

***IN VITRO* ANTI-MYCOBACTERIAL AND
BIOACTIVE COMPOUNDS OF
Pluchea indica (L.) LESS. AGAINST
SURROGATE TUBERCULOSIS ORGANISMS**

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SURROGATE TUBERCULOSIS ORGANISMS**

by

AZIRAH INEZ BINTI JAMALUDIN

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LIST OF ABBREVIATIONS

| | |
|-----------------|---|
| TB: | Tuberculosis |
| MDR-TB: | Multi-drug resistant tuberculosis |
| XDR-TB: | Extensively-drug resistant tuberculosis |
| PTB: | Pulmonary tuberculosis |
| EPTB: | Extrapulmonary tuberculosis |
| HIV: | Human Immunodeficiency Virus |
| BCG: | Bacillus Calmette-Guerin |
| PG: | Peptidoglycan |
| AG: | Arabinogalactan |
| mAGP: | Mycolyl arabinogalactan–peptidoglycan |
| PIMs: | Phosphatidylinositol mannosides |
| LM: | Lipomannan |
| LAM: | Lipoarabinomannan |
| GC: | Guanine-cytosine |
| MTBC: | <i>Mycobacterium tuberculosis</i> complex |
| rRNA: | Ribosomal ribonucleic acid |
| PCR: | Polymerase chain reaction |
| ELISpot: | Enzyme-linked immunospot |
| ELISA: | Enzyme-linked immunosorbent assays |
| IFN- γ : | Interferon-gamma |
| DOT: | Directly observed therapy |
| ADC: | Albumin Dextrose Catalase |
| OADC: | Oleic Albumin Dextrose Catalase |
| ATCC: | American Type Culture Collection |

| | |
|--------------------|---|
| NCTC: | National Collection of Type Cultures |
| CFU/mL: | Colony Forming Unit per millilitre |
| CO ₂ : | Carbon dioxide |
| DMSO: | Dimethyl sulphoxide |
| MTT: | Tetrazolium salt, 3-(4,5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide |
| TEMA: | Tetrazolium microplate assay |
| MIC: | Minimum inhibitory concentration |
| MBC: | Minimum bactericidal concentration |
| EMB: | Ethambutol |
| STR: | Streptomycin |
| RIF: | Rifampicin |
| INH: | Isoniazid |
| FICI: | Fractional inhibitory concentration index |
| GC-MS: | Gas Chromatography-Mass Spectrometry |
| TEM: | Transmission electron microscope |
| HCl: | Hydrochloric acid |
| BPE: | Bovine pituitary extract |
| rEGF: | Human recombinant Epidermal Growth Factor |
| NP69: | Nasopharyngeal epithelial cell line |
| 2D: | Two dimension |
| EDTA: | Ethylene-diamine-tetra-acetic acid |
| IC ₅₀ : | 50 % inhibitory concentration |

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***IN VITRO* ANTI-MIKROBAKTERIA DAN SEBATIAN BIOAKTIF**

***Pluchea indica* (L.) LESS. TERHADAP ORGANISMA PENGGANTI**

TUBERKULOSIS

ABSTRAK

Pluchea indica (L.) Less. merupakan tumbuhan ubatan Asia yang digunakan secara meluas dalam perubatan tradisional untuk merawat pelbagai penyakit termasuk gejala tuberkulosis (TB) dan oleh itu, dapat menyediakan sumber alternatif sebatian anti-TB. Penyaringan awal *in vitro* tiga bahagian berbeza *P. indica* menggunakan asai pencairan mikro tetrazolium menunjukkan bahagian n-heksana daun dan batang dan kloroform daun mempamerkan aktiviti rencatan terhadap *Mycobacterium smegmatis*, *M. fortuitum* and *M. kansasii* dengan kepekatan perencat minimum (MIC) berjulat antara 200 - 3200 µg/mL. Aktiviti paling tinggi ditunjukkan oleh n-heksana daun terhadap *M. kansasii* dengan MIC 200 µg/mL. Pemecahan berpandu bioasai bahagian n-heksana daun dilakukan untuk analisis selanjutnya menggunakan kromatografi lapisan nipis dan kromatografi turus yang menghasilkan lapan fraksi (Fraksi A - H). Fraksi tersebut mempamerkan aktiviti terhadap *M. kansasii* dengan MIC berjulat antara 12.5 - 800 µg/mL. Fraksi yang sangat aktif (Fraksi A, C, D, dan E) juga merencat *M. tuberculosis* H37Ra dengan MIC berjulat antara 50 - 100 µg/mL. Pengenalpastian fitokimia fraksi paling aktif, Fraksi C menggunakan gas kromatografi-spektrometri jisim menghasilkan 10 sebatian yang dikenal pasti, yang mana asid n-heksadekanoik, asid 9,12-oktadekadienoik (Z,Z)-, dan asid oktadekanoik adalah sebatian utama. Saringan sebatian tersebut terhadap *M. tuberculosis* H37Ra menunjukkan aktiviti sebatian tulen adalah rendah daripada aktiviti Fraksi C asalnya.

Oleh itu, Fraksi C dikaji selanjutnya berkenaan interaksinya dengan empat drug anti-TB barisan pertama terhadap *M. kansasii* dan *M. tuberculosis* H37Ra. Satu interaksi sinergi telah dihasilkan oleh gabungannya dengan rifampisin terhadap *M. tuberculosis* H37Ra dengan nilai indeks kepekatan perencatan pecahan (FICI) 0.375. Interaksi antagonistik telah diperhatikan dalam gabungan dengan etambutol terhadap *M. kansasii* dengan nilai FICI 4.125. Kajian selanjutnya mengenai kadar pembunuhan gabungan Fraksi C dengan rifampisin pada nilai MICnya menunjukkan aktiviti bakterisid (kadar pembunuhan 95%) terhadap pertumbuhan *M. tuberculosis* H37Ra, manakala, Fraksi C adalah bakteriostat (kadar pembunuhan 67%). Pemerhatian di bawah mikroskop elektron transmisi ke atas struktur-ultra sel *M. tuberculosis* H37Ra yang dirawat, menunjukkan Fraksi C menyebabkan perpecahan sel terutamanya pada dinding sel. Kajian sitotoksik Fraksi C terhadap warisan sel epitelium nasofarinks (NP69) menunjukkan bahawa Fraksi C adalah toksik dengan nilai IC₅₀ rendah sebanyak $2.68 \pm 0.1 \mu\text{g/mL}$ tetapi kesan fraksi tersebut kemungkinan bergantung kepada dos atau masa. Keputusan kajian ini memberikan asas saintifik terhadap penggunaan *P. indica* secara tradisional untuk gejala TB dan tumbuhan ini boleh menjadi sumber sebatian anti-TB berpotensi yang layak untuk kajian lanjut.

