ANTI-MYCOBACTERIAL ACTIVITY OF *Labisia pumila* Benth. & Hook. f. AND ITS BIOACTIVE CONSTITUENTS AGAINST SURROGATE TUBERCULOSIS ORGANISMS

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by

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DEDICATION

Dedicated to my beloved mother, Daharah; my late father, Jamaludin; my dear husband, Muhammd Firdaus and our beloved daughter, Nur Insyirah. I am truly thankful for having all of you in my life and may Allah grant us with Jannah. Amin.

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Bismillahirrahmanirrahim. In the name of Allah, The Most Gracious, Most Merciful. Selawat and salam for our Holy Prophet Muhammad s.a.w.

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LIST OF ABBREVIATIONS

µg	Microgram
µL	Microlitre
µm	Micrometer
ADC	Albumin, dextrose and catalase
AG	Arabinogalactan
AIDS	Acquired immune deficiency syndrome
ATCC	American Type Culture Collection
BCG	Bacillus of Calmette-Guerin
CDC	Centers for Disease Control and Prevention
cfu	Colony forming unit
CO_2	Carbon dioxide
DMSO	Dimethyl sulphoxide
EMB	Ethambutol
EPTB	Extra pulmonary TB
FICI	Fractional inhibitory concentration index
g	Gram
GC-MS	Gas chromatography-mass spectrometry
HIV	*Human immunodeficiency virus*
INH	Isoniazid
L	Litre
LTBI	Latent TB infection
MA	Mycolic acids
MAPc	Mycolyl-arabinogalactan-peptidoglycan complex

MBC	Minimal bactericidal concentration
MB 7H9	Middlebrook 7H9 broth
MB 7H10	Middlebrook 7H10 agar
MDR-TB	Multi drug resistant-TB
MIC	Minimal inhibitory concentration
mL	Mililitre
MTT	Thiazolyl blue tetrazolium bromide
NCTC	National Collection of Type Cultures
OADC	Oleic acid, albumin, dextrose and catalase
O_2	Oxygen
^{o}C	Degree celcius
PBS	Phosphate buffer saline
PG	Peptidoglycan complex
PTB	Pulmonary TB
R_f	Retention factor
RIF	Rifampicin
RT	Retention time
STR	Streptomycin
TB	Tuberculosis
TEM	Transmission electron microscopy
TEMA	Tetrazolium microplate assay
TLC	Thin layer chromatography
UV	Ultra-violet
v/v	Volume by volume
XDR-TB	Extensively-drug resistant TB

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AKTIVITI ANTI-MIKOBAKTERIA *Labisia pumila.* Benth. & Hook. f. DAN SEBATIAN BIOAKTIFNYA TERHADAP ORGANISMA PENGGANTI TUBERKULOSIS

ABSTRAK

Kemunculan strain tuberkulosis (TB) rintang pelbagai drug menjadi faktor utama yang menyumbang kepada peningkatan jangkitan TB secara global. Oleh yang demikian, keperluan yang mendesak bagi drug anti-TB yang efektif, selamat dan murah telah mendorong banyak kajian dijalankan terhadap produk semulajadi termasuk tumbuhan. Bahagian daripada *Labisia pumila* telah dikaji untuk mengenal pasti potensi anti-mikobakterianya terhadap organisma pengganti *Mycobacterium tuberculosis* (*M. smegmatis, M. fortuitum* dan *M. kansasii*) menggunakan asai pencairanmikro tetrazolium. Bahagian paling aktif telah dihasilkan oleh n-heksana batang-akar dan daun terhadap *M. kansasii* dengan kepekatan perencat minima (MIC) 50 - 200 μg/mL. Bahagian ini telah dipilih untuk proses fraksinasi berpandukan bioasai menggunakan teknik kromatografi turus yang telah menghasilkan 20 jenis fraksi yang berbeza. Fraksi ini telah diuji terhadap *M. kansasii* dan fraksi R2, R6 dan L7 telah mempamerkan aktiviti paling memberangsangkan (MIC: 25 - 50 μg/mL). Ujian saringan lanjutan terhadap fraksi aktif ini telah dijalankan terhadap strain *M. tuberculosis* H37Ra dan nilai MIC yang dihasilkan masing – masing adalah 12.5, 50 dan 100 μg/mL. Analisis fitokimia terhadap fraksi paling aktif, R2 menggunakan komatografi gas-spektrometri jisim telah mengenalpasti tiga sebatian utama; asid vasenik, asid oktadekanoik dan asid 10-heptadesenoik. Aktiviti sebatian ini terhadap *M. tuberculosis* H37Ra telah

mempamerkan nilai MIC 100 – 400 µg/mL, menunjukkan aktiviti yang rendah berbanding fraksi asal R2 (MIC: 12.5 µg/mL). Kajian interaksi pecahan R2 dengan drug anti-TB barisan pertama terhadap *M. kansasii* dan *M. tuberculosis* H37Ra menggunakan kaedah papan catur telah menghasilkan satu interaksi sinergistik dengan rifampisin terhadap *M. tuberculosis* H37Ra dengan nilai indek kepekatan perencat fraksi (FICI) 0.375, aktiviti individu fraksi dan drug meningkat masing – masing sebanyak lapan dan empat kali ganda. Interaksi sinergi ini diuji secara lanjut menggunakan kaedah masa membunuh dan telah menghasilkan interaksi tambahan dengan 94.46 % kadar pembunuhan pada akhir tempoh kajian. Pemerhatian terhadap sel *M. tuberculosis* H37Ra yang dirawat dengan Fraksi R2 menggunakan mikroskop elektron tranmisi (TEM) menunjukkan pemecahan struktur sel luar dan perubahan bentuk sel tuberkel. Kajian kesitotoksikan Fraksi R2 terhadap warisan sel epitelium nasoforinks, NP69 menunjukkan ia amat sitotoksik dengan nilai IC_{50} sebanyak 7.57 ± 1.75 (purata ± SD) µg/mL. Kesimpulannya, aktiviti anti-mikobakteria yang memberangsangkan oleh *L. pumila* menunjukkan potensinya sebagai sumber ubat untuk rawatan TB.

