

ANTITUBERCULOSIS, DRUG-FRACTION INTERACTION AND CYTOTOXICITY
ACTIVITIES OF *Tetracera macrophylla* STEM EXTRACT

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DEDICATION

First and foremost, all praises to Allah s.w.t for all the blessings and strength given to me to complete my Master's Degree Project with the title of Antituberculosis Activities and Bioassay-guided Fractionation of *T. macrophylla* Stem Extracts.

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ABSTRACT

Tetracera macrophylla is a climbing woody vine that has been used by several ethnics of indigenous people around Malaysia for various ethnomedicinal purposes. With the emergence of resistance strains of *Mycobacterium tuberculosis*, *T. macrophylla* could be a potential reservoir in quest of novel anti-TB compounds. This research hence embarks on achieving three objectives which are to assess the anti-TB activities of *T. macrophylla* stem fractions against *M. tuberculosis* H37Ra, to evaluate the synergistic interactions of *T. macrophylla* stem fractions with anti-TB drugs (Rifampicin and Isoniazid) and to assess cytotoxicity of stem fractions of *T. macrophylla* to Human Embryonic Kidney (HEK-293) cell line. Fractionation via column and thin-layer chromatography of the plant ethyl acetate extract has led to the elution of 29 fractions. Anti-TB activities assessment of all *T. macrophylla* fractions via Tetrazolium Microplate Assay (TEMA) has revealed that seven of the fractions (S2, S3, S5, S6, S11, S12 & S15) inhibited the growth of mycobacteria at Minimum Inhibition Concentration (MIC) ranges from 400 to 800 µg/ml though with Minimum Bactericidal Concentration (MBC) that were more than 800 µg/ml. Furthermore, the evaluation of synergistic interactions of selected bioactive fractions (S5, S6 & S12) with selected anti-TB drugs (Rifampicin and Isoniazid) via checkerboard assay has showed that the fractions exhibited additive effects with Fractional Inhibition Concentration Index (FICI) ranges from 0.625 to 1.125, suggesting that the fractions may possess a mechanism-of-action and drug target which resemble that of the anti-TB drugs. On the other hand, the selectivity index (SI) as calculated from the cytotoxicity assay via tetrazolium colorimetric-based method has displayed that all of the test fractions were more selective towards the mammalian cells than the mycobacterial cells. Conclusively, present study has provided insight into anti-TB potential of *T. macrophylla* and further scientifically consolidate the species as an anti-TB ethnomedicinal importance.

ABSTRAK

Tetracera macrophylla ialah tumbuhan kayu jenis memanjang yang telah digunakan oleh beberapa etnik orang asli di Malaysia untuk pelbagai tujuan perubatan ethno. Dengan kemunculan jenis *M. tuberculosis* yang kebal terhadap ubat anti-TB sedia ada, *T. macrophylla* mampu menjadi sumber penyimpanan semulajadi dalam pencarian sebatian anti-TB yang baharu. Penyelidikan ini maka bertujuan untuk mencapai tiga objektif: untuk menilai aktiviti anti-tuberkulosis pecahan ekstrak batang *T. macrophylla*; untuk menilai kesan interaksi antara pecahan ekstrak batang *T. macrophylla* dengan ubat anti-TB (Rifampicin dan Isoniazid) dan; untuk menilai sitotoksiti pecahan ekstrak batang *T. macrophylla* terhadap Sel Embrio Ginjal Manusia (HEK-293). Pemecahan ekstrak tumbuhan itu melalui kromatografi kolumn dan lapisan-nipis telah menghasilkan 29 pecahan. Penilaian aktiviti anti-TB pada semua pecahan *T. macrophylla* melalui Ujian Mikroplat Tetrazolium (TEMA) telah mendedahkan bahawa tujuh daripada pecahan-pecahan itu (S2, S3, S5, S6, S11, S12 & S15) telah menghalang pertumbuhan bacteria itu pada Kepekatan Perencatan Minimum (MIC) berjulat 400 hingga 800 $\mu\text{g}/\text{ml}$. Penilaian kesan interaksi antara pecahan *T. macrophylla* yang bioaktif dan ubat anti-TB melalui kaedah ujian papan checker telah menunjukkan kesan “aditif” dengan Indeks Kepekatan Perencatan Pecahan (FICI) berjulat 0.625 hingga 1.125, mencadangkan bahawa pecahan tumbuhan itu mempunyai mekanisma tindakan yang sama dengan ubat anti-TB itu. Tambahan itu, Indeks Selektiviti Index (SI) yang dikira daripada ujian sitotoksiti pula telah menunjukkan bahawa pecahan tumbuhan itu lebih selektif terhadap sel mamalia itu berbanding sel mikobakteria. Kesimpulannya, kajian ini telah memberikan pengetahuan ke dalam potensi anti-TB *T. macrophylla* dan seterusnya menguatkan lagi nilai spesis tumbuhan ini dalam kepentingan perubatan anti-TB ethno.