**IN VITRO ANTI-MYCOBACTERIAL AND BIOACTIVE COMPOUNDS OF
Pluchea indica (L.) LESS. AGAINST SURROGATE TUBERCULOSIS
ORGANISMS**

ABSTRACT

Pluchea indica (L.) Less. has been used widely in traditional medicine as a remedy of a variety of illnesses including symptoms of tuberculosis (TB) and thus, could provide an alternative source of anti-TB compounds. Preliminary *in vitro* screening of three different *P. indica* partitions, using tetrazolium microdilution assay showed that leaf and stem n-hexane and leaf chloroform partitions exhibited inhibitory activity against *Mycobacterium smegmatis*, *M. fortuitum* and *M. kansasii* with minimum inhibitory concentrations (MICs) of 200 - 3200 µg/mL. The highest activity was shown by leaf n-hexane against *M. kansasii* with MIC of 200 µg/mL. The leaf n-hexane partition was selected for further bioassay guided fractionation process using thin layer and column chromatographic techniques, which yielded eight fractions (Fractions A - H). These fractions exhibited activity against *M. kansasii* with MICs in the range of 12.5 – 800 µg/mL. The highly active fractions (A, C, D, and E) also inhibited *M. tuberculosis* H37Ra with MICs in the range of 50 – 100 µg/mL. Phytochemical identifications of the most active fraction, Fraction C using gas chromatography-mass spectrometry produced 10 identified compounds, of which, n-hexadecanoic acid, 9,12-octadecadienoic acid (Z,Z)-, and octadecanoic acid were the major compounds. Screening of these compounds against *M. tuberculosis* H37Ra showed that their activities were lower than the activity of their original Fraction C. Thus, Fraction C was further assessed on its interactions with four first-line anti-TB

drugs against *M. kansasii* and *M. tuberculosis* H37Ra. One synergistic interaction was produced by the combination with rifampicin against *M. tuberculosis* H37Ra with fractional inhibitory concentration index (FICI) value of 0.375. An antagonistic interaction was observed in the combination with ethambutol against *M. kansasii* with FICI value of 4.125. Further study on the killing rate of the combination of Fraction C with rifampicin at their MIC values showed a bactericidal activity (95 % killing rate) towards the growth of *M. tuberculosis* H37Ra, whereas, Fraction C alone was bacteriostatic (67 % killing rate). Observation of the ultra-structure of the treated *M. tuberculosis* H37Ra cells under transmission electron microscope showed that Fraction C caused general disintegration of the cells particularly, the cell wall. The cytotoxicity study of Fraction C on nasopharyngeal epithelial cell line (NP69) showed that Fraction C was toxic with low IC₅₀ value of $2.68 \pm 0.1 \mu\text{g/mL}$ but the effects could be dose-dependent or time-dependent. The findings of this study gave a scientific basis to the traditional use of *P. indica* for symptoms of TB and this plant could be a potential source of anti-TB compounds worthy of further investigation.

CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION

Mycobacterium tuberculosis, the etiologic agent of tuberculosis (TB) is one of the most infectious human pathogen that causes high morbidity and mortality and infects one-third of the world's population (Stewart *et al.*, 2003; Agarwal, 2004; Gomes-Flores *et al.*, 2008). TB is an ancient human scourge that remains a major cause of death worldwide (Miller, 1994; McDermott *et al.*, 1997; Galagan, 2014). The theories of the origin of TB continue to change as new discoveries are made with the development of molecular technologies (Davis, 2000). TB disease commonly affects the lungs, which is termed as pulmonary TB, and can also occur in sites other than the lungs, which is referred as extrapulmonary TB (Centres for Disease Control and Prevention, 2013a). The common symptoms of pulmonary TB are cough, fever, and weight loss. *M. tuberculosis* is also able to persist in the form of long-term asymptomatic infection (latent TB) (Stewart *et al.*, 2003).

Recently, TB disease has been a topic of global concern as the increase of incidence rate among both immunocompetent and immunocompromised patients becomes escalated (Harisinghani *et al.*, 2000). The epidemiology of TB has become more serious by the emergence of multi-drug resistant *M. tuberculosis* (MDR-TB) strains against both the first-line and second-line anti-TB drugs (Singh, 2007; Leite *et al.*, 2008). Unfortunately, no new anti-TB drugs have been introduced in the past 30 years mainly because TB drug discovery had been confronted with many limitations such as a slow growth rate of the mycobacteria, biosafety concerns and other issues that are related to *M. tuberculosis* (Primm and Franzblau, 2007). With the present

advance technologies, there are positive possibilities of discovering potential new drugs, which are affordable and effective from the natural sources such as plants as they contain enormous chemical diversity (Gautam *et al.*, 2007). Natural product remedies from plants are therapeutic alternatives that have been used to treat many diseases for centuries and are now extensively explored for pure compounds or crude materials for drug discovery (Gupta *et al.*, 2010). Many review publications have reported that many plant species have been shown to possess anti-TB activity (Copp, 2003; Gautam *et al.*, 2007; Negi *et al.*, 2010; Arya, 2011). In view of these findings, the present study was concerned with the anti-TB activity of a local ethnopharmacological plant, *Pluchea indica* (L.) Less., which has been used traditionally to treat symptoms of TB (Mohamad *et al.*, 2011; Suriyaphan, 2014; Radji *et al.*, 2015).

Pluchea indica has been reported in previous studies to possess anti-mycobacterial activity against *Mycobacterium* species (Caldwell *et al.*, 2000; Stavri *et al.*, 2004; Mohamad *et al.*, 2011; Mohamad, 2014). A recent study by Mohamad (2014) observed that the non-polar n-hexane partition of *P. indica* exhibited promising anti-TB activity against *M. tuberculosis* H37Rv with MIC of 50 µg/mL, worthy for further investigations. Hence, based on the follow-up of anti-mycobacterial activity reports, the main objective of this research was to further investigate the anti-mycobacterial activity of *P. indica* against several surrogate TB organisms focussing on its active chemical constituents, interaction with anti-TB drugs, and cytotoxic level.

1.2 AIM AND OBJECTIVES OF THE RESEARCH

In view of the urgent need for new TB drugs, which are effective, cheaper, and readily available from plants, this research was carried out to investigate the anti-mycobacterial activity of *P. indica* against surrogate TB organisms (*M. smegmatis*, *M. fortuitum*, *M. kansasii*, and *M. tuberculosis* H37Ra) and to identify its active chemical constituents.

The specific objectives were:

1. To screen the anti-mycobacterial inhibition of the *P. indica* partitions against the test *Mycobacterium* species using a colorimetric tetrazolium microplate assay (TEMA) and to identify the bioactive fractions using bioassay-guided chromatographic fractionation techniques.
2. To identify the potential chemical constituents from the most active fraction using gas chromatography-mass spectrometry (GC-MS) techniques and to evaluate their anti-mycobacterial inhibition.
3. To study the interaction of the most active fraction with front-line anti-TB drugs using checkerboard and time-kill assay methods against *M. kansasii* and *M. tuberculosis* H37Ra.
4. To study the effects of the most active fraction on the ultra-structures of the test organism under transmission electron microscope (TEM) and to investigate its cytotoxicity level using human cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 TUBERCULOSIS

2.1.1 Overview

Tuberculosis (TB) is an infectious respiratory disease caused by *M. tuberculosis* (Arya, 2011). The TB bacteria is spread through airborne droplets when people with TB cough or sneeze. The risk of contracting TB is high based on the frequency of contact with infected person, living in crowd population or unhygienic environments, and being an immunocompromised person. The symptoms of TB are characterized with persistent cough, fever, weight loss, and sweats during the night (Grandjean *et al.*, 2015).

