas such as multiplicity of infection (MOI) and time point for measurement of luminescence (Banaiee et al., 2001; Bardarov et al., 2003). Using improved LRP constructs where latency gene promoters are made to express luciferase gene and using a modified assay format Diagnostic LRP Assay (DLRPA) was
found to be more sensitive than the smear and culture methods picking up additional true positives as confirmed by RT PCR (Dusthackeer et al., 2012).

There has been profound improvement in using molecular based diagnosis for infectious diseases over the last century. Both in-house and commercial assays are currently available for the same (Pai et al., 2004; Daley et al., 2007; Ling et al., 2008). Nucleic acid amplification tests (NAAT) are available for detection of *M. tuberculosis* from clinical specimens (Shetty et al., 2000). They are tailor made to be genus or species specific, where both forms of genetic material namely ribo and deoxy ribo nucleic acid (RNA and DNA) could be used. Many regions in *M. tuberculosis* had been exploited for amplification-insertion sequence (IS) 6110, heat shock proteins (hsp) 60, 16S ribosomal RNA (rRNA), region of deletion (RD) (Bergmann and Woods, 1998; Gill et al., 2006). Some of the commercial assays used for detection and susceptibility profiling include,

- Amplicor MTB from Roche Molecular Diagnostics, USA,
- Amplified *M. tuberculosis* Direct Test from Gen-Probe Inc, USA,
- Light Cycler reaction (LCx) from Abbott Labs, Illinois,
- BD-Probe Tec Direct from BD Diagnostic systems, USA,
- Loop Mediated Isothermal amplification (LAMP) from Eiken Chemical Co., Japan,

Even though molecular based detection methods provide bed side solution to the problem, they pose limitations in certain areas like cost of commercial kits, dedicated infrastructure, expertise and need for region specific re-standardization or evaluation. Moreover, highly variable performance characteristics, inability for quantification of bacteria are other reasons that
need attention. Diagnostic accuracy of these tests may not correlate clinically rendering them as adjunct test for TB diagnosis (Pai et al., 2004).

Serological testing using interferon gamma based tests are promising but cannot distinguish between active and latent infection. Low specificity due to cross reactivity with environmental mycobacteria makes them not useful in clinical settings. Recently WHO and Indian government had issued a notification stating the ban on the use of serological testing for tuberculosis diagnosis (http://tbcindia.nic.in/pdfs/Letter_Serodiagnosis.pdf).

1.7. Treatment

In the late 19th and early 20th centuries sanatoria were developed for the treatment of patients with tuberculosis. The rest provided there was supplemented with pulmonary collapse procedures designed to rest infected parts of lungs and to close cavities. The modern era of tuberculosis treatment and control was heralded by the discovery of streptomycin in 1944 and isoniazid in 1952. Even though tuberculosis is a curable disease it requires a lengthy treatment extending up to a period of six months or more with the cocktail of first line drugs Rifampicin (RIF), Isoniazid (INH), Ethambutol (EMB), Pyrazinamide (PZA) (Wade and Zhang, 2004).

Due to rampant usage of anti-tuberculosis drugs, the bacteria had adopted mechanisms to evade the drug action through various mechanisms leading to the development of drug resistance (Raviglione, 2006). The multi drug resistance defined as resistance to RIF and INH with or without resistance to other first line drugs pose a major threat to TB treatment. The choice of drugs for MDR-TB patients is limited. Thioamide, Fluorquinolones,
aminoglycoside and peptide are the class of drugs used for treatment of MDR-TB. These drugs are broad spectrum antibiotics with limited efficacy and thus the bacteria develop resistance to these drugs establishing themselves as the deadly extensively drug resistant (XDR) TB.

Despite increasing knowledge about the pathogenicity and epidemiology of the disease, several problems still remain with prevention and treatment of *M. tuberculosis* (Russell *et al.*, 2010). By the end of 2012, data on TB drug resistance was available for 136 countries (70% of 194 WHO Member States), either by continuous surveillance or by special surveys.