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ANTI-MYCOBACTERIAL ACTIVITY OF *Labisia pumila* Benth & Hook. f. AND ITS BIOACTIVE CONSTITUENTS AGAINST SURROGATE TUBERCULOSIS ORGANISMS

ABSTRACT

The development of multidrug resistant tuberculosis (TB) strains becomes a major factor contributing to the rise of global TB incidence. Therefore, the urgent need of effective, safe and inexpensive anti-TB drugs has geared many researches towards natural products including plants. Partitions from *Labisia pumila* were investigated to evaluate their anti-mycobacterial potential against *Mycobacterium tuberculosis* surrogate organisms (*M. smegmatis, M. fortuitum*, and *M. kansasii*) using tetrazolium microdilution assay. The most active partitions were produced by n-hexane of stem-root and leaf parts against *M. kansasii* with minimum inhibitory concentrations (MICs) of 50 - 200 µg/mL. These partitions were selected for bioassay guided fractionation process using column chromatographic techniques, which produced 20 different fractions. These fractions were screened against *M. kansasii* and fractions R2, R6, and L7 exhibited the most promising activity (MIC: 25 – 50 µg/mL). Further screening of these active fractions was carried out against *M. tuberculosis* H37Ra strain and the MIC values produced were 12.5, 50 and 100, µg/mL, respectively. Phytochemical analysis of the most active R2 fraction using gas chromatography-mass spectrometry resulted in the identification of three major compounds; vaccenic acid, octadecanoic acid, and 10-heptadecenoic acid. The activity of these compounds against *M. tuberculosis* H37Ra exhibited MIC values 100 - 400 µg/mL, displaying lower activity compared to their original fraction R2

(MIC: 12.5 µg/mL). Interaction study of R2 fraction with first-line anti-TB drugs against *M. kansasii* and *M. tuberculosis* H37Ra using checkerboard method produced one synergistic interaction with rifampicin against *M. tuberculosis* H37Ra, with fractional inhibitory concentration index (FICI) value of 0.375, increasing the individual activity of fraction and drug by eight and four folds, respectively. This synergistic interaction was further verified using time-kill assay and produced an additive interaction of 94.46 % killing rate at the end of the study period. The observation of *M. tuberculosis* H37Ra cells treated with R2 fraction using transmission electron microscopy (TEM) showed the breakage of the outer cell structure and deformation of the tubercle cells. Cytotoxicity study of R2 fraction against nasopharyngeal epithelial cell line, NP69 revealed that it was highly cytotoxic with IC_{50} value of 7.57 ± 1.75 (mean ± SD) µg/mL. In conclusion, the promising anti-mycobacterial activity of *L. pumila* indicates its potential as a source of remedy for the treatment of TB.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB) remains one of the most common infectious pathogens of mankind that infects one third of the world's population (Stewart *et al.*, 2003). *M. tuberculosis* usually infects the lungs (pulmonary TB) and can disseminate to other sites of the body (extrapulmonary TB). These bacteria can also remain dormant in the body for years asymptomatically before causing active infection. Now, TB infection becomes a new leading cause of death globally together with *human immunodeficiency virus* (HIV). The World Health Organization (WHO) estimated that, there were 1.2 million deaths and 9.6 million people became sick with TB worldwide in 2014 (WHO, 2015a). Out of these estimates, six million were new TB cases and about 480,000 cases of multidrug-resistant TB (MDR-TB).

The global emergence of MDR-TB and extensively drug-resistant TB (XDR-TB) strains becomes a serious threat to human health (de Araujo-Filho *et al.*, 2008). The percentage of patients infected with TB that cannot be cured with current therapeutic regimen is increasing annually. Unfortunately, there have been no new drugs being developed against TB since the 1960s (Todar, 2004). These limited discoveries of new drugs from natural and synthetic compounds are mainly caused by the slow growth rate, biosafety concern and other issues that are related to *Mycobacterium* species (Primm & Franzblau, 2007). Therefore, urgent actions must be taken in order to combat this disease, including antibiotic control usage, continuous research on the genetic mechanisms of the bacterial resistance and

development of new drugs, either synthetic or from natural products (Nascimento *et al.,* 2000).

Nowadays, studies based on the extraction of biologically active compounds from plant species for medicinal purpose are becoming prominent. Natural products from plants contain a wide range of substances and continue to play significant role in drug discovery and development (Alam *et al.,* 2009). About 80 % of the world's population rely on the use of traditional medicine, which are predominantly plant based (Obeidat *et al.,* 2012). There are more than 100 plant species in Malaysia reported to have medicinal properties as remedies in the treatment of human diseases and some microbial infections (Perumal *et al.,* 2012). One famous plant is *Labisia pumila* (Benth).

L. pumila is commonly known as Kacip Fatimah in Malaysia. It belongs to the Mysinaceae family. It is a small sub-herbaceous plant with leafy appearances that can be found mainly in the low land and hill forest of Malaysia and Indonesia (Shahrim *et al.,* 2006). Almost all parts of *L. pumila* are used in folk medicine. It is commonly used to induce childbirth, ease the pain of delivery, and to treat a variety of illnesses including dysentery, flatulence, rheumatism, gonorrhoea and dysmenorrhoea (Ibrahim & Jaafar, 2011). Extracts from *L. pumila* have been shown to possess inhibitory activity against many aerobic microorganisms (Karimi *et al.,* 2011). However, there is limited finding based on scientific research to claim the anti-mycobacterial activities of this plant. In a recent study, Mohamad (2014) observed that the non-polar n-hexane partition of root and leaf parts of *L. pumila* exhibited promising anti-TB activity (MIC: 50 µg/mL and 100 µg/mL, respectively) against *M. tuberculosis* H37Rv, which was worthy of further investigation. Therefore, based on the approach "follow-up of anti-microbial activity reports", the

main aim of this study was to further investigate the anti-mycobacterial activity of this plant against non-pathogenic surrogate TB organisms, *M. smegmatis*, *M. fortuitum*, *M. kansasii*, and *M. tuberculosis* H37Ra. This study opted for use of surrogate organisms, since *M. tuberculosis* H37Rv is a virulent strain and requires strict containment facilities for its handling. In order to achieve the main objective of this study several key activities were undertaken including the identification of the active chemical constituents, their interactions with anti-TB drugs, and their cytotoxicity studies.

