ANTI-*MYCOBACTERIUM* PROPERTY OF *ANACARDIUM OCCIDENTALE*

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THIS DISSERTATION IS SUBMITTED TO FULFILL PARTIAL OF THE REQUIREMENT TO OBTAIN A DEGREE IN BACHELOR OF SCIENCE WITH HONOUR

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DECLARATION

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

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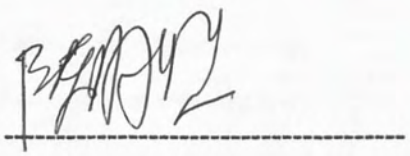

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ABSTRACT

ANTI-*MYCOBACTERIUM* PROPERTY OF *ANACARDIUM OCCIDENTALE*

Tuberculosis (TB) disease is caused by *Mycobacterium tuberculosis*. Despite the development of anti-TB drugs, but still unable to treat persistent TB, there were 3 million deaths in the 1990s due to this disease. Therefore, TB remains a leading cause of mortality worldwide into the 21st century. In this study, leave extracts were obtained and subjected to agar-diffusion screening systems with acetate and glucose utilization of H8000 *Mycobacterium smegmatis* mc^2155 targeted on glyoxylate cycle and two-component signal transduction. The screening result showed that all the extract fractions showed inhibition against glyoxylate cycle with the n-butanol showed the most promising potential to be develop as anti-persistent drugs for treating persistent *mycobacteria* with cytotoxic effect. In two-component screening system, the dichloromethane extract and aqueous extract showed promising activity targeting two-component system.

ABSTRAK

CIRI-CIRI ANTI-*MIKOBAKTERIA* DARIPADA *ANACARDIUM OCCIDENTALE*

Mikrobakteria tuberkulosi *merupakan punca kepada penyakit tuberkulosi (TB).Walaupun terdapat perkembangan dari segi perubatan untuk merawat penyakit ini, tetapi TB masih mengakibatkan 3 million kematian pada 1990-an. TB merupakan salah satu punca kematian secara global menjelang abad ke-21. Dalam kajian ini, kaedah pengekstrakan refluks dijalankan ke atas sampel daun Anacardium occidentale dan kaedah bioesei fraksinasi dilakukan ke atas ekstrak mentah daun tersebut. Bioesei yang digunakan merupakan sistem penyaringan yang sasar kepada putaran glyoxylate dengan menggunakan kultur H8000* Mikrobakteria smegmatis *mc^2155 dalam sumber karbon asetat dan glukosa serta sistem penyaringan yang sasar kepada transduksi isyarat. Keputusan penyaringan putaran glyoxylate mendapati semua ekstrak menunjukkan keputusan anti-*mikrobakteria *dengan n-butanol merupakan ekstrak yang terbaik tetapi kandungan cytotoksik juga tinggi dalam ekstrak tersebut. Dalam sistem penyaringan transduksi isyarat, ekstrak akueous dan diklorometana menunjukkan keputusan yang memberangsangkan dalam menyasar kepada sistem transduksi isyarat.*

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LIST OF SYMBOLS, UNITS AND ABBREVIATION

M.	*Mycobacterium*
wv^{-1}	weight over volume
vv^{-1}	volume over volume
%	percent
s	second
mL	mililiter
µL	microliter
°C	degree Celcius
$mgmL^{-1}$	miligram per mililiter
μgmL^{-1}	microgram per mililiter
M	Molar
mM	miliMolar
µM	microMolar
Mg^{2+}	Magnesium Ion
mm	milimeter
µm	micrometer
nm	nanometer
TB	Tuberculosis
TCA	tricarboxylic acid
BCG	Bacille Calmette-Guerin
HIV	Human Immunodeficiency Virus
AIDS	Acquired Immuno Deficiency Syndrome
°	Degree
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
MXF	moxifloxacin
DOT	Directly Observed Therapy
$MgSO_4$	Magnesium sulphate
A. occidentale	*Anacardium occidentale*

No.	number
ICL	Isocitrate Lyase
MS	Malate Synthase

CHAPTER 1

INTRODUCTION

Tuberculosis (TB) is caused by *Mycobacterium tuberculosis* (*M. tuberculosis*). It is an illness of the respiratory system caused by the *M. tuberculosis*. The *mycobacterium* can be spreaded easily by coughing and sneezing. A person will be infected by inhaling the airborne *M. tuberculosis.*

TB is the most common cause of death especially among the third-world countries, and the first trace of the infection can be backtracked to 3000 years ago in the Egyptian Empire (Black, 2002). Before 1900, there was no effective treatment of TB and this caused approximately one-third of population in US died because of TB before reaching adult stage (Black, 2002). Even now, TB continues to be a devastating pathogen throughout the world, particularly in developing nations (Jamshidi & Palsson, 2007).