TB disease occurs in two sites: pulmonary and extrapulmonary organs. Pulmonary TB (PTB) refers to TB disease that affects the lungs with common signs such as cough, chest radiograph abnormality and may be infectious. Extrapulmonary TB (EPTB) is a TB disease that occurs in other parts of the body such as the brain, kidneys, larynx, lymph node, bones, or plurea. However, EPTB disease can occur together with PTB in human immunodeficiency virus (HIV)-infected person. EPTB is not infectious unless the person is also having PTB. EPTB can also occur in the oral cavity, or involves an open lesion with high concentration of organisms (CDC, 2013a). Miliary TB, a rare type of TB is a widespread lymphohaematogenous dissemination of *M. tuberculosis*, which is a lethal disease if not treated early (Ray *et al.*, 2013). Tuberculous meningitis is another form of TB disease that occurs when the tissues surrounding the brain and spinal cord are infected with TB (CDC, 2013a).

Historically, TB is an ancient disease based on the skeletal abnormalities of TB found in ancient Egyptian mummies about 3000 years old (Zink *et al.*, 2001). In the 17th and 18th centuries, TB was the feared White Plague in Europe (Todar, 2012). In the 19th century, TB was widespread in East Africa and reached America, brought by the early migrants from Africa via the Bering Strait (Daniel, 2006). The pathogenesis of TB was beginning to be understood by the demonstration of the transmissibility of *M. tuberculosis* infection in 1865 and the discovery of the tubercle bacillus as the etiologic agent of TB by Robert Koch in 1882 (Daniel, 2006). In the late 19th and early 20th centuries, hospitals were established for the treatment of TB patients and the bacillus of Calmette-Guerin (BCG) vaccination was widely used after World War I. The modern age for the treatment and control of TB was established by the discovery of streptomycin, the first TB antibiotic in 1944 and isoniazid in 1952 (Daniel, 2006).

2.1.2 Epidemiology of tuberculosis

Epidemiology is the study of causes, distribution, and control of disease in a population. TB is the most common cause of human mortality worldwide (Arya, 2011). It is an extremely infectious disease with about one third of the world's population estimated to be infected with it (Gupta *et al.*, 2010). It is estimated that about eight million new cases and two million deaths occur each year throughout the world due to TB (Kishore *et al.*, 2007).

In 2014, about 9.6 million new cases of TB were recorded and 1.5 million patients died, in which, 1.1 million and 0.4 million were people who were HIV-negative and HIV-positive, respectively (World Health Organization, 2015). The incidence toll comprised of 5.4 million men, 3.2 million women, and 1.0 million children. Of the 480 000 cases of multidrug-resistant TB (MDR-TB) estimated to have

occurred, 190 000 died of MDR-TB. The South-East Asia and Western Pacific regions had more than half of the TB cases (58 %) (Figure 2.1). Whereas, the African region had 28 % of the world's TB cases (WHO, 2015). Nevertheless, this region had the most severe burden relative to population: 281 incident cases per 100 000 on average population, which was more than double the global average of 133. India, Indonesia and China accounted the largest number of cases of the global total with 23 %, 10 %, and 10 %, respectively (WHO, 2015).

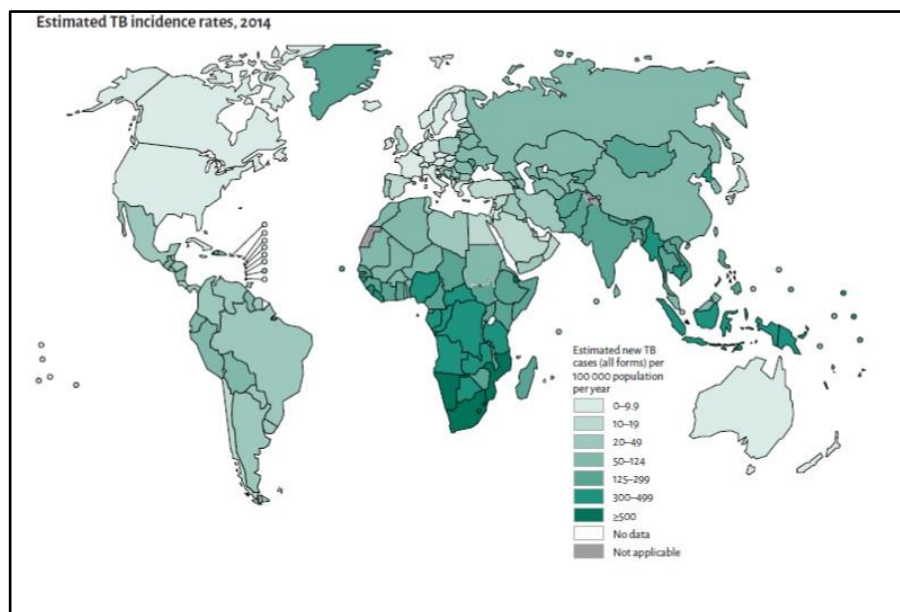


Figure 2.1. Global tuberculosis incidence rate in 2014.
(WHO, 2015)

Incidence of TB becomes worst with the emergence of multi-drug resistant (MDR) and extensively-drug resistant (XDR) strains of *M. tuberculosis* worldwide due to *M. tuberculosis* developing resistance to both the first-line and second-line anti-TB drugs (Singh, 2007; Gupta *et al.*, 2010). MDR strains are defined as resistant to at least one of the first-line drugs of isoniazid or rifampicin, while, XDR strains are resistant to both isoniazid and rifampicin, fluoroquinolone and to at least one of the three injectable second-line drugs (amikacin, capreomycin or kanamycin) (Galagan, 2014).

In 2014, it was estimated that about 3.3 % of new cases and 20 % of previously treated cases developed MDR-TB (Figure 2.2), where 9.7 % of MDR-TB cases had XDR-TB (WHO, 2015). Eastern European and central Asian countries continue to have the highest levels of MDR-TB.

