SCREENING OF MICROBIAL AND PLANT EXTRACTS FOR NEW ANTI-*MYCOBACTERIUM* DRUGS

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DECLARATION

I declare that this dissertation is the results of my own independent work, except where otherwise stated.

20 April 2007

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AUTHENTICATION

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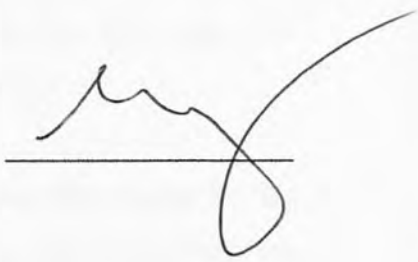

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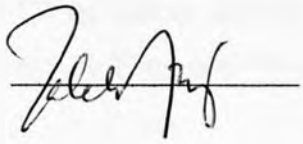

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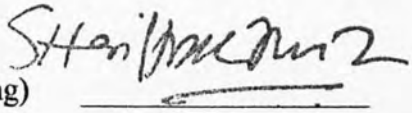

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PENYARINGAN EKSTRAK MIKROB DAN TUMBUHAN UNTUK PENEMUAN UBAT BARU ANTI-MIKOBAKTERIUM

Tuberkulosis merupakan penyakit batuk kering yang dijangkiti oleh *Mycobacterium tuberculosis*. Isositrat liase (ICL) merupakan salah satu enzim dalam laluan metabolisme gliosilat yang diperlukan oleh *Mycobacterium smegmatis* dalam jangkitan berterusan dan patogenesis. Dalam kajian ini, ekstrak mentah aseton mikrob daripada aktinomiset H7763 dan 29 ekstrak daripada 11 species tumbuhan telah disaring untuk mencari perencat penggunaan sumber karbon asetat dan glukosa dalam pertumbuhan *Mycobacterium smegmatis* mc^2155, H8000. Ekstrak mentah aseton H7763 telah menunjukkan zon perencatan separa yang luas (48 mm) pada media asetat manakala tiada zon perencatan pada media glukosa. Ekstrak mentah aseton H7763 itu telah diekstrakkan dengan menggunakan kaedah pengekstrakan pelarut-pelarut (air-etil asetat). Pemecahan telah dijalankan terhadap lapisan etil asetat tersebut dengan menggunakan kaedah pemfasaan songsang kromatografi cecair tekanan tinggi (RP-HPLC). Pecahan dikutip dari pemeringkatan itu telah disaring di mana F7, F8, F9, F10, F11 dan F12 telah menunjukkan keputusan positif. Dalam penyaringan tumbuhan pula, ekstrak-ekstrak mentah *Alpinia galangal*, n-butanol *Anacardium occidentale* dan n-butanol *Chromolaena odorata* telah menunjukkan keputusan positif yang terbaik. Kepekatan minimum perencat (MIC) bagi ekstrak-ekstrak ini yang merencatkan pertumbuhan H8000 adalah sebanyak 1, 4 dan 10 $mgml^{-1}$ masing-masing. Akan tetapi, ketiga-tiga ekstrak tumbuhan ini tidak merencatkan activiti ICL pada kepekatan rendah sebanyak 1 dan 2 $mgml^{-1}$. Kesimpulannya, lapisan etil-asetat (F7, F8, F9, F10, F11 dan F12) daripada ekstrak mentah aseton H7763, ekstrak mentah *Alpinia galangal*, ekstrak n-butanol *Anarcardium occidentale* dan ekstrak n-butanol *Chromolaena odorata* merupakan perencat *Mycobacterium smegmatis* mc^2155 yang berpotensi.