1.2 Aim and objectives of research

The main purpose of this study was to investigate the anti-mycobacterial property of *L. pumila* using surrogate TB organisms. The specific objectives were:

1. To screen the anti-mycobacterial activities of *L. pumila* partitions against 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 phytochemical constituents of the most active fractions by using gas chromatography–mass spectrometry (GC–MS) and to determine their anti-mycobacterial inhibition.
3. To study the interaction activity of the most active fraction with first-line anti-TB drugs using checkerboard and time-kill methods against *M. kansasii* and *M. tuberculosis* H37Ra.
4. To study the effects of the most active fraction on the ultra-structure of the test organism under transmission electron microscope (TEM) and to investigate their cytotoxicity levels using nasopharyngeal epithelial cell line.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

2.1.1 Overview

Tuberculosis is a serious respiratory disease, which is a major cause of morbidity and mortality worldwide (Chakrabarti *et al.,* 2007). The causative agent of TB, *M. tuberculosis* was first isolated by Robert Koch in 1882 (Cambau & Drancourt, 2014). This contagious disease can be transmitted by airborne particles from throat and lung droplets of active infected person to anyone nearby. Only a few small droplets nuclei containing these bacilli are required for a person to become infected. The inhalation of the tubercle bacilli may lead to one of these possible effects: a) immediate elimination of bacilli, b) latent infection, c) development of primary disease, and d) reactivation of disease after many years of infection (Robert & Wani, 2013).

TB infection can be classified into pulmonary TB (PTB) and extra pulmonary TB (EPTB) (Sharma & Mohan, 2004). PTB commonly affects the lungs and is a highly contagious disease. The infected patients with active PTB may undergo asymptomatic disease, mild or progressive cough, or develop several common symptoms such as fatigue, fever, chill, coughing with bloody sputum, night sweats, and loss of appetite and weight (Crane, 2013). Unlike PTB infection, EPTB occurs in organs other than the lungs and is usually not infectious. The common sites of EPTB infection include lymph nodes, osteoarticular areas, pleura, and bones meninges (Golden, 2005). The infection of EPTB has become more prevalent in patients with HIV infection. The symptoms of EPTB vary by site of infection, but commonly

include abdominal pain, confusion, seizures, and persistently swollen gland and headache (Rieder *et al.*, 1990, National Health Service (NHS), 2014).

The infection of *M. tuberculosis* commonly results in either latent infection or active TB infection. In latent TB infection, the infected persons do not develop any clinical signs or symptoms and are not contagious to others. The latency period signifies the majority of infected individuals in a state, in which the host manage to control the infection from developing inside the body (Lin & Flynn, 2010). Once the immune system of infected persons fails in some ways, the reactivation of TB occurs, resulting in the spread of this contagious pathogen (WHO, 2009a). The uncontrolled misused and mismanagement of drug treatment can result in the development of drug-resistant TB. The emergence of MDR-TB and XDR-TB has further become a serious threat to the global public health (Centres for Disease Control and Prevention (CDC), 2015).

2.1.2 Epidemiology of tuberculosis

Epidemiology study of TB is crucial for effective measure in controlling the complexity of disease outbreak. TB is declared as a major cause of death around the globe and becomes a global health burden. According to Global Tuberculosis report (2015), in 2014, there were 9.6 million new TB cases, with 1.5 million deaths inclusive of 1.1 million among HIV-negative people and 0.4 million among HIV-positive people (WHO, 2015a). From these values, over 95 % of the death cases occur in low and middle income countries. Globally, the largest burden of TB with 58 % of new cases occurred in the South East Asian and Western Pacific regions (Figure 2.1). African region accounted for the highest TB burden, with 281 cases for every 100,000 population compared to global average of 133 cases. These reports

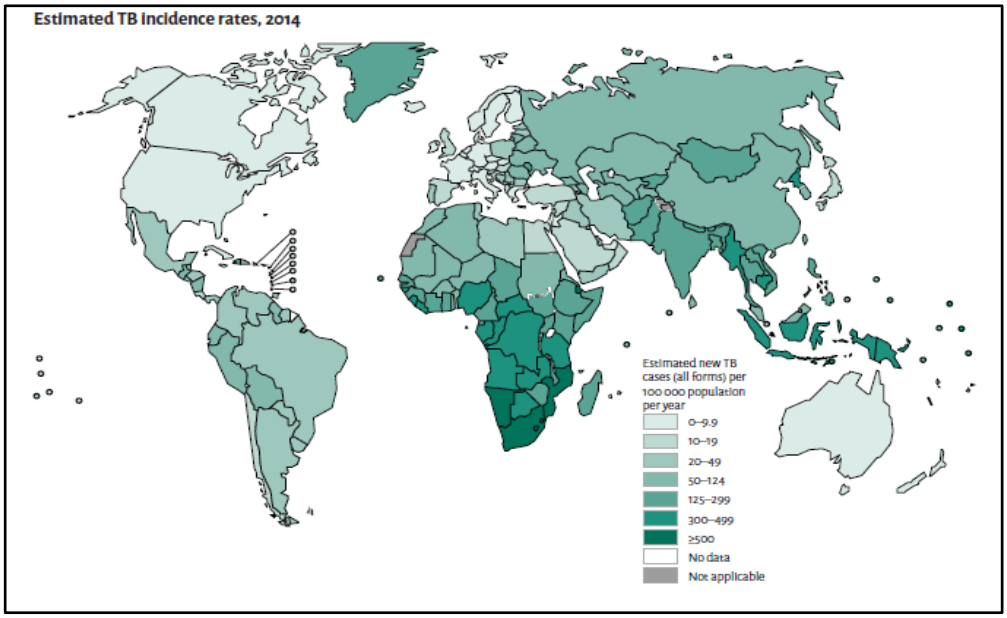

Figure 2.1. Estimated global TB incidence rate, 2014.
(WHO, 2015a)

also estimated that over one million children became sick and 140,000 children died of TB. In addition, almost 480,000 people who developed MDR-TB were reported worldwide. The latest report in 2015 showed that the statistic of death ratio among TB co-infection with HIV positive patients was 1 in every 3 deaths, making TB as one of the leading killers among the HIV patients (WHO, 2015a).