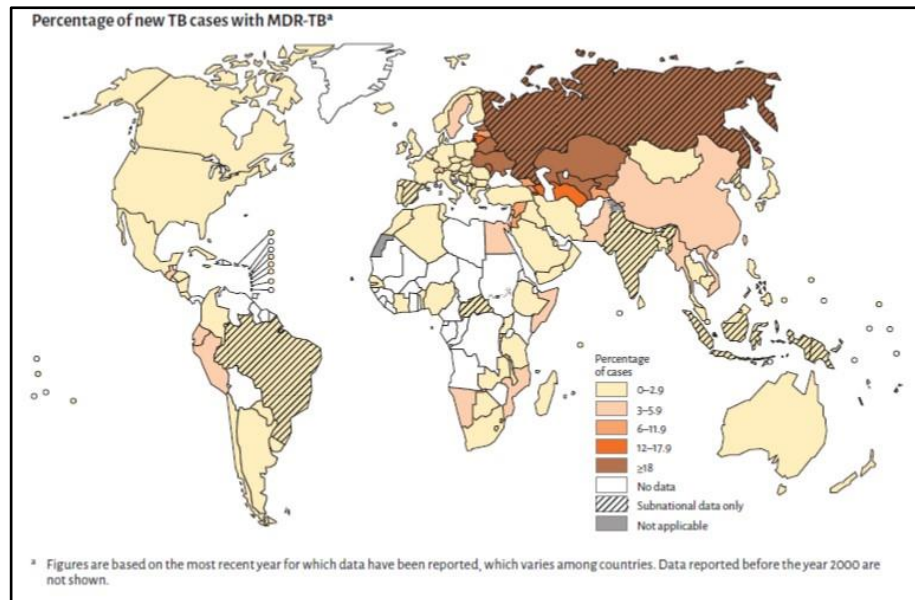


Figure 2.2. Global incidence of multi-drug resistant-tuberculosis in 2014.
(WHO, 2015)

2.1.3 The tubercle bacilli

Mycobacterium tuberculosis, also called tubercle bacillus, is an obligate aerobe, which has the ability to survive under hypoxia (Berney and Cook, 2010; CDC, 2013a). It is a non-spore forming rod-shaped bacteria measuring 0.5 μm to 3 μm (Knechel, 2009). The genus *Mycobacterium* possesses distinctive staining property called acid-fastness (Ziehl-Neelsen). This property is due to the presence of mycolic acids, found only in *Mycobacterium* species (Madigan *et al.*, 2012a). Mycolic acids are a group of complex branched-chain fatty acids covalently bound to peptidoglycan in the mycobacterial cell wall (Bhatt *et al.*, 2007; Madigan *et al.*, 2012a).

Mycobacterial cells cannot be stained with normal Gram stain technique due to the presence of the complex waxy surface cell envelope (Madigan *et al.*, 2012a).

The features of the tubercle bacilli are characterized by their slow growth, dormancy, complex cell envelope, intracellular pathogenesis and genetic homogeneity (Cole *et al.*, 1998). The generation time of *M. tuberculosis* is about 24 hours either in synthetic medium or in infected animals. This contributes to the chronic nature of the disease, prolong treatment regimen and causes an obstacle for researchers (Cole *et al.*, 1998).

The cell envelope of *M. tuberculosis* contains an additional layer beyond the peptidoglycan that is exceptionally rich in lipids, glycolipids and polysaccharides (Cole *et al.*, 1998). The cell wall is composed of two segments: upper and lower segments (Figure 2.3) (Brennan, 2003). Beyond the membrane is peptidoglycan (PG), which is covalently attached to arabinogalactan (AG), which is successively attached to the mycolic acids with long meromycolate and short α -chains. These layers make up the cell wall core known as mycolyl arabinogalactan–peptidoglycan (mAGP) complex. The upper segment contains the free lipids, which complement the α -chains. The cell wall is also interspersed with some of the cell wall proteins such as phosphatidylinositol mannosides (PIMs), phthiocerol-containing lipids, lipomannan (LM), and lipoarabinomannan (LAM). When the cell wall is disrupted (i.e. extracted with various solvents), the free lipids, proteins, LAM, and PIMs are solubilized, and the mycolic acid–arabinogalactan–peptidoglycan complex remains as the insoluble residue.

The bacterium has a genome of 4.4 Mb, about 4000 genes, which is rich in guanine-cytosine (GC)-content (Galagan, 2014). The genome sequence has been used to probe the gene content of closely related mycobacteria that led to the identification

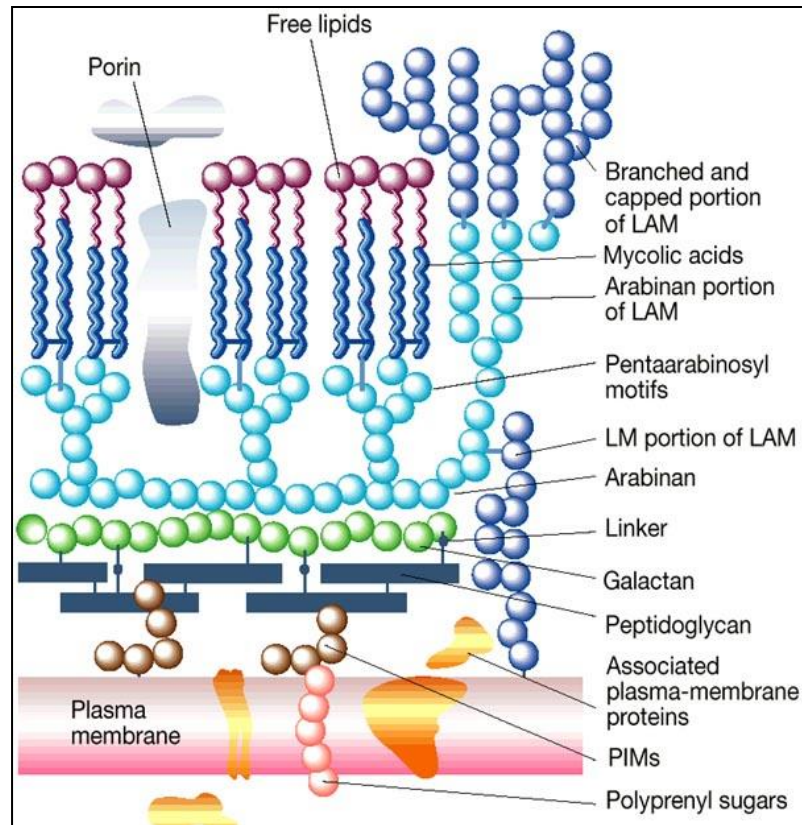


Figure 2.3. The cell envelope of *Mycobacterium tuberculosis*.
(Park and Bendelac, 2000)

of variable genomic regions that are present in some *M. tuberculosis* complex (MTBC) strains. These regions resulted in the construction of MTBC species phylogenetic tree, which is important to study about the origins of TB (Brosch *et al.*, 2002; Galagan, 2014). *M. tuberculosis* is a one of the member of MTBC and the members in this group are very closely related at the nucleotide level and have identical 16S ribosomal ribonucleic acid (rRNA) sequences (Boddinghaus *et al.*, 1990). Their differences are in terms of their host tropism, phenotypes, and pathogenicity (Brosch *et al.*, 2002). All MTBC members are obligate pathogens and causative agent of TB (Ahmad, 2011).

2.1.4 Pathophysiology of tuberculosis

According to CDC (2012), there are two TB-related conditions: latent TB infection and active TB diseases. Patients with latent TB infection do not show the symptoms and do not have TB disease. The progression from latent state of TB infection will result in development of TB disease. Latent TB infection and TB disease occur when unaffected people inhaled droplet nuclei containing *M. tuberculosis* exhaled by infected person.