### 1.8. Need for alternate therapeutics

With increasing prevalence of multi (MDR) and extensively drug resistant (XDR) tuberculosis strains more complicated, expensive and longer duration of treatment becomes inevitable. In some cases treatment options are also limited. Another reason for slow progress in TB control is due to less interest shown by pharmaceutical companies for drug discovery and development of lead compounds. Apparently no new antibiotics have been developed against mycobacteria since 1970 after discovery of rifamycin. Hence there is an urgent need to develop alternative and effective drugs that shorten the course of chemotherapy and counteract the spread of drug resistant tuberculosis (WHO 2006). Primary requisites for the novel drugs in need are such as, unique mode of action, minimal expectation of development of resistance, maximum tolerance, less adverse reaction, bactericidal effect at lowest concentration and stability during treatment. In the pharmacological point of view, the shelf life of the drug or its formulation should be prolonged and it should not have antagonistic activity with any of the existing anti TB
drug. Higher absorption maxima, low affinity to proteins and extended serum half-life with minimal excretion will be ideal for therapy.

1.9. Phytochemicals: The living natural pharmacy

Plants are the oldest source of pharmacologically active compounds and their extracts were used by human beings for thousands of years (Cordell, 1981). Plants may be considered to be a biodynamic laboratory for producing chemical constituents of primary metabolites and secondary natural substances (Haensel et al., 1999). Secondary metabolites mainly function as defense tools against predators and microbial pathogens.

Dating back to Vedic period, usage of plants and other natural products remains a template for the development of new scaffolds of drugs (Newman and Cragg, 2007). Drugs from natural sources have novel structural features and biological activity. Potent molecules can be used as lead structures for synthetic modification and optimization of bioactivity. Recent data suggests that 80% of the drug molecules is derived from natural products or natural compound inspired (Harvey, 2008).

India is one of the few countries in the world that is equipped with unique wealth of medicinal plants. The vast traditional knowledge for their use in the treatment of various diseases has been reported in the classic texts like Ayurveda and Charak Samhita eventually contributing as ‘boom’ in drug discovery (Patawardhan, 2007). Herbal medicines and their purified compounds have shown to possess varied pharmacological activity for the treatment of many illness starting such as cold and fever from time immemorial (Prabuseenivasan et al., 2006).
Over 2,48,000 species of higher plants have been identified and among these, about 12,000 plant species are identified to have medicinal properties. However, from phytochemical and or pharmacological point of view, only less than 10% of these plants have been investigated (Harborne et al., 1999). With high throughput screening methods becoming more advanced and accessible, the number is expected to increase. Approaches for selecting plants as candidates for drug discovery programs have been reviewed extensively and published (Verpoorte, 2000).

A considerable number of plant species have been mentioned in Ayurveda for the treatment of TB, leprosy and related disorders. Among these, 60 and 91 plant species were specific for treatment of TB and leprosy respectively (Sharma 1998). Although, plant species serves as rich source for many novel biologically active compounds, only very few species have been investigated against *M. tuberculosis* (Heinrich and Gibbons, 2001).

Many studies have revealed that a wide range of natural products exhibited promising activities against mycobacteria (Cantrell et al., 2001). More than 350 natural products have been evaluated for their antimycobacterial activities (Newton et al., 2000). Two of the vital drugs used as first choice in tuberculosis treatment are the synthetic compounds: isoniazid and rifampicin that are derived from the natural products nicotinamide (the amide of vitamin B3) and rifamycin a byproduct of actinomycetes (Salomon and Schmidt, 2012).

In recent years, with the help of advancement of science, the isolation; identification and elucidation of chemical principals from natural sources have become uncomplicated and have contributed to the development of new drugs.
from medicinal plants. A number of studies have been reported for their antibacterial (Kumar et al., 2006), antifungal (Jain et al., 2010), antiviral (Mukhtar et al., 2008) and anticancer (Baskar et al., 2012) properties and are presently used in pharmacological preparations. Introduction of novel mechanism-based *in vitro* bioassays is virtually limitless, and therefore any plant, regardless of the extent of prior biologic or chemical study, could prove interesting as a potential new drug source (Cragg et al., 1997). Higher plants with their secondary metabolites form a reservoir of low molecular weight organic compounds that is largely untapped as a source of pharmaceuticals.

Primary benefits of using plant derived medicines are that they are relatively safer than synthetic derivatives, offering profound therapeutic benefits and more affordable treatment. For these reasons, medicinal plants have gained much attention as alternatives to antibiotics. However, not much attention has been given to the laboratory evaluation of their antimycobacterial activity. The present study was undertaken to evaluate the efficacy of selected medicinal plants against *M. tuberculosis* for identification of the active compounds using bioassay-guided fractionation.