SCREENING OF MICROBIAL AND PLANT EXTRACTS FOR NEW ANTI-*MYCOBACTERIUM* DRUGS

Tuberculosis is one of the world's most pernicious disease that infected by *Mycobacterium tuberculosis*. Isocitrate lyase (ICL) is one of the enzymes in the glyoxylate metabolic pathway that is required in persistent infection and pathogenesis of *M. tuberculosis*. In this study, a microbial acetone crude extract of actinomycete H7763 and 29 plant extracts of 11 plant species were screened using a cell-based screening system against the acetate and glucose utilization of *Mycobacterium smegmatis* mc^2155, H8000. The H7763 acetone crude extract showed a wide partial inhibition zone (48 mm) on the acetate plate and no inhibition zone on the glucose plate. A partial purification of the H7763 crude extract was carried out by water-ethyl acetate extraction and the organic layer obtained was further purified using Reversed Phase High Performance Liquid Chromatography (RP-HPLC). Fractions collected from the RP-HPLC fractionation were screened in which the fractions of F7, F8, F9, F10, F11 and F12 showed positive results. For plant extracts, *Alpinia galangal* crude extract, *Anacardium occidentale* n-butanol extract and *Chromolaena odorata* n-butanol extract showed the most promising positive results. MIC of these plant extracts that prevented the growth of H8000 in the cell-based screening system was 1, 4 and 10 $mgml^{-1}$ respectively. However, these three plant extracts did not inhibit ICL activity at the low concentration of 1 and 2 $mgml^{-1}$. In conclusion, the ethyl acetate layer (F7, F8, F9, F10, F11 and F12) of the H7763 acetone crude extract, *Alpinia galangal* crude extract, *Anacardium occidentale* n-butanol extract and *Chromolaena odorata* n-butanol extract had been shown to be potential inhibitors against the growth of *Mycobacterium smegmatis* mc^2155, H8000.

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LIST OF ABBREVIATIONS

%	percent
°C	Celsius
Δ*icl1*	*icl1* gene mutant
Δ*icl2*	*icl2* gene mutant
w/v	weight-to-volume ratio
g	gram
min	minute
ml	milliliter
mm	millimeter
nm	nanometer
r.p.m.	revolutions per minute
s	second
µl	microliter
µm	micrometer
M	molar
ATP	adenosine triphosphate
BCG	Calmette-Guérin bacillus
BSA	Bovine serum albumin
$CD4^+$	helper T cell
$CD8^+$	cytotoxic T cell with CD8 surface protein
CFU	colony forming units
CoA	coenzyme A
DOTS	direct observed therapy, short course
DOTS-Plus	DOTS plus second line anti-TB drugs
DPA	decaprenolphosphoarabinose
DNA	deoxyribonucleic acid
GFPMA	green fluorescent protein reporter microplate assay
HIV	human immunodeficiency virus

HIV/AIDS	acquired immunodeficiency syndrome caused by HIV
ICL	isocitrate lyase
IFN-γ	interferon gamma
IR	infrared spectroscopic method
LJ	Löwenstein-Jensen
MAPTB	Malaysian Association for The Prevention of Tuberculosis
MDR-TB	multi-drug resistant tuberculosis
MDR-TB/HIV	Co-infection of MDR-TB and HIV
MIC	minimum inhibitory concentration
MS	malate synthase
MS	mass spectroscopic method
NMR	nuclear magnetic resonance spectroscopic method
pH	potential of hydrogen
REMA	resazurin microtiter assay
TB	tuberculosis
TB/HIV	Co-infection of TB and HIV
TCA	tricarboxylic acid cycle
TNF-α	tumor necrosis factor-alpha
UV	ultra violet spectroscopic method
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) was a contagious disease that reached epidemic proportions with emergence of cities at the start of Industrial Revolution. It was due to the poor socioeconomic conditions, overcrowding and improper management.

TB is caused by *Mycobacterium tuberculosis* infection. *Mycobacterium tuberculosis* was found by Robert Koch in 1882. He was awarded the Nobel Prize in Physiology or Medicine for his tuberculosis findings in 1905. Around the middle of 18th Century, there was a decline in the rates of TB in industrialized countries due to the improvement of socioeconomic condition and development of anti-TB agents (Newton *et al.*, 2002).

The TB treatment with a single drug was ineffective after the advent of multi-drug resistant tuberculosis (MDR-TB). MDR-TB is defined as tuberculosis caused by *Mycobacterium tuberculosis in vitro* to the effects of at least isoniazid and rifampicin, with or without resistance to any other drugs (WHO, 2006). From a microbiological perspective, resistance is caused by genetic mutation that makes a drug ineffective against the mutant bacilli. Thus, drug-resistant TB is basically a man-made phenomenon because this genetic mutation is generally due to inadequate of anti-TB treatments.

Briefly, MDR-TB treatment is complex and no single strategy will fit all situations. Consideration such as epidemiological, financial and operational factors must be taken into the strategy. Moreover, special conditions and situations such as pregnancy, breastfeeding, contraception, children, diabetes mellitus, renal indefficiency, liver disorders, seizure disorders, psychiatric disorders and HIV infection had worsen the situation of being infected with MDR-TB.