Once the infectious droplets are inhaled, the bacilli are trapped in the airways where the mucus-secreting goblet cells exist to catch foreign substances (Knechel, 2009). Meanwhile, the cilia on the surface of the cells constantly beat the mucus and the entrapped particles travelled upwards for removal. This system is an initial physical defense of the body to prevent infection. Bacilli that bypass this system and reached the alveoli are quickly engulfed by the macrophages. This process is a part of the innate immune system of host defense to destroy the invading bacilli and prevent infection. Macrophages are phagocytic cells that combat pathogens without requiring earlier exposure to that pathogens (van Crevel *et al.*, 2002; Knechel, 2009). The phagocytosis by macrophages initiates the cascade events that result in either successful control of TB infection, development of latent TB infection, or progression to active TB disease (Frieden *et al.*, 2003).

Regarding the successful control or progress to infection, their initial development involves secretion of proteolytic enzymes and cytokines by macrophages to degrade the bacilli (Knechel, 2009). The released cytokines attract T-lymphocytes to the site and the macrophages then present the mycobacterial antigens to the T-cells. This process is called the cell-mediated immune response. As the bacilli continue to multiply intracellularly, a barrier shell known as granuloma develops from the

accumulation of activated T-cells and macrophages, which keeps the mycobacterial cells in dormant condition. The macrophages are then destroyed and produce necrosis in the lesion.

However, when the immunity is decreased due to ageing or immune suppression, the dormant bacilli reactivate, causing an outbreak of disease long after the initial infection (Cole *et al.*, 1998). In this case, granuloma formation is unsuccessful in bearing the bacilli (Knechel, 2009). The necrotic tissue loses structural integrity and the semi-liquid material of the necrotic tissue can then leak into a bronchus or nearby blood vessel. The droplets coughed up from patients infected with *M. tuberculosis* will infect other persons and extrapulmonary tuberculosis occurs if the bacilli is released into a vessel (Knechel, 2009). If the bacilli are drained into the lymphatic system, it will be collected in the tracheobronchial lymph nodes of the affected lung, thus, causing the formation of new granulomas (Dheda *et al.*, 2005).

2.1.5 Diagnosis of tuberculosis

The diagnosis of TB largely depends on clinical suspicion and radiographic evidence (Khomeenko *et al.*, 1996). The diagnostic test of TB can be divided into two groups: isolation of the bacterium by Ziehl-Neelsen staining, sputum culture, and polymerase chain reaction (PCR); and detection of host response towards *M. tuberculosis* exposure by Mantoux test and serodiagnosis (Chan *et al.*, 2000; Lodha *et al.*, 2000). The radiograph of the chest is also an important key tool for diagnosis of TB (Bates, 1979).

Ziehl-Neelsen stain

Ziehl-Neelsen stain is also known as acid-fast stain, which is a specialized staining applied for acid-fast bacteria due to its distinct property (Madigan *et al.*, 2012a). The stain is driven into the mycobacterial cell by slow heating and penetration of fuchsin into the mycolic acids is enhanced by the addition of phenol. After rinsing with distilled water, the decolourization of the cells with 3 % solution of HCl-ethanol is done before the cells are counterstained with methylene blue (Murohashi and Yoshida, 1968; Madigan *et al.*, 2012a). Cells of acid-fast bacteria stain red, while non-acid-fast bacteria stain blue (Figure 2.4) (Madigan *et al.*, 2012a). Acid-fast staining of sputum smears is a simple and relatively rapid for detection of active TB. However, acid-fast staining possesses low sensitivity because reliable detection needs higher than 10^4 bacilli per mL of sputum (Chan *et al.*, 2000).

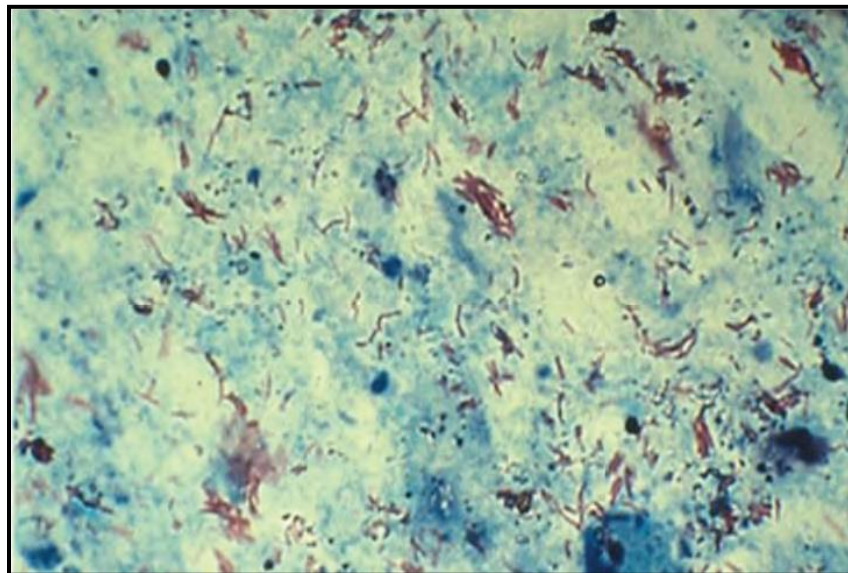


Figure 2.4. Acid-fast staining of *Mycobacterium tuberculosis*.
(CDC, 2013b)

Note: The acid-fast stains depend on the ability of mycobacteria to retain dye when treated with mineral acid or an acid-alcohol solution such as the Ziehl-Neelsen, or the Kinyoun stains that are carbolfuchsin methods specific for *M. tuberculosis*.

Culture of *Mycobacterium tuberculosis*

There are two types of media that are primarily used to culture *M. tuberculosis*: 1) Middlebrook medium, an agar based medium and 2) Lowenstein-Jensen medium, an egg based medium. Both of these media are specialized media containing inhibitor to prevent bacteria other than *M. tuberculosis* from growing (Todar, 2012). *M. tuberculosis* colonies appear compact and wrinkled on these media (Figure 2.5) (Madigan *et al.*, 2012a).