Before the issue of the first anti-TB drugs at 1944, the only way to combat *M. tuberculosis* was improving the body immune system by improving sanitary and

providing adequate nutrition. After two decades, the drugs for treatment of TB were developed. These include *p*-aminosalicylic acid, isoniazid, pyrazinamide, *D*-cycloserine, ethambutol, ethionamide, rifampicin and along with the development of BCG (Khasnobis *et al.*, 2002). Despite the development of anti-TB drugs, there were 3 million deaths in the 1990s due to this disease. Therefore TB remains a leading cause of mortality worldwide into the 21st century (Smith *et al.*, 2004).

The high number of mortality was due to the development of Multi-Drug Resistant TB (MDR-TB) due to long term treatment by a cocktail of first line and second line anti-TB drugs. The persistent phase of infection that is recalcitrant to conventional anti-TB drugs (Smith *et al.*, 2004), and the HIV related tuberculosis (Burman & Jones, 2001). Therefore there is a need to develop new anti-TB drugs.

Although there are many conventional anti-TB drugs that can be used for the treatment, but the drawback of these drugs are the side effects of them (Winstanley, 1995) and the drugs only target actively growing bacteria in cell process such as cell wall biogenesis and chromosome replication. There are still persistent bacteria left inside the body. These bacteria will eventually become active and more resistant to the anti-TB drugs that used to treat the bacteria before (Smith *et al.*, 2004). Therefore study has to be done to treat the persistent bacteria to ensure the successful of the TB treatment.

Isocitrate Lyase (ICL) and Malate Synthase (MS) are two key enzymes in the glyoxylate shunt (Bentrup *et al.*, 1999; Smith *et al.*, 2003). It is potential to inhibit the two

enzymes to prevent growth of the *Mycobacteria* (Bentrup *et al.,* 1999; Smith *et al.,* 2003). This is due to the fact that the *Mycobacteria* need acetate or fatty acids as their carbon source employed in the glyoxylate bypass for the biosynthesis of cellular material (Bentrup *et al.,* 1999; Smith *et al.,* 2003). The key enzymes of this bypass are isocitrate lyase and malate synthase (Bentrup *et al.,* 1999).

ICL helps to cleave isocitrate to succinate and glyoxylate and the malate synthase is an enzyme that condenses glyoxylate with acetyl coenzyme A (acetyl-CoA) to yield malate (Bentrup *et al.,* 1999). Muñoz-Elías & McKinney (2005) reported that the growth of *M. Tuberculosis* were disturbed when the ICL were inhibited in mice. Smith *et al.* (2003) reported that the disturbance of growth of *M. tuberculosis* was observed when MS was inhibited. The inhibition of these two enzymes holds a key to inhibit the *mycobacteria* growth (Vivek, 2006).

Two-component system is a signal transduction system in a cell that response to external stimulus (Parkinson, 1994). This system has a specific sensor kinase and a response regulator protein. The sensor kinase detects a signal from the environment, autophosphorylates at a specific histidine residue using energy from ATP hydrolysis and transmits a phosphoryl group to the response regulator. The response regulator is thus activated and this DNA-binding protein will bind to DNA to regulate transcription. To complete this regulation, this system has to be terminated by a phosphatase. In some bacteria, this reaction is carried out by the sensor kinase itself and some even has a third

protein for this termination. Some antibacterial agents were identified to inhibit the two-component signal transduction system (Barrett *et al.,* 1998).

Cashew nut (*Anarcadium occidentale*) is a heart like shaped fruit widely grown in Africa and West Indies. In Nigeria about 5000 - 7000 tones are produced annually and mainly as an export crop. There was limited information in the nutritional composition, utilization and physicochemical properties of the cashew nut leaves (Aremu *et al.,* 2006).

Researches had shown that the fruit juice produce by *A. occidentale* contains anti-tumor agents against BT-20 breast cancer, the anacardic acids found in the fruit juice of *A. occidentale* exhibit moderate cytotoxic activity against both BT-20 breast and HeLa epithelioid cervix carcinoma cells (Kubo *et al.*, 1993). A research done by Mota *et al.* (1985) found out properties of anti-inflammatory actions of tannins isolated from the bark of *A. occidentale*. Furthermore, Kubo *et al.* (1994) found out that anacardic acids, 2-methylcardols, and cardols isolated from various parts of the *A. occidentale* exhibited tyrosinase inhibitory activity.

A previous study in our group by Ch'ng (2007) & Teoh (2007) showed inhibiting effect of the crude extract, n-butanol and aqueous extracts of *A. occidentale* against *Mycobacteria.* In this study, the *Mycobacteria* used was *M. smegmatis,* this was due to the *mycobacteria* was not hazardous to human, easy to grow and contain the similarity pathway of metabolism and two-component signal transduction system to *M. tuberculosis* (Etienne *et al.,* 2005).