CONTENTS

TITLE	i
DECLARATION	ii
DEDICATION	iii
ACKNOWLEDGEMENT	iv
ABSTRACT	v
ABSTRAK	vi
CONTENTS	vii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiv
LIST OF APPENDICES	xvi
LIST OF PUBLICATIONS	xvii
CHAPTER 1 INTRODUCTION	1
1.1 Research background	1
1.2 Problem statements	3
1.3 Research objectives	4
1.4 Research significance	4
CHAPTER 2 LITERATURE REVIEW	6

2.1	Tuberculosis (TB)	6
2.2	<i>Mycobacterium tuberculosis</i> strain	8
2.2.1	Mycobacterial cell envelope	9
2.2.2	Treatment	10
2.3	Plant secondary metabolites with anti-TB activities	13
2.3.1	Terpenoids	14
2.3.2	Phenolics	15
2.3.3	Flavonoids	16
2.3.4	Alkaloids	17
2.4	<i>Tetracera macrophylla</i> Wall. ex Hook. f. & Thoms	19
2.5	Bioassays in <i>in-vitro</i> antituberculosis study	21
2.5.1	Broth dilution assay	21
2.5.2	Checkerboard assay	23
2.5.3	Cytotoxicity assay	25
CHAPTER 3	METHODOLOGY	27
3.1	Sampling area and design	27
3.1.1	Study design	27
3.1.2	Sampling area: Taman Negara Johor Endau Rompin (TNJER)	29
3.2	Plant materials	30
3.2.1	Collection and preparation of plant materials	30
3.3	Extraction of plant raw materials for crude extracts	31
3.3.1	Maceration	31
3.3.2	Determination of extraction yield	31

3.4 Fractionation of crude extract using chromatographic techniques	32
3.5 <i>In-vitro</i> antimycobacterial assay	33
3.5.1 Inoculum preparation	33
3.5.2 Fraction preparation	33
3.5.3 Tetrazolium {3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide} preparation	34
3.5.4 Positive control preparation, Isoniazid (INH)	34
3.5.5 TEMA plate preparation	34
3.6 Checkerboard assay in assessing interaction effects of of selected <i>T. macrophylla</i> fractions and selected Tuberculosis antibiotics	36
3.6.1 Fraction plate (Plate A) setup	37
3.6.2 Drug plate (Plate B) setup	38
3.6.3 Combination plate setup	38
3.7 Cytotoxicity evaluation of selected fractions	40
3.7.1 Cell culture conditions	40
3.7.2 Cytotoxicity of fractions	40
3.8 Statistical analysis	42
CHAPTER 4 RESULTS AND DISCUSSIONS	43
4.1 Extraction of <i>T. macrophylla</i> stems for crude extracts	43
4.2 Fractionation of <i>T. macrophylla</i> crude extract chromatographic techniques	44
4.2.1 Column chromatography	44

4.2.2 Thin-layer chromatography	46
4.3 Minimum Inhibition Concentration (MIC) and Minimum Bactericidal Concentration (MBC)	50
4.4 In-vitro interactions of selected <i>T. macrophylla</i> fractions with two anti-TB first-line drugs against <i>M. tuberculosis</i> H37Ra	54
4.5 Cytotoxicity of selected <i>T. macrophylla</i> fractions	57
CHAPTER 5 CONCLUSION AND RECOMMENDATIONS	60
5.1 General conclusion	60
5.2 Recommendations	61
REFERENCES	62
APPENDICES	79
VITA	91