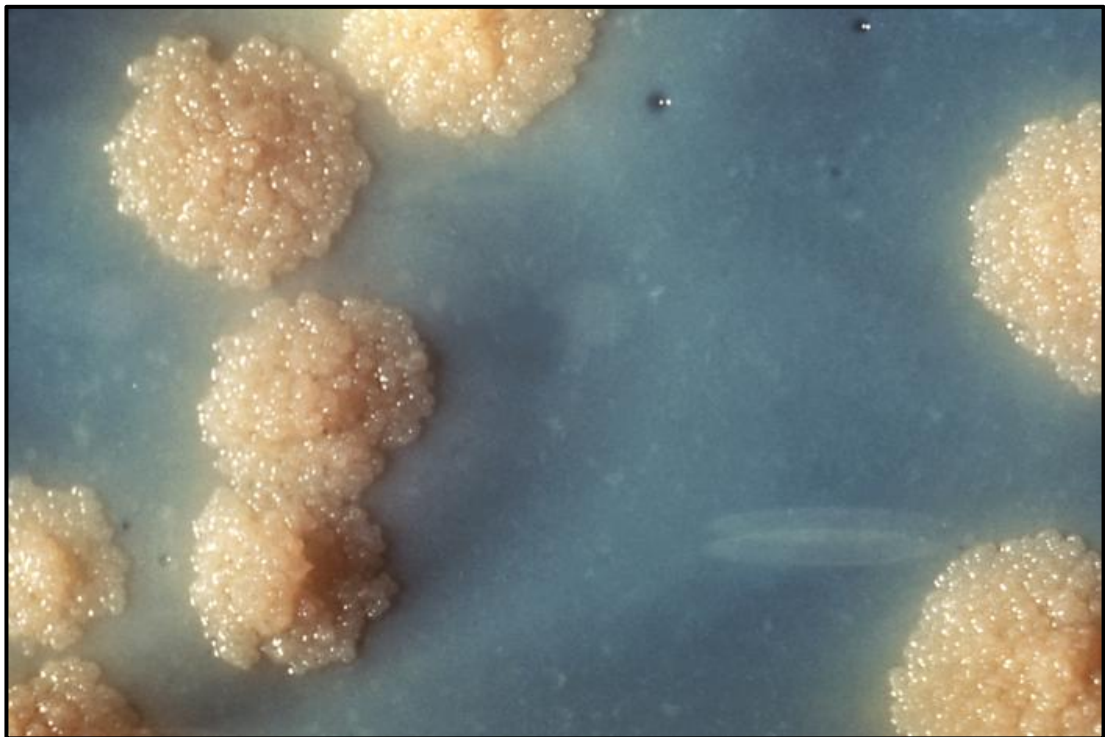


Figure 2.5. Compact and wrinkled colonies of *Mycobacterium tuberculosis*.
(CDC, 2016)

Note: A close-up of a *M. tuberculosis* culture revealing its colourless rough surface colonial morphology, which are typical morphologic characteristics for macroscopic identification.

Polymerase chain reaction

Nowadays, more rapid diagnostic techniques are also utilized in an attempt to improve the accuracy of diagnosis of TB and one of them is polymerase chain reaction

(PCR) (Chan *et al.*, 2000). This assay employs pan-*Mycobacterium* amplification primers, which ease in identification of *Mycobacterium* species from a single amplification reaction of nucleic acid. The amplification is very sensitive and specific (Tevere *et al.*, 1996). The numbers of bacilli present through PCR are considerably higher than those counted by microscopic smear of *M. tuberculosis* (Fukunaga *et al.*, 2002).

Mantoux tuberculin skin test

Tuberculin skin test was developed by Koch in 1890 but this intradermal technique was introduced by Charles Mantoux in 1912 (Nayak and Acharjya, 2012). The Mantoux tuberculin skin test measures the degree of hypersensitivity to tuberculin (Nayak and Acharjya, 2012). Tuberculin testing is also targeted for latent TB infection (Richeldi, 2006; Trajman *et al.*, 2013). Tuberculin is a purified protein to activate delayed-type hypersensitivity reaction in a person who is infected by *M. tuberculosis* after the protein was intracutaneously injected (Nayak and Acharjya, 2012; Trajman *et al.*, 2013). In this reaction, T-cells are recruited to the skin area where tuberculin is injected and lymphokines are released and resulted in local induration of the skin due to the vasodilatation, oedema, fibrin deposition, and recruitment of other inflammatory cells to the area (Huebner *et al.*, 1993; Trajman *et al.*, 2013). The induration size is correlated with the future risk of developing TB disease (Nayak and Acharjya, 2012). The test is accepted as a positive reaction when the skin induration is greater than 5 mm within 48 hours. However, the sensitivity of tuberculin skin test may be reduced by malnutrition, severe TB diseases and immunodeficiency particularly related to HIV infection (Nayak and Acharjya, 2012; Trajman *et al.*, 2013).

Interferon-gamma release assay

Interferon-gamma release assay (IGRA) is also used to detect latent TB infection and it is designed to detect the immune response to specific *M. tuberculosis* antigens, which are absent in Bacillus Calmette-Guerin (BCG) or other non-tuberculous mycobacteria (Richeldi, 2006; Trajman *et al.*, 2013). This new generation of immune-based rapid blood test is commercially available in two types of test, which are enzyme-linked immunospot (ELISpot) assay (T-SPOT.TB, Oxford Immunotec, UK) and the enzyme-linked immunosorbent assay (ELISA) technique (QuantiFERON-TB Gold-in-Tube, Cellestis, Australia, QFT-GIT). ELISpot assay was developed in late 1990s, while QuantiFERON-TB Gold test was developed in 1980s (Richeldi, 2006). The principle of both tests is that the T-cells of an individual who have TB infection will respond by secreting the cytokine interferon-gamma (IFN- γ) when stimulated with *M. tuberculosis* antigens (Trajman *et al.*, 2013).

Radiographs

The chest radiograph is useful in diagnosis of TB disease especially the pulmonary TB by revealing the chest abnormalities. The abnormalities can be observed in the upper lobe (apical and posterior segments) or in the lower lobe (superior segments). However, lesions may appear anywhere in the lungs and differ in size, shape, and density especially in immunocompromised and immunosuppressed persons. The chest radiographs are also used to exclude pulmonary TB disease in HIV-negative person who has a positive tuberculin skin test or IGRA and who possesses no symptoms of TB disease (CDC, 2013b).

2.1.6 Treatment and prevention of tuberculosis

Due to the highly contagious nature of TB, strict requirements have been imposed by Occupational Safety and Health Administration worldwide for the protection of healthcare workers who are involved in TB patient care (Jensen *et al.*, 2005). Infected patients must be hospitalized in negative-pressure rooms and the healthcare workers who have patient contact must be provided with high-efficiency particulate air (HEPA) filter face mask to prevent the droplet passage containing of *M. tuberculosis* (Jensen *et al.*, 2005; Madigan *et al.*, 2012b).

Besides that, proper prescribed treatment need to be followed to avoid the reactivation of infection that can lead to the emergence of resistant *M. tuberculosis* to the original drug treatment. Anti-TB drugs are divided into two groups, which are first-line drugs used to treat TB patients with susceptible *M. tuberculosis* and second-line drugs, which are used for MDR-TB treatment. Both of these anti-TB drugs have adverse effects, but second-line anti-TB drugs have many more severe side effects (Ghosh *et al.*, 2010; Arya, 2011). Effective regimens for TB treatment must contain multiple drugs to which, the bacteria are susceptible because administration of single drugs may lead to the resistant of the bacteria (Madigan *et al.*, 2012b). A four-drug regimen with first-line drugs: isoniazid, rifampicin, pyrazinamide, and streptomycin or ethambutol is favoured for the initial treatment of TB (CDC, 1993). To promote effective treatment of TB, it is crucial to take the anti-TB drugs exactly as prescribed in accurate dosage. Patients with pulmonary TB should receive a regimen containing six months of isoniazid and rifampicin throughout the treatments in both intensive and continuation phases (WHO, 2010; CDC, 2012). The intensive phase of treatment is given for two months (56 doses) with isoniazid, rifampicin, pyrazinamide, and ethambutol taken daily. Omission of ethambutol is needed in case when the drug

susceptibility studies demonstrated susceptibility. Ethambutol should be replaced by streptomycin in patients with tuberculous meningitis. The continuation phase is given for four months with isoniazid and rifampicin taken daily. However, dosing frequency may vary depending on the treatment conditions. Patients that receive directly observed therapy (DOT) have to take the drugs daily and three times per week in the intensive and continuation phases, respectively. Whereas, patients who receive DOT and either living or not living with HIV prevalent setting have to take the drugs three times per week for both intensive and continuation phases.