In Malaysia, an estimated number of 2400 deaths caused by TB infection, excluding co-infection with HIV patients were reported in 2014 (WHO 2015a). Approximately 78,000 cases of all form of TB were notified and 24,054 new TB cases and relapse were reported (Table 2.1).

Table 2.1. Estimated TB burden in Malaysia, 2014.
(WHO, 2015a)

Population 2014		**30 million**
Estimates of TB burden *2014	Number (thousand)	Rate (per 100 000 population
Mortality (excludes HIV + TB)	24 (1.3-3.7)	8 (4.5-12)
Mortality (HIV + TB only)	0.63 (0.38-0.93)	2.1 (1.3-3.1)
Prevalence (includes HIV +TB)	40 (19-69)	135 (63-232)
Incidence (includes HIV + TB)	31 (25-37)	103 (83-124)
Incidence (HIV + TB only)	22 (1.8-2.6)	7.3 (5.9-8.8)
Case detection, all form	**78 (65-96)**	

Estimates of MDR-TB butden *2014	New	Retreatment
% of TB cases with MDR-TB	0.4 (0.24-0.63)	1.1 (0.24-0.33)
MDR-TB case among notified pulmonary TB cases	80 (48-120)	20 (4-56)

TB case notification 2014	New**	Relapse
Pulmonary, bacteriologically confirmed	14099	749
Pulmonary, clinically diagnosed	5743	300
Extrapulmonary	3055	108
Total new and relapse	24054	
Previously treated, excluding relapse	657	
Total case notified	**24711**	

Among 24 054 new and relapse cases:
691 (3%) cases aged under 15 years; male:female: 1: 7

2.1.3 The tubercle bacteria

Mycobacterium species belong to the family of Mycobacteriaceae and was first discovered by Hansen in 1868, who isolated *M. leprae* and followed by Robert Koch in 1882, who isolated *M. tuberculosis* (Cambau & Drancourt, 2014). *M. tuberculosis* is a non-motile, rod-shaped, slow-growing and facultative intracellular pathogen, which can survive and thrive in the host's macrophages for a long period and under different stress conditions. Under favourable condition, the generation time of *M. tuberculosis* is between 18 - 24 hours and produces white to light-yellow colony within 3 - 4 weeks on media plates (Gengenbarcher & Kaufmann, 2012).

The term 'myco' means fungus to illustrate the characteristics of mycobacteria that grow in mould-like fashion in liquid culture media (James, 1994). The growth features of mycobacteria are significantly different compared to other

bacteria species, which are probably influenced by their highly packed hydrophobic cell wall. The cell wall structures are divided into inner and outer layers that surround the plasma membrane, which make up a rigid cell wall. The outer layer compartments consist of mainly proteins and lipids component, which are freely linked with the cell wall (Brennan & Nikaido. 1995; Draper, 1998).

The inner compartments are divided into three components; peptidoglycan (PG), arabinogalactan (AG), and mycolic acids (MAs), which are covalently linked together to form mycolyl-arabinogalactan-peptidoglycan complex (MAPc). These components are arranged accordingly starting from PG and ending with MAs, which surround the plasma membrane (Brennan, 2003). Figures 2.2 illustrates the structural model of the mycobacterial cell wall. PG or murein, is a polymer that consists of peptides and glycan strands attached to the plasma membrane. PG forms a rigid skeletal backbone of the cell envelope by maintaining the cell shape and thus, protects the cell from osmotic pressure. AG is made up of biopolymer polysaccharides, which consist of arabinose and galactose monosaccharaides. AG helps in maintaining the cell wall integrity and acts as an attachment site for MAs and peptidoglycan by covalent bonding. MAs are long chain fatty acid, which may represent up to 40 % of the cell dry mass (Crick *et al.*, 2001). The structures are arranged compactly as monolayer and consist of aliphatic side chain at the position C_2 and high molecular weight of 3-hydroxy fatty acid. The molecular mass of mycolic acids of mycobacterial varies from C_{60} to C_{90} (Dworkin & Falkow, 2006). The special constituents of mycolic acids affect the permeability of the cell surface, which contributes to the hydrophobic nature of the cell wall. These characteristics also contribute to its distinctive property of acid fastness due to their impermeability

by certain dyes and stains such as Gram staining and required special staining method of the Ziehl-Neelsen (Daffe & Reyrat, 2008).

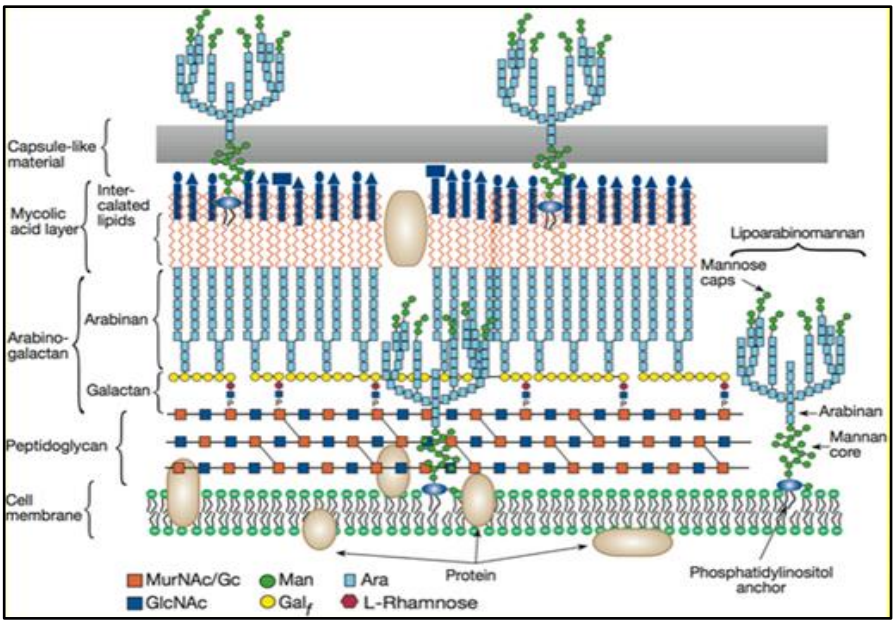

Figure 2.2. Structural model of the mycobacterial cell wall.
(Rolfe, 2015)