LIST OF TABLES

2.1	Common anti-TB drugs, their mode of actions and adverse effects from use of them	12
4.1	Silica 60 gel column chromatography solvent systems, fractions and combined fractions number	45
4.2	MCI® gel column chromatography solvent systems, fractions and combined fractions number	45
4.3	TLC profiles of the Si 60 gel combined fractions	48
4.4	TLC profiles of the MCI® gel combined fractions	49
4.5	Antimycobacterial activity of the fractions eluted from Si 60 gel column chromatography	53
4.6	Antimycobacterial activity of the fractions eluted from MCI gel column chromatography	53
4.7	Fractional Inhibition Concentration Index (FICI) of selected <i>T. macrophylla</i> fractions combined with RIF	56
4.8	Fractional Inhibition Concentration Index (FICI) of selected <i>T. macrophylla</i> fractions combined with INH	56
4.9	Antimycobacterial activity of selected fractions, when used alone and in combination with anti-TB drugs	56
4.10	MIC, IC ₅₀ and SI values of six selected fractions	59

LIST OF FIGURES

2.1	Incidence rates of TB across countries worldwide in 2018	7
2.2	Layout of <i>M. tuberculosis</i> cell envelope (CM: cytoplasmic membrane; PG: peptidoglycan; AG: arabinogalactan; MA: mycolic acids)	10
2.3	Moretenol, an example of triterpenoid with an anti-TB activity	15
2.4	Tetraceranoate, an example of non-flavonoid phenolic compound with an anti-TB activity	16
2.5	Basic structure of flavonoid	17
2.6	Lupinofolin, an example of flavonoid with an anti-TB activity	18
2.7	3-hydroxy-1, 5, 6-trimethoxy-9-acridone, an example of alkaloid with an anti-TB activity	19
2.8	<i>Tetracera macrophylla</i> as seen on-field at Taman Negara Johor Endau-Rompin	21
2.9	A brief illustration of a typical checkerboard assay with 6-by-6 configuration, in a 96-well plate	25
3.1	Flowchart of the study design	28
3.2	The location of Endau-Rompin Johor National Park (Labis District Council, 2015)	29

3.3	A brief depiction of TEMA assay layout used in this study	35
3.4	A depiction of fraction plate (Plate A) setup procedure as used in this study	37
3.5	A depiction of drug plate (Plate B) setup procedure as used in this study	38
3.6	A depiction of combination plate setup procedure as used in this study	39
4.1	MIC and IC ₅₀ of selected plant fractions in graph lines	57



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PERPUSTAKAAN TUNKU TUN AMINAH

LIST OF ABBREVIATIONS

AG	-	Arabinogalactan
BCG	-	Bacillus Calmette–Guérin
CM	-	Cytoplasmic Membrane
CFU	-	Colony-Forming Unit
CHCl ₃	-	Chloroform
EMEM	-	Eagle's Minimum Essential Medium
ETB	-	Ethambutol
dH ₂ O	-	Distilled water
HEK-293	-	Human Embryonic Kidney Cell Line
HIV	-	Human Immunodeficiency Virus
INH	-	Isoniazid
FBS	-	Fetal Bovine Serum
FICI	-	Fractional Inhibition Concentration Index
FRIM	-	Forest Research Institute of Malaysia
JAKOA	-	Jabatan Kemajuan Orang Asli
MA	-	Mycolic Acids
MABA	-	Microplate Alamar Blue Assay
mAGP	-	Mycolyl-Arabinogalactan-Peptidoglycan Complex
MaHTAS	-	Malaysian Health Technology Assessment Section
MBC	-	Minimum Bactericidal Concentration
MCI®	-	Mitsubishi Chemical Ion
MDR-TB	-	Multi-Drug Resistance Tuberculosis
MIC	-	Minimum Inhibition Concentration