Treatment of MDR-TB requires an immediate diagnosis of the disease, fast accurate susceptibility results, and prompt administration of retreatment regimen of sensitive first-line drugs supplemented by second-line drugs (kanamycin, amikacin, capreomycin, ethionamide, ciprofloxacin, ofloxacin, cycloserine) (Bastian and Colebunders, 1999). However, this therapy is prolonged maybe up to 24 months, more expensive, and has multiple adverse effects. Therefore, prevention of MDR-TB is prime importance (Bastian and Colebunders, 1999).

Directly observed therapy strategy had been implemented as a prime management strategy for all TB patients (CDC, 1993). DOT involves providing the anti-TB drugs directly to the patients and watching them as the patients take the medicines (Bastian and Colebunders, 1999). Lastly, prevention with regards to vaccination has been established by the use of the Bacillus Calmette-Guerin (BCG) vaccination, which had been introduced since 1920s in many countries to combat TB (WHO, 1992; Madigan *et al.*, 2012b).

2.2 PLANTS AS A SOURCES OF ANTI-TUBERCULAR AGENTS

Over the years, plants have been used widely as a major source of medicines to cure a variety of diseases in almost all cultures worldwide (Arya, 2011). All plants containing active compounds are useful as remedies of ailments and different plant species possess their own medicinal actions (Parekh *et al.*, 2005). Pure compounds of natural products from medicinal plants provide wide opportunities for new drug leads due to the availability of phytochemical diversity. The advantageous medicinal effects of plant materials result from the merger of secondary metabolites that are present in the plants such as alkaloids, steroids, tannins, and phenol compounds (Balandrin *et al.*, 1985; Parekh *et al.*, 2005).

Compounds from natural products that possess anti-TB activity have the potential for new drugs discovery that could be active against multiplying and dormant bacilli (Palomino *et al.*, 2009). Numerous research reports on the variety of anti-tubercular plant species have been shown to display anti-TB activity (Copp, 2003; Gautam *et al.*, 2007; Arya, 2011). Large numbers of plant extracts and pure compounds isolated have been reported to exhibit inhibitory effect on the growth of *M. tuberculosis* and also to its related species (Newton *et al.*, 2002; Copp and Pearce, 2007).

2.3 *Pluchea indica*

2.3.1 General characteristics

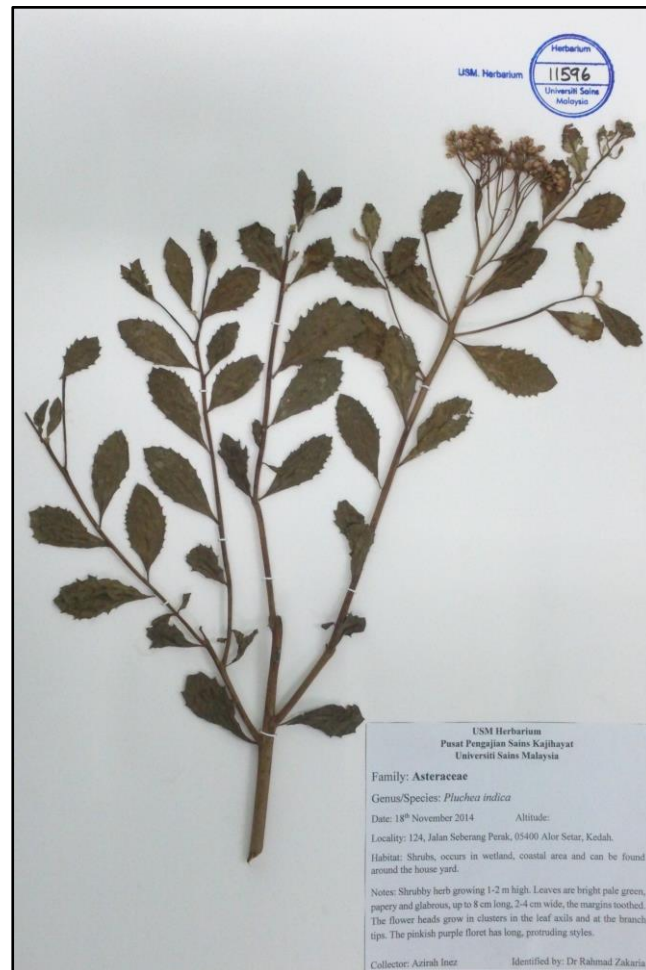
Pluchea indica (L.) Less. (Figure 2.6) or locally known as ‘beluntas’, belongs to family of Asteraceae (Noridayu *et al.*, 2011). It is a perennial shrub, which is commonly distributed in South-East Asian countries and has been used widely as a



a



b



c

Figure 2.6. *Pluchea indica* (a) plant, (b) leaves and flowers, and (c) herbarium specimen.

medicinal plant (Sen *et al.*, 2002; Ohtsuki *et al.*, 2008; Noridayu *et al.*, 2011; Cho *et al.*, 2012). It branches up to 2 metres tall and the leaves are bright pale green, papery and glabrous, up to 8 cm long, 2-4 cm wide, with toothed oval leaf blades. The flowers grow in clusters in the leaf axils at the branch tips and the pinkish purple florets have long, protruding styles. *P. indica* mainly grows in wet saline coastal habitat such as brackish marshes and mangrove swamps and can easily colonize the coastal habitat ("*Pluchea indica* (L.) Less., Asteraceae," 2013). In Peninsular Malaysia, the plant is cultivated in courtyard for its young shoots, which can be eaten raw as a vegetable or called as 'ulam' (Sen *et al.*, 2002; Noridayu *et al.*, 2011; Cho *et al.*, 2012).

2.3.2 Traditional therapeutic uses

P. indica is a traditional remedy used for the treatment of haemorrhoids, lumbago, leucorrhoea and inflammation (Buapool *et al.*, 2013). *P. indica* is also used as medicinal supplement to treat and prevent diabetes by consuming the leaf as a tea or as a health promoting drink among Indonesians (Arsiningtyas *et al.*, 2014). The leaves and roots of *P. indica* possess anti-ulcer, astringent and antipyretic properties and are also used as a diaphoretic in fevers (Bandaranayake, 2002). The poultices of *P. indica* fresh leaves are used against atonic and gangrenous ulcers (Mukhopadhyay and Cordell, 1983). The cigarettes prepared from the chopped stem bark are smoked to relieve sinusitis pain (Bandaranayake, 2002). In Indo-China, the leaves and young shoots are crushed and mixed with alcohol before applying to the back for lumbago cases and are also used to relieve pain of rheumatic, and used in baths to treat scabies (Mukhopadhyay and Cordell, 1983). The leaves of *P. indica* are also used traditionally to cure symptoms of TB such as cough, decrease fever and increase appetite (Mohamad *et al.*, 2011; Suriyaphan, 2014).