2.1.4 Pathophysiology of tuberculosis

The progression of the disease depends mainly on prior exposure, type of strain, vaccination, infectious dose, and the immune system of the host (Todar, 2004). The inhalation of droplets containing tubercle cells may or may not cause the infected person to develop the clinical symptoms (Table 2.2). Basically, the TB infected persons may encounter several possible outcomes including: a) immediate destroyal of TB bacilli by the host's primary immune system responses, b) the development of active TB, probably by a person with lack of ability to control the infection, and c) development of latent stage infection (Flynn & Chan, 2001).

Table 2.2. The similarities and differences of tuberculosis infection and disease. (Todar, 2004)

Tuberculosis infection	Tuberculosis disease in lungs
M. tuberculosis present	*M. tuberculosis* present
Tuberculin skin test positive	Tuberculin skin test positive
Chest X-ray normal	Chest X-ray usually reveals lesion
Sputum smears and cultures negative	Sputum smears and cultures positive
No symptoms	Symptoms such as cough, fever, weight loss
Not infectious	Often infectious before treatment
Not defined as a case of TB	Defined as a case of TB

Initially, after inhalation of airborne droplet nuclei, the infection of *M. tuberculosis* occurs in an alveolar macrophage where the replication occurs (American Thoracic Society (ATS), 2000; Frieden *et al.,* 2003). This replication triggers the cytokine formation and thus, initiates the production of inflammatory response. In response, host's macrophages and lymphocytes migrate to the infection site and form granulomas to prevent further dissemination of *M. tuberculosis* to other organs. The formation of granuloma also helps to establish direct immune reaction at the site of infection. At this stage, the granulomatous lesion consists of *M. tuberculosis*, which is in inactive state and remains dormant for some time (Knechel, 2009).

M. tuberculosis can exist in different states during infection (Nguta *et al.,* 2015). The different states describe the ability of *M. tuberculosis* to survive in host's primary immune cell for a long period and under different stress conditions. The latency period or latent TB infection (LTBI) is a condition when the infected person with TB does not produced any symptoms or disease. The inactive TB infection occurs in the early stage of infection when the host's cell-mediated immune response protects and limits the progression and dissemination of *M. tuberculosis*. In addition, the triggered host's immune also help in latency period of infection and sustaining

the long term immune protection (Ewer *et al.,* 2006). The treatment for LTBI is slow and challenging to accomplish. Without suitable prescription, the risk of progression from LTBI to active symptomatic TB infection will increase. The reactivation of LTBI occurs after several years or decades after subclinical infection. About 10 % of people infected with LTBI will develop the active TB in their life time (WHO, 2015a). However, the risk of activation is higher in immunocompromised patients with co-infection with HIV/acquired immunodeficiency syndrome (AIDS), diabetes, obesity, and chronic inflammatory disease (Barry *et al.,* 2009). Active pulmonary TB occurs when the *M. tuberculosis* is able to overcome the immune response and begins to multiply and spread, resulting in the development of the symptomatic TB (CDC, 2016).

2.1.5 Diagnosis of tuberculosis

According to WHO, it is estimated that 43 million lives were saved from successful TB diagnosis and treatment between 2000 and 2014 (WHO, 2015a). The selection of suitable diagnosis for TB infection should include the purpose for testing, overall cost, effectiveness of examination, and test availability (CDC, 2012a). A complete diagnosis for TB infection includes medical history, chest radiograph, physical examination, tuberculin skin test, diagnostic microbiology tests, and serological tests (Jeong & Lee, 2008).

The information about the medical history of the infected patients helps the clinicians to specify a suitable diagnosis, depending on the type of TB suspected. The history includes: a) prolong symptoms of active TB, e. g. cough, fatigue, chest pain, fever, or night sweats; b) recent exposure with a person who might be infected with TB; c) recent travel to high-incidence countries; d) medical condition, e. g. HIV

infection, e) demographic factors, e. g. occupation, age, ethnic or country of origin, and f) past TB history (National Institute for Health and Clinical Excellence (NICE), 2011; WebMD, 2014).

A physical examination provides useful information about the overall condition of the patient and helps to identify the disease progression that may determine the TB treatment plan. Chest radiography is an essential diagnosis method to identify the possibility of pulmonary TB infection. Radiographic of posterior and anterior segment of the lungs is used to identify chest abnormalities related to TB infection (Leung, 1999). These abnormality changes are usually associated with reactivation of TB infection, which causes fibronodular infilterate, specifically in the upper posterior parts of the lungs. However, the radiographic abnormalities of primary TB infection are more difficult to distinguish from other pulmonary infections (Wilkins *et al.,* 2007). Although abnormalities are detected on chest radiography, these results may suggest TB infection, but not as a confirmation of the disease. Thus, chest radiographs may be important to detect the probability of pulmonary TB infection, especially in a person with positive reaction towards tuberculin skin test with asymptomatic TB (CDC, 2011).

The Mantoux tuberculin skin test (TST) is the standard method that is used widely for the detection of LTBI (Nayak & Acharjya, 2012). This method involves the intracutaneous injection of purified protein derivatives (PPD) into inner part of the arm. The reaction is measured in millimetres of the induration, which involves visible raised, hardened area or swelling after 48 - 72 hours of administration. The size of induction between 5 – 15 mm is classified as a positive result, which indicates TB infection (CDC, 2012b).