MOM	-	Mycobacterial Outer Membrane
MTb	-	<i>Mycobacterium tuberculosis</i>
		(3-(4,5-dimethylthiazol-2-yl)-5-(3-
MTS	-	carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-
		tetrazolium
MTT	-	Tetrazolium Bromide
OADC	-	Oleic acid-Albumin-Dextrose-Catalase
PERHILITAN	-	Jabatan Perlindungan Hidupan Liar dan Taman Negara
PG	-	Peptidoglycan
PIP	-	Piperine
PTNJ	-	Perbadanan Taman Negara Johor
REMA	-	Resazurin Microplate Assay
RIF	-	Rifampicin
rRNA	-	Ribosomal Ribonucleic Acid
Si gel 60	-	Silica gel 60
TB	-	Tuberculosis
TEMA	-	Tetrazolium Microplate Assay
TK	-	Traditional Knowledge
TLC	-	Thin-Layer Chromatography
TNJER	-	Taman Negara Johor Endau Rompin
UV	-	Ultra-Violet
WHO	-	World Health Organization
XDR-TB	-	Extensively-Drug Resistance Tuberculosis

LIST OF APPENDICES

APPENDIX	TITLE	PAGE
A	Cleaning plant sample stem bark and storage of the sample	79
B	Maceration and filtration of plant sample	80
C	Column and thin-layer chromatography of crude extract	81
D1-D3	TEMA visual results of several Silica 60 fractions	82
E1-E6	The raw results of the cytotoxicity assay of selected Silica 60 fractions	85

LIST OF PUBLICATIONS

Journal:

- i) **Mazlun, M. H.**, Sabran, S. F., Mohamed, M., Abu Bakar, M. F., & Abdullah, Z. (2019). Phenolic Compounds as Promising Drug Candidates in Tuberculosis Therapy. *Molecules*, 24(13). doi: 10.3390/molecules24132449
- ii) Harith, S. S., **Mazlun, M. H.**, Mydin, M. M., Nawi, L., & Saat, R. (2018). Studies on Phytochemical Constituents and Antimicrobial Properties of *Citrullus lanatus* Peels. *Malaysian Journal of Analytical Science*, 22(1), 151-156. doi: 10.17576/mjas-2018-2201-19
- iii) Sabran, S. F., **Mazlun, M. H.**, Mohamed, M., Abu Bakar, M. F., Pa'ee, F., Linatoc, A. C., & Abdullah, Z. (2020). Study on antimycobacterial activity and phytochemical constituents of *Dipterocarpus sublamellatus* foxw. Hexane extract (DSHE). *International Journal of Pharmaceutical Research*, 12(1), 651-659. doi: 10.31838/ijpr/2020.12.01.133

Conference:

- i) **Mazlun, M. H.**, Sabran, S. F., Abdullah, Z., & Parumasivam, T. (2021). A comparative study of antituberculosis activities of *Tetracera macrophylla* Wall. Ex Hook. f. & Thoms. stem fractions using different chromatographic stationary phases. *IOP Conference Series: Earth and Environmental Science*, 736(1), 012036. doi: 10.1088/1755-1315/736/1/012036.



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CHAPTER 1

INTRODUCTION

1.1 Research background

Tuberculosis (TB) is a communicable and fatal pulmonary disease which has been alarming mankind for millennia, and it remains a major health concern worldwide (WHO, 2019). As reported by World Health Organization (WHO) in Global Tuberculosis Report 2019, in 2018, the estimated incidence cases of TB were 10.0 million (range, 9.0- 11.1 million) cases globally, which was equivalent to 132 cases (range, 118-146) of 100,000 populations. Most estimated number of cases in 2018 occurred in WHO Southeast Asia Region, which constituted 44% of total cases reported, followed by WHO African Region (24%), WHO Western Pacific Region (18%), WHO Eastern Mediterranean Region (8.1%), WHO Americas Region (2.9%), and the remaining WHO European Region (2.6%) (WHO, 2019). In the same year 2018, TB alone has claimed approximately 1.2 million of lives (range, 1.1- 1.3 million), from which 85% of the deaths occurred in WHO African Region and South-East Asia Region (WHO, 2019). Standing at tenth place, TB is one of the top leading causes of death worldwide, and since 2007, it has been the leading cause of death from a single infectious agent, ranking above Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS) (WHO, 2019).