2.3.3 Phytochemical contents

Phytochemical studies have revealed that extracts of *P. indica* plant parts contain saponins, tannins, flavonoids, alkaloids, glycosides, antocyanins, β -carotene, carotenoids, and phenolic compounds (Arya, 2011; Suriyaphan, 2014; Radji *et al.*, 2015). A study done by Cho *et al.* (2012), showed that phytochemical compounds such as tannins, saponins, flavonoids and phenol existed in appreciable amount in *P. indica* root extracts. Quercetin and kaemferol are the major flavonoids, whereas, chlorogenic and caffeic acid are the major phenolic acids found in *P. indica* leaves.

2.3.4 Pharmacological activities

Recent pharmacological studies have demonstrated that aqueous and alcohol extracts of *P. indica* leaves possess significant pharmacological activities such as anti-inflammatory (Buapool *et al.*, 2013), anti-oxidant (Sen *et al.*, 2002; Noridayu *et al.*, 2011), anti-tuberculosis (Mohamad *et al.*, 2011), anti-diabetic (Arsiningtyas *et al.*, 2014), anti-cancer activities (Cho *et al.*, 2012), and anti-venom activity (Gomes *et al.*, 2007).

Buapool *et al.* (2013), studied the anti-inflammatory effect of ethanol extract of *P. indica* leaves and the results showed promising activities on the carrageenan-induced rat hind paw oedema through NF- κ B pathway. NF- κ B is a transcription factor, which is responsible in regulating the genes expressions involved in inflammatory responses (Buapool *et al.*, 2013). Carrageenan-induced rat hind paw oedema acts as a model of acute inflammation and had been widely used in the study of anti-inflammatory agents (Panthong *et al.*, 2007; Sae-wong *et al.*, 2009).

Srisook *et al.* (2012), reported that extract from *P. indica* herbal tea exhibit anti-oxidant and anti-inflammatory activities. The *P. indica* herbal tea showed potent

inhibitory effects against lipopolysaccharide-induced nitric oxide and prostaglandin E₂ production in RAW 264.7 macrophages. *P. indica* leaf had been reported to show collagenase inhibitory activity due to 3,4,5-Tri-Ocaffeoylquinic acid and 1,3,4,5-tetra-O-caffeoylquinic acid, which were isolated as constituents from methanol extract of *P. indica* leaf (Ohtsuki *et al.*, 2008).

Anti-TB activity of *P. indica* was justified in previous study by Mohamad *et al.* (2011), which demonstrated that methanol extract of *P. indica* leaf and flower were active against *M. tuberculosis* H37Rv standard strain with minimal inhibitory concentration (MIC) of 800 µg/mL each. The extracts were further assayed, and the results showed that the n-hexane fractions exhibited promising anti-tubercular activity with MIC of 50 µg/mL (Mohamad, 2014). Other study by Radji *et al.* (2015) showed that aqueous extract of *P. indica* also demonstrated bactericidal effects against *M. tuberculosis* H37Rv and MDR strain.

Caffeoylquinic acid derivatives isolated from *P. indica* leaf were shown to be responsible for inhibitory activity of intestinal maltase, which is involved in anti-diabetic activities (Arsiningtyas *et al.*, 2014). The aqueous extract of *P. indica* leaves and roots also showed potential anti-cancer agent by involving in suppression of proliferation and migration activities of the cancer cells (Cho *et al.*, 2012). In addition, the methanol extract of *P. indica* roots was also able to neutralize viper venom and counter venom-induced lethality (Gomes *et al.*, 2007), and exhibited anti-amoebic activity (Biswas *et al.*, 2007).

2.4 STRATEGIES FOR ANTI-TUBERCULOSIS DRUG DEVELOPMENT FROM PLANTS

The existence of standard regimens of TB treatment together with the implementation of DOT strategy are core components of success to control TB (WHO, 2014). However, TB remains one of the world's fatal contagious diseases with the evidence of the latest year's report by (WHO, 2015), which showed an increase in global incidences for new TB cases and deaths in 2014.

A growing problem is that the etiologic agent of TB becomes resistant to the major drugs used in TB treatment such as rifampicin and isoniazid due to the ineffective treatment, which fosters the emergence of drug-resistant strains of *M. tuberculosis* (WHO, 1992). The obstacle towards successful TB treatment with current TB drugs is the duration and complexity of the treatment regimens such as drugs quantity, dosages and their adverse reactions, which negatively influence patient adherence and cause the emergence of drug resistant TB (Laurenzi *et al.*, 2007). Hence, development of new drug targets is needed to treat the *Mycobacterium* resistance strains so that TB epidemic is under control (Rohini and Srikumar, 2013). The strategies in the development of drugs is by initially screening against the organisms and then screening against specific biochemical targets, which involves *in vitro* assay against the target organism (Mohamad, 2014).

2.4.1 Target organisms

Basically, the etiologic agent of TB, *M. tuberculosis* is the best target organism. The target organism that is extensively used in biomedical research is the standard strain of *M. tuberculosis* H37Rv because it retains full virulence in animal models of TB and susceptible to drugs and amenable to genetic manipulation (Cole *et*

al., 1998). However, it has many limitations such as its slow generation time (about 24 hours), which needs about three to four weeks to yield visible colonies on a plate (Reyrat and Kahn, 2001) and it is highly virulent (Smith, 2003). In the absence of strict containment facilities of at least biosafety level 3 laboratory to handle such infectious bacteria, other options of target surrogate organisms have to be selected. Surrogate organism is defined as an indicator or substitute for an organism of interest (Sinclair *et al.*, 2012). Surrogate microorganisms for *M. tuberculosis* should be harmless microbes, easy to handle, with correlated survival and growth parameters and display a profile similar to the target pathogen. In most research studies, the alternative targets organisms used are slow-growing mycobacteria such as *M. tuberculosis* H37Ra and *M. kansasii*; and rapid-growing mycobacteria such as *M. smegmatis* and *M. fortuitum* (Soto *et al.*, 2002; Philips *et al.*, 2005; Nguyen Thi *et al.*, 2010; Wang *et al.*, 2015).

Mycobacterium smegmatis

Mycobacterium smegmatis is a model mycobacterial system, a non-pathogenic and fast-growing soil bacterium that shares many features and identical genomic sequences with the pathogenic *M. tuberculosis* (Wallace *et al.*, 1988; Wang *et al.*, 2005; Cayabyab *et al.*, 2006). *M. smegmatis* also displays a profile similar to MDR *M. tuberculosis* in terms of susceptibility for two first-line anti-TB drugs: isoniazid and rifampicin (Chaturvedi *et al.*, 2007). *M. smegmatis* is suitable for research in normal laboratories, cost effective, time-saving with a short generation time (about 3 to 4 hours) and also has to have the advantages for TB vaccine development (Reyrat and Kahn, 2001; Nguyen Thi *et al.*, 2010).