Diagnostic microbiological tests of TB diagnosis are necessary to detect the presence of acid-fast-bacilli (AFB) from sputum smear and other specimens (Mathew *et al.,* 2012). The microbiological testing of TB involves the isolation, detection and identification of mycobacteria species, type of drug resistance, and observation after prescription. Even though acid-fast microscopy is relatively a rapid and easy method, it is not specific because the results produced are unable to distinguish between MTB and non-tuberculous strain (Hale *et al.,* 2001).

New advanced serological and molecular diagnostic techniques for TB detection include polymerase chain reaction (PCR) and interferon-gamma release assays (IGRAs). In PCR assay, nucleic acid of infected patients are amplified and used to distinguish between *M. tuberculosis* and other mycobacteria strains based on their genetic information (Knechel, 2009). Meanwhile, in IGRAs, whole-blood tests from infected patients are used to determine the immune reactivity towards *M. tuberculosis* (CDC, 2012a).

2.1.6 Treatment and prevention of tuberculosis

Mycobacterium tuberculosis remains a major cause of infectious disease worldwide although chemotherapy and vaccination are available as control measures. An estimated 47 % of TB death rates dropped between 1990 and 2015 (WHO, 2015a). The standard treatment for TB infection requires multiple drugs treatment, which consists of several antibiotics in fixed-dose combination. The multiple drug therapy is important to prevent the development of drug-resistant mutants that might occur during the treatment period. In addition, multiple drug therapy is able to increase the bactericidal effect and also reduce the toxicity level of drugs by lowering the drug consumption dosage (Blomberg *et al.,* 2001).

Treatment regimen for new TB patients with drug susceptible TB requires an intensive initial phase of two months, which consists of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA), and ethambutol (EMB) followed by a continuation phase of INH and RIF of either four or seven months. Once the drug susceptibility test is known and fully susceptible, EMB can be excluded from the continuation phase (Morbidity and Mortality Weekly Report (MMWR), 2003; WHO, 2009b). However, a more complex and longer treatment period are needed to manage resistant strains of MDR-TB and XDR-TB (Gupta & Bakhta, 2012). MDR-TB strains are resistant to RIF and INH while XDR-TB strains have additional resistance to any fluoroquinolones and to at least one of the three injectable second-line anti-TB drugs (amikacin, kanamycin, or capreomycin) (Youm & Saier, 2012).

A successful treatment is important in the effort to control and manage TB. Effective vaccination strategy is needed to prevent incidence of TB. The invention of TB vaccine in 1921 called bacille Calmette-Guérin (BCG) may help in preventing severe TB infection in children. However, BCG is unable to protect adults from TB infection especially against pulmonary TB (Barreto *et al.*, 2006). Therefore, there is an urgent need for new vaccine, which is safer and effective to protect against all forms of TB including infection, disease, and relapse (Tiwari *et al.*, 2011). Until recently, 16 different new TB vaccine candidates are now in clinical trials and preclinical development (WHO, 2015b). These vaccines were generated from whole cell mycobacteria and various subunits of *M. tuberculosis* antigens, which are expressed as recombinant proteins.

2.2 Natural products as source of therapeutic agents

Plants and herbs are the earliest sources of medicine and have been used for thousands of years with great contributions as therapeutic properties. In Asian countries, the traditional therapies include ayurvedic medicine (India), traditional chinese medicine, jamu (Indonesia), korean chinese medicine and japanese medicine (kampo) (Itokawa *et al.,* 2009). According to WHO, it is estimated that 80 % of some Asian and African countries depend on traditional medicine as primary health care, while in developed countries, the traditional medicine is used as alternative or complementary medicine (WHO, 2008).

There are many reasons for using plants as natural source of therapeutic agents, which include a) as herbal remedy by using whole or part of plant, e.g. garlic; b) as direct drug usage by isolation of bioactive compounds, e.g. morphine, digoxin; c) as lead compounds for designing of semi-synthetic drugs, which produce patentable drugs with lower toxicity and/or higher activity from bioactive compounds of novel or known compounds structures, e.g., metformin, nabilone; and d) as pharmacologic tools agents, e.g., mescaline lysergic acid diethylamide (Daniel *et al.,* 2001). The common advantages of using medicines derived from plant product are because they are safer than other synthetics prescription, more economical and cover wider therapeutic benefits.

Plant derived medicines offer great contribution towards human health. The abundant distributions of plant diversity especially higher plant in tropical countries provide most of these therapeutics agents (Owolabi *et al.,* 2007). The presence of secondary metabolites such as, flavonoids, tannins, terpenoids, and alkaloids are the most important active substances with anti-microbial properties (Obeidat *et al.,* 2012). These substances are believed to act as constitutive chemical barriers of plant

defence mechanism against herbivores and insects or even protect the plants from microbial attack and other inducible anti-microbials (Cowan, 1999). Plant based anti-microbial property becomes the most important target source for new drug development. However, further research of their therapeutic potential needs to be carried out to identify their benefits and side effects. The multiple reactions from plant therapeutics on the human body should be investigated before using any plant as treatment. This is mainly due to the multiple actions of plant metabolites, which usually react beyond the treatment of diseases (Iwu *et al.*, 1990).

Based on several studies, therapeutic value of ethnobotanical plants regains much interest in many researches to identify and isolate natural compounds that exhibit certain activity against mycobacteria (Jimenez-Arellanes *et al*., 2003; Ramos *et al*., 2008; Gupta *et al*., 2010). In a previous study, selected Malaysian plants that are traditionally used to treat respiratory diseases and symptoms of TB were screened to investigate their therapeutics property against *M. tuberculosis* (Mohamad *et al.*, 2011). The results of this study showed that 36 local plants exhibited promising anti-TB activity against *M. tuberculosis* H37Rv. This research has provided a good TB database of Malaysian plants that can be selected for further phytochemical and pharmacological investigations.