Since their first discovery in 1950s, TB-afflicted patients have since been relying on the current anti-TB drugs which are divided into three main groups: First-line anti-TB drugs, second-line anti-TB drugs and third-line anti-TB drugs (Keshavjee & Farmer, 2012; Zumla, Nahid and Cole, 2013). By possessing different drug targets which resulted

in different antimycobacterial effects, first-line TB drugs were proven efficacious for TB therapy, with more than 95% cure rates under ideal conditions of direct observation by the patients' carers (Zumla, Nahid & Cole, 2013). Despite high level of efficacy of aforementioned anti-TB drugs, they faced several challenges such as compliance issues and arising adverse side effects within TB patients (Zumla, Nahid & Cole, 2013; Zumla *et al.*, 2013). In addition, the emergence of new strains of *Mycobacterium. tuberculosis*; multi-drug resistance tuberculosis (MDR-TB) and extensively-drug resistance tuberculosis (XDR-TB) have since exacerbated this global health problem (Tabarsi & Mardani, 2012; Gupta *et al.*, 2011). Therefore, an effort of discovering novel anti-TB drug should be commenced to overcome the said problems, and as a sign of support to both Sustainable Development Goal 3.3: includes ending the TB epidemic by 2030 and End TB Strategy (WHO, 2016).

Plants are usable for medicinal purposes, particularly for TB therapy, owing to its wide array of secondary metabolites within them (Sharma & Yadav, 2016). Plants secondary metabolites, also known as phytochemicals, are chemicals that present naturally in plants. Unlike primary metabolites, these chemicals do not directly take part in the growth, reproduction and development of plants (Ahmed *et al.*, 2017). However, their absence may disrupt the plants survivability on a long-term basis (Irchhaiya *et al.*, 2015). Phytochemicals are also distinct from primary metabolites in a way that their distribution is limited in the plant kingdom (Anulika *et al.*, 2016). In other words, particular phytochemicals are present only in a specific species or related species of plant (Anulika *et al.*, 2016). Plants which are sessile organisms that lacks immunity system, rely chiefly on the arsenal of these secondary metabolites to defend themselves against both biotic and abiotic stresses (Mazid, Khan & Mohammad, 2011). Antimycobacterial activity displayed by particular plants are actually closely related with the phytochemicals contained within the plants as these chemicals are the ones responsible in characterizing such activity (Compean & Ynalvez, 2014; Kumar, Banik & Sharma, 2010). In most of the times, however, plant extracts are naturally complex in the sense that they contain a huge number of phytochemicals. Hence, bioassay-guided fractionation approach is a practical way that helps to separate these groups of phytochemicals into their individual entities while

simultaneously only selecting compounds with bioactivity (Weller, 2012; Nothias *et al.*, 2018).

Tetracera macrophylla, locally known as “Hempelas” is used traditionally by drinking plant sap from its root to treat malaria (Ismail *et al.*, 2015). *T. macrophylla* extracts from Sarawak also shown antiviral activity against H3N1 and H1N1 strains (Sambhara *et al.*, 2013). The most recent study by Sabran, Mohamed and Abu Bakar (2016) reported that the stem decoction of *T. macrophylla* is utilized by the Jakun community, indigenous people residing in Johor, to treat TB-related symptoms. They also reported that the ethyl acetate extract of *T. macrophylla* exhibited the highest antimycobacterial activity. The promising result shown by *T. macrophylla* extracts from the study by Sabran, Mohamed and Abu Bakar (2016) has therefore prompted this research to further investigate into its phytochemical fractions with anti-TB activity.