1.2 The objective of this study were:

- To prepare extracts and fractions of *A. occidentale.*
- To screen the extracts and fractions of *A. occidentale* against *M. smegmatis* mc^2155, H8000 targeting the glyoxylate cycle and the two-component signal transduction system using an agar-diffusion screening system.

1.3 Scope of Study:

Leaves sample of the *A. occidentale* was extracted to obtain the crude extract. Then the crude extract of the plant was evaluated for its biological inhibitory activity through an agar-diffusion screening system.

The crude extract with positive activity using bioassay-guided fractionation was partitioned into petroleum ether, dichloromethane, n-butanol and aqueous extracts fractions. The partitioned fractions were evaluated for its biological activities using an agar-diffusion screening system.

The study focuses on the biological activities of inhibition against persistent latent TB infection targeting on glyoxylate cycle and the two-component signal transduction system in *M. smegmatis* mc^2155, H8000.

CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

TB can be acquired by the inhalation of droplet nuclei of respiratory secretions or particles of dry sputum containing tubercle bacilli. TB often infects the lungs of the host, but in some cases, TB can also infect bones, urogenital tract, meninges, lymphatic system, and peritoneum. These infections are classified as extrapulmonary tuberculosis (Black, 2002).

When a person inhales *M. tuberculosis,* the *mycobacterium* will undergo a rapid expansion under limited host immune respond in the lungs before the host body immune system detects the invading bacteria. After a period of time when the host immune system finally realize the bacteria infection, the host immune system will eventually responds and limits dissemination of infection by developing granulomas or 'tubercles' around the infection site (Bentrup & Russell, 2001).

There are two types of pulmonary tuberculosis; the primary tuberculosis and secondary tuberculosis. Primary tuberculosis consists of three stages of infections. Primary tuberculosis is due to the first infection of an unsensitized host. At the stage one of the primary tuberculosis disease, most of the inhaled *mycobacterium* will be phagocytized by the neutrophils and white blood cells initially and macrophages later. The stage two of the disease occurs when some of the bacilli survive and replicate slowly in the cell that phagocytized them. Eventually the cell will die off and more bacilli will produce. At the third stage, the overwhelming bacilli in the lungs caused the immune system to respond by secrete more fluid in the lungs. These fluids will eventually surround the infection site and solidify to become chronic granulomas or tubercles (Black, 2002; Sheffield, 1994).

Secondary tuberculosis infection occurs when the host has some surviving bacilli left in the body and host resistant is impaired due to immunosuppression from any cause, including malnutrition, alcoholism, malignant disease, silicosis, diabetes and acquired immune deficiency syndrome (AIDS). These infections are mainly occurring in the post-primary tuberculosis patients, old folks and drug addict (Sheffield, 1994).

2.2 *M. tuberculosis*

M. tuberculosis is a member of *Mycobacteriaceae*. The *mycobacterium* was first described by Robert Koch in 1905 (Kanai, 1991; Snewin, 2001). The complete genome

sequence and annotation of *M. tuberculosis* strain was published in 1998 (Cole *et al.*, 1998).

M. tuberculosis is a large slender or slightly-curved rod bacterium, its shape can be around 1-4 μm in length and 0.3-0.6 μm in breadth. It is a gram-positive bacterium and can grow optimally at 37 ^{0}C, pH ranging from 6.4-7.0 is also a suitable condition for growth. But it has a typically slow replication rate ranging from 14-20 hours under optimum conditions. Furthermore *M. tuberculosis* growth tends to make serpentine and cord-like pattern of bacillary arrangement due to a parallel orientation of mutual contact. The bacteria itself can adapt themselves to microaerophilic environment or in the host body hostile environment by changing their metabolic machinery and continue surviving (Kanai, 1991; Manabe & Bishai, 2000).

The notable characteristic of *M. tuberculosis* is its "acid-fastness" characteristics, it is due to when the *mycobacteria* once stained, they resist to decolourization by mineral acids (Kanai, 1991; Snewin, 2001). There is also a very characteristic feature that appears in *M. tuberculosis* but not other *Mycobacteria,* the characteristic is the feature of the cell wall of *M. tuberculosis.* In the insoluble cell wall core are chemically composed of three covalently linked macromolecules, they are highly cross-linked peptidoglycan, arabinogalactan (AG) and mycolic acids. The *mycobacteria* peptidoglycan is distinct with other bacteria peptidoglycan due to the muramic acid residues are *N*-glycolylated with glycolic acids and the cross-linking bonds are found between two residues of diaminopimelic acids as well as between diaminopimelic acid and D-alanine residues.

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