2.3 *Labisia pumila* as a therapeutic plant

2.3.1 Botanical characteristics

Labisia pumila (Benth) or locally known as Kacip Fatimah (Figure 2.3) is a small herbaceous and slow growing creeping rhizome herb. *L. pumila* belongs to the family of Myrsinaceae, which consists of 30 genera and 1000 species and about 40 species are used in medical field in Asia-Pacific region especially for treatment of

a

b

c

Figure 2.3. *Labisia pumila* (a) whole plant, (b) flowers, and (c) fruits.
Forest Research Institute Malaysia (FRIM), (2012)

inflammatory ailments (Shah *et al.,* 2011). It can be found widely in the lowland and shady areas of forest floors in South East Asian countries.

There are three varieties of *L. pumila* documented in Malaysia, i.e. *L. pumila* var. *alata*, *L. pumila* var. *pumila,* and *L. pumila* var. *lanceolata* (Stone, 1998). Different varieties of *L. pumila* have different petiole and leaf structures (Sunarno, 2005). *L. pumila* var. *pumila* has slightly marginated petiole with ovate leaf blade structure, which is broad rounded at the base and tapered pointed towards the tip. In addition, it has lighter green colour on the abxial surface and dark green colour on adaxial surface. *L. pumila* var. *alata* has broadly winged petiole with distinctive red veins and *L. pumila* var. *lanceolata* has long lanceolate leaf shape with terete petiole characteristics (Abdullah *et al.,* 2013).

2.3.2 Traditional therapeutic uses

Labisia pumila has been widely used by ethnic Malay women to treat many ailments and also for health supplements (Hussain & Kadir, 2013). A water decoction is normally prepared by boiling the leave, root, or all plant parts in plain water, and is consumed as a tonic. *L. pumila* is also consumed to induce and ease the pain of delivery, as a post-partum medication to contract the birth channel and to regain body strength, help to delay fertility, regulate normal menstrual cycle and to prevent menopausal symptoms. Some other folkloric uses include, treating a variety of illnesses such as dysentery, flatulence, rheumatism, gonorrhoea, dysmenorrhoea, and "sickness in the bones" (Jamal *et al.,* 2003; Ibrahim & Jaafar, 2011). This plant is also used for firming and toning the abdominal muscles and to relieve stomach discomfort of babies by rubbing the abdomen with the leaf and coconut oil paste (Bodeker *et al.,* 2009).

2.3.3 Phytochemical contents

Labisia pumila has been reported to contain high levels of bioactive compounds including alkyl compounds, saponins, flavonoids, benzoquinone derivatives, isoflavonoid, and phenolics acids (Abdullah *et al.,* 2013). These bioactive phytochemical contents are responsible towards its wide range of pharmacological activities and medical values. The flavonoid and phenolic contents were reported to be higher in the leaves compared to the other plant parts (Abdullah *et al.,* 2013).

Various flavonoids and phenolic acids are scientifically proven for its anti-oxidative potentials that can protect body cells from free radical effects of reactive oxygen species (Ali & Khan, 2011; Chua *et al.,* 2011). If the anti-oxidant content is imbalanced, the excessive amount of free radicals will enhance the cellular damage of the body that can develop into many diseases such as, autoimmune diseases, inflammation, cancer, arteriosclerosis, cataract, Parkinson's disease, and aging (Kukic *et al.,* 2006). The anti-oxidant properties of these compounds have been reported to be higher than vitamin C, E and carotenoids (Tsao & Deng, 2004; Dai & Mumper, 2010). The anti-oxidant activities from flavonoids also provide important protection against ultra-violet (UV) irradiation that might affect the human dermal fibroblasts (Norhaiza *et al*., 2009; Avula *et al*., 2011). The saponins contents can be found in many parts of the plant including root, leaves, fruits, and stems, which are specifically higher in stem and root parts (Abdullah *et al.*, 2013). Saponin compounds have been reported to be good chemopreventive agents that act as anti-neoplastic, anti-bacterial, and anti-cancer (Laszczyk, 2009).

2.3.4 Pharmacological activities

Previous studies on the activity of methanolic extracts from different varieties of *L. pumila* have shown that the extracts possess active anti-bacterial activity at low concentration against Gram-positive and Gram-negative bacteria (Karimi *et al.,* 2013). However, these plant extracts showed moderate anti-fungal activity. In addition, the same group of researchers also reported the potential *in vitro* effects of three different varieties of *L. pumila* as anti-inflammatory, anti-cancer and cytotoxic effects by using microwave extraction method at different extract concentrations.

L. pumila was also shown to exert phytoestrogen (plant estrogen) property that can be used as an alternative treatment for estrogen replacement theraphy (ERT) (Lelovas *et al.*, 2008). The combining effects of *L. pumila* as anti-inflammatory, phytoestrogenic, and anti-oxidative properties help to overcome the estrogen deficiency problem and thus, it will prevent development of postmenopausal osteoporosis (Nadia *et al.,* 2012).

2.4 Strategies for anti-tuberculosis drug development from plants

2.4.1 Target organisms

The development of new anti-TB drugs requires a high-throughput screening procedure, which is comprehensive and much easier to carry out. Ideally, the test organism should be the etiological agent itself. However, the use of virulent strain of *M. tuberculosis* as the test organism is highly dangerous because it is highly infectious in nature. Hence, the handling of *M. tuberculosis* virulent strain requires a complex and strict containment facility and this poses a major obstacle towards drugs development because many non-clinical laboratories do not have such facilities (Griffths *et al.*, 1998). This has led to the search of suitable candidate organism as a

'surrogate', which can be used as an alternative to substitute the target organism based on its ideal attributes and genetic profiles. In the scope of surrogate TB organisms, the surrogate organisms should be non-virulent strains and safer to handle in biosafety level 2, less hazardous, and more practical for primary screening of inhibitors in early development stages of drug development. The common TB surrogates include *M. smegmatis, M. fortuitum, M. kansasii and M. tuberculosis* H37Ra (Gupta & Bhakta, 2012; Ibekwe & Ameh, 2014).