1.2 Problem statements

The currently used four-drug treatment regimen of the first-line anti-TB drugs (rifampicin, isoniazid, ethambutol and pyrazinamide) were proven to be highly efficacious with more than 95% cure rates (Zumla *et al.*, 2013; 2014). However, these drugs came with shortcomings. One primary challenge associated with current therapy is the drug intolerance, pharmacokinetic drug-drug interactions (particularly antiretroviral treatment in patients co-infected with HIV) and various adverse effects which result in the need of interruptions and changes to the regimen. Moreover, treatment with the current therapy is rather lengthy; minimum of six months duration which in turn, led to the adherence and compliance issues in TB patients (Pai *et al.*, 2016). Furthermore, the emergence of resistance strains of tuberculosis; multidrug-resistant (resistance to at least rifampicin and isoniazid) and extensively-drug resistant (resistance to rifampicin, isoniazid, any fluoroquinolones, plus at least one of three injectable second-line drugs) is now a widespread problem around the globe. Patients with multidrug-resistant tuberculosis requires a combination of second-line and third-line anti-TB drugs, which are more expensive, more toxic, and less effective when compared with that of standard regimen (Tiberi *et al.*, 2017)

Existing treatment of resistance strains of TB has a success rate of only 56% on a global scale (WHO, 2019). Besides, unless fully eliminated, *M. tuberculosis* may be contained in their host as asymptomatic latent TB infection which pose risks of reactivation, and an estimate of 1.7 billion people have already been latently infected (Zumla *et al.*, 2013, WHO, 2019). On the other hand, while there have been several studies conducted to evaluate antimycobacterial activity of other species in genus *Tetracera* (Fomogne-Fodjo *et al.*, 2014; Lawal *et al.*, 2011; Liliwirianis *et al.*, 2011), the in-depth analysis of antimycobacterial activities of *Tetracera macrophylla* is still an unanswered question. Therefore, the aforementioned problems have necessitated the efforts to search for novel anti-TB compounds, and drug discovery from plants is seen as one of the ways forward for tuberculosis study.

1.3 Research objectives

The objectives of the study are:

- i. To assess the anti-tuberculosis activities of fractions from *T. macrophylla* stem extract against *Mycobacterium tuberculosis* H37Ra.
- ii. To evaluate the interaction effects of fractions from *T. macrophylla* stem extract with anti-tuberculosis drugs (rifampicin and isoniazid).
- iii. To assess cytotoxicity of fractions from *T. macrophylla* stem extract to Human Embryonic Kidney cell line (HEK-293).

1.4 Research significance

The significance of this study is that it would provide an additional body of knowledge regarding genus *Tetracera*, particularly *Tetracera macrophylla*. Findings of this study, particularly pertaining to the bioactive fractions from *T. macrophylla* would provide a crucial insight into its overall potential as anti-TB drug. Apart from that, the effort of this study in obtaining a greater clarity in the interaction effect between bioactive fractions and current antibiotics would significantly help on how to combat *M. tuberculosis* more

effectively using natural products from plants. Moreover, since this study involves an ethnobotanical approach to acquire the plant samples, the result as yielded from this study would indirectly aid in uplifting the value of medicinal plants used by the indigenous people, particularly the Jakun ethnic. In addition, as this study is a continuation from a recent ethnobotanical study, the outcomes of this study provide a further scientific validation for claims made by the Jakun community: the use of *T. macrophylla* to treat TB-related symptoms. Ultimately, findings of this study therefore play a significant role in providing a stepping stone for further researches on discovering a novel nature-derived anti-TB drug while preserving the values of ethnobotanical medicine in indigenous people.



CHAPTER 2

LITERATURE REVIEW

2.1 Tuberculosis

Tuberculosis (TB) is the tenth leading cause of death globally and since 2007, it has been the leading cause of death from a single infectious agent which ranked just above Human Immunodeficiency Virus (HIV)/Acquired Immunodeficiency Syndrome (AIDS)(WHO, 2019). Due to its fatal threat and high mortality rate, World Health Organization (WHO) has declared TB as a global public health emergency in 1993 (WHO, 2014). On a global scale, an estimated of 10 million people were infected with TB which is equivalent to 132 cases per 100,000 population (WHO, 2020). The worldwide incidence of TB in 2019 occurred mostly in WHO South-East Asia Region which accounted for 44 % of total incidence, followed by the WHO African Region (24%), the WHO Western Pacific Region (18%), the WHO Eastern Mediterranean Region (8.1%), the WHO Region of Americas (2.9%) and the WHO European Region (2.6%). Furthermore, in 2018 alone, TB has claimed lives of approximately 1.2 million HIV-negative people with an additional of 251,000 lives among HIV-positive people (WHO, 2020). From these numbers, in 2018, 82% of death in HIV-negative people caused by TB occurred in the WHO African Region and the WHO South-East Asian Region in 2016 (WHO, 2020). Incidence caused by TB around the globe is shown in Figure 2.1.