Directly observed therapy, short course (DOTS) is an international recommended strategy for TB control by WHO based on case-finding and cure with its five elements. In Malaysia, DOTS with its five main components can be summarized as government support, microscopes, observers, medicines and records. In fact, one third of MDR-TB cases had resistance to all four first line drugs (isoniazid, pyrazinamide, ethambutol and rifampicin) reported on the anti-TB drug resistance surveillance (WHO, 2006). Thus, DOTS-Plus is an adaptation of the DOTS strategy respond to MDR-TB (Zhang, 2005; WHO, 2006).

TB/HIV is a term used for two epidemics co-infection which is TB and HIV/AIDS. The interaction of TB with HIV infection has pernicious effects where patients have the most potent risk factor for a latent TB infection to convert into active TB. The occurrence of MDR-TB/HIV co-infection has exacerbated the disease because these patients cannot have treatments without being individually special diagnosed (WHO, 2006).

Mycobacterium tuberculosis can seed any organ via hematogenous spread. Therefore, latent TB occurs when the patient is infected with *Mycobacterium tuberculosis* but does not have active tuberculosis disease. However, the main point is that this patient will go on to develop active tuberculosis at later stage of his life. Almost all known anti-TB drugs efficiently kill the replicating microbe but they perform poorly on non-replicating *Mycobacterium tuberculosis* (Balganesh, 2004).

During persistency and virulence, fatty acid metabolism is carried out in *Mycobacterium* to replace pentose phosphate pathway in carbohydrate metabolism because of glucose shortage. Glyoxylate pathway is an enzymatic pathway that allows the bacteria to convert fatty acids to carbohydrates. Here, isocitrate lyase (ICL), an essential enzyme that operates fatty acid metabolism in the glyoxylate pathway. Glyoxylate pathway is only occurring for *Mycobacterium tuberculosis* viability in long-term persistence and virulence in host. Thus, an ICL inhibitor will enhance the specific killing of *Mycobacterium tuberculosis* in a persistency and virulence state.

The World Health Organization declared TB as a global emergency disease in 1993 when it killed nearly two million people a year, five thousand every day, afflicts one third of the world's population and nearly nine million new cases develop every year (WHO, 2006). Ten highest burdens of TB countries reported in Global Tuberculosis Report by WHO (2004) are India, China, Indonesia, Nigeria, Bangladesh, Pakistan, Ethiopia, Philippines, South Africa and DR Congo.

The aims of The Global Plan to Stop TB 2006-2015 are to produce the first new anti-TB drug in 40 years by 2010 and develop a new vaccine by 2015. In addition, it must save 50 million people from TB and treat 800,000 people from MDR-TB. A total amount of US$56 billion is enabling in The Global Plan for the Stop TB Strategy implementation towards a TB-free world (WHO, 2006). Furthermore, The Global Alliance for TB Drug Development has announced in May 25, 2006 that $104 million from Bill & Melinda Gates Foundation is used to advance a pipeline of new TB drugs over the next five years to find a faster and more effective TB cure.

Secondary metabolites (natural products) are metabolism compounds that are not essential for normal growth but usually of ecological nature as defense against predators. Plants have exceptional ability to produce cytotoxic agents. These agents represent an ecological rationale that synthesized *de novo* in plants. They protect the producer from pathogenic infection in its environment (Gibbons, 2005). Over 350 plants have been evaluated for their anti-TB activities (Billo *et al.*, 2005). Compounds such as alkaloids,

chalcones, flavonoids or terpenoids have demonstrated *in vitro* anti-TB activity (Billo *et al.*, 2005).

Soil microbes represent an important source of biologically active compounds because they produce original and unexpected structure secondary metabolites. The variety of existing strains enable different new bioactive compounds being discovered. This ability of producing a large number of chemically different secondary metabolites is mostly comprised of the filamentous actinomycetes, the myxobacteria, the pseudomonads and the cyanobacteria within the prokaryotic world (Monciardini *et al.*, 2002).

There are 10 – 100 million species or organisms living on earth. Besides, higher plants are forming a group of 250000 species. However, only 6% of the 250000 species has been investigated for biological activities and 15% for their chemical constituents (Gurib-Fakim, 2006). Thus, there is an exigency to carry out effort to find potential anti-TB drugs from these natural products.

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