My*cobacterium smegmatis*

Mycobacterium smegmatis is a unicellular, non-motile and rod shaped bacterium with thick hydrophobic cell wall (Sattar, 2007). *M. smegmatis* can be found in normal human-genital secretions, and also in animal and environments such as, soil, water and plants (Newton *et al.,* 1993). The pathogenicity related to *M. smegmatis* only occurs in patients with weak immune system (Pennekamp *et al.,* 1997). Thus, *M. smegmatis* is classified as non-pathogenic and also a fast growing mycobacteria as its generation time is about three hours under favourable conditions. *M. smegmatis* has been used widely as a model organism to study the basic cellular pattern and physiology process of other pathogenic *Mycobacterium* species such as *M. tuberculosis, M. leprae,* and *M. paratuberculosis* (Provvedi *et al.,* 2008). *M. smegmatis* is also widely used to study the genetic analysis of *M. tuberculosis* due to its ability to grow rapidly and ease of growth and cultivation (Jacobs, 2000). The natural resistance of *M. smegmatis* towards INH and RIF was reported to be identical to MDR-TB strain from clinical isolates with similar sensitivity profile (Wallace *et al*., 1988; Li *et al*., 2004). *M. smegmatis* is also commonly used as a primary screen to select active compounds, which could be active against *M. tuberculosis* (Lu &

Drlica, 2003; Andries *et al*., 2005). Furthermore, the close related protein expression and biosynthetic pathway with other related *Mycobacterium* species especially *M. tuberculosis,* makes *M. smegmatis* a good surrogate to study the behavioural properties of other *Mycobacterium* species (Chaturvedi *et al.,* 2007).

Mycobacterium fortuitum

Mycobacterium fortuitum is a rapid to moderate growing nontuberculous mycobacteria, which are classified in the Runyon group IV and can be found widely in water, soil and dust environment (Choi *et al.,* 1993). *M. fortuitum* produces non-pigmented colonies with three to four hours generation time. *M. fortuitum* is an opportunistic mycobacteria that can cause lung disease resembling TB, lymphadenitis, soft skin and joint infection, catheter infections, eye disease after trauma, and disseminated infection in severe immosuppression, especially in HIV patients (Mary *et al.,* 2006). According to previous studies, *M. fortuitum* had been used widely as surrogate organism based on the good correlations shown between *M. fortuitum* and MTB in susceptibility testing (O'Donnell & Gibbons, 2007; Andrea *et al.,* 2009). In addition, *M. fortuitum* is usually susceptible towards commonly used anti-TB drugs. The rapid growing of *M. fortuitum* allows the test to be accomplished in a short time (Stephen *et al.,* 2005).

Mycobacterium kansasii

Mycobacterium kansasii is a slow growing nontuberculous photochromogenic mycobacteria with doubling time is about one day, and classified in a Runyon class I, which produces yellow-orange colonies when exposed to light and dark red crystals of β-carotene colonies when grown in light incubator or

prolonged exposure to light source (Parija, 2012; Huitt *et al.*, 2015). *M. kansasii* can cause serious opportunistic infection and is categorized as one of the most common opportunistic nontuberculous mycobacteria causing lung pulmonary infection resembling TB (Bloch *et al.*, 1998). *M. kansasii* can also cause osteomyelitis, arthritis, dermatitis, cervical lymphadenitis, and disseminated infection, which are usually related to immunosuppressed patients (Sherer *et al.*, 1986; Valainis *et al.*, 1991). The isolates of *M. kansasii* are susceptible to RIF, INH, streptomycin (STR), EMB, rifabutin (RFB), amikacin (AMK), ethionamide (ETH), clarithromycin (CLR) and ciprofloxacin (CIP) (Shitrit *et al.*, 2007). Although *M. kansasii* can spread by aerosol route, the infection is not transmitted by direct contact from person-to-person because it only can be found in water or soil environment and the major reservoir for *M. kansasii* is the tap water (Evans *et al.*, 1996).

***Mycobacterium tuberculosis* H37Ra**

Mycobacterium tuberculosis H37Ra strain is an attenuated tubercle bacillus, which originated from the parental virulent strain of *M. tuberculosis* H37 (Steenken *et al.*, 1934). This slow growing avirulent strain has a generation time of between 18 - 24 hours and is classified as Biosafety Level 2 (BSL) organism (Gengenbarcher & Kaufmann, 2012). Even though the variance in virulence between strain H37Ra and H37Rv are still being debated, several data have reported the differences in characteristics between the strains H37Ra and H37Rv, including the ability of strain H37Ra to form raised colony morphology (Steenken, 1934), reduced ability to survive under anaerobic condition or inside the macrophage (Heplar *et al.*, 1954), unable to form cord formation (Middlebrook *et al.*, 1947), loss ability to interrupt phagosomal membranes (Hart & Armstrong, 1974), and avirulent in guinea pig and

mice (Steenken, 1938). The genomic differences between H37Rv and H37Ra, which involved gene insertions, deletions, and single nucleotide polymorphisms have been reported previously. Zheng *et al*. (2008) reported that 57 of different genetic variations, which may account to its pathogenicity differences and other phenotypic changes from virulent strain of H37Rv were identified. However, Malen *et al.* (2011) observed that the similar relative abundance of the proteins was identified in both strains and suggested that the two strains are highly related at protein level. Thus, H37Ra strain is widely used as an alternative strain to replace the virulent strain of H37Rv also due to other factors, including similar slow growing pattern, non-pathogenic and more correlated with H37Rv strain compared to other rapid growing mycobacteria (Nguta *et al.,* 2015). Moreover, the close relation between H37Ra with H37Rv in terms of genetic composition and drug susceptibility profile makes *M. tuberculosis* H37Ra the best alternative option in less equipped laboratories (Ibekwe & Ameh, 2014).

2.4.2 Bioassays for *in vitro* evaluation of anti-tubercular activity

The emergence of resistant strains together with complicated and long course regimens increases the urgent need for new therapeutics against TB. Development of new drugs requires screening of large group of compounds for identification of suitable drug candidates. Therefore, a simple and reliable method for the determination of minimum inhibitory concentration (MIC) is required to determine the relationship between *in vitro* susceptibility testing and clinical test (Fabry *et al.,* 1996). Rapid detection and identification of mycobacterial susceptibility are important to control the outbreak of multiple-drug resistant strain and for the