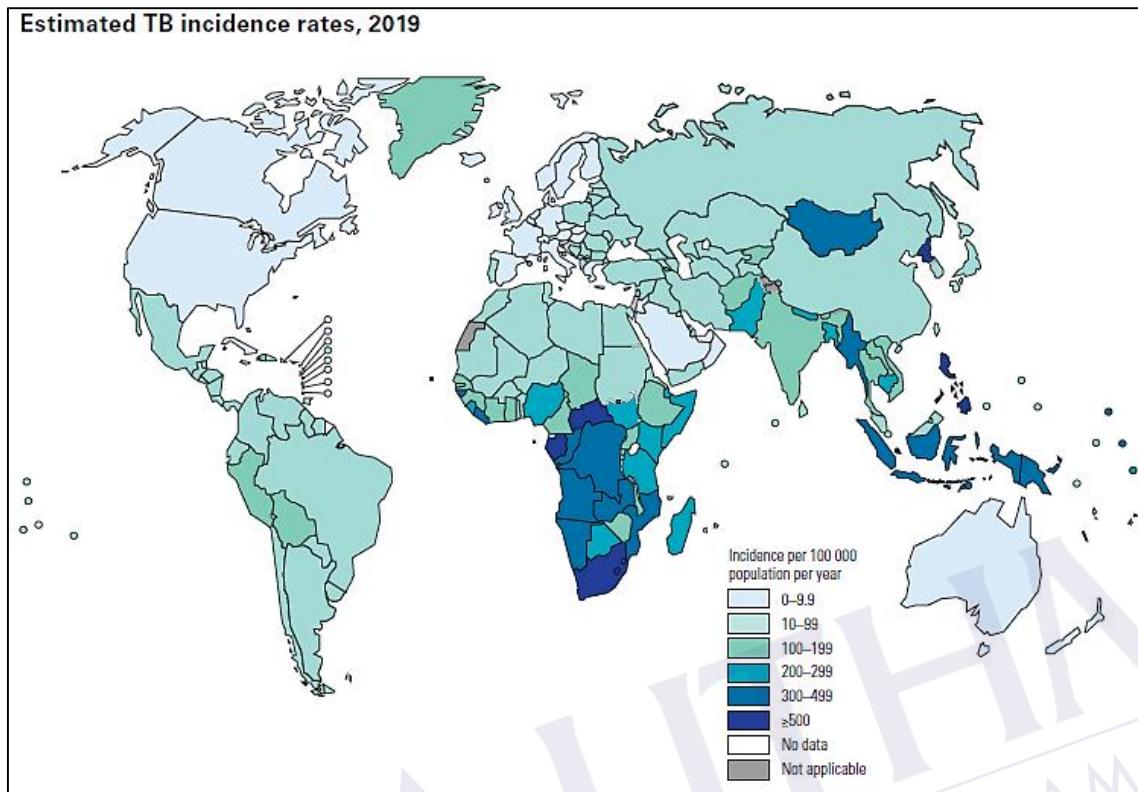


Figure 2.1: Incidence rates of TB across countries worldwide in 2019 (WHO, 2020)

As TB has been one of the primary causes of mankind death globally for many years, particularly across South-East Asian Region in which the incidence and mortality rate of TB was the highest, Malaysia was no exception. In 2001, TB was the second highest in terms of notification of communicable diseases in the country (Dony, Ahmad & Khen Tiong, 2004). From 2005 to 2011, the number of TB incidences in the country increased by 28 %, that was from 15,000 to 19,251. In 2016, it was best estimated that the incidence of TB in Malaysia was approximately 29,000 cases of a total population of 32 million people (WHO, 2019). Among the 15 states of Malaysia, Sabah, Selangor, Sarawak and Johor appeared to be the four states with the highest number of TB cases in 2011.

The incidence and prevalence of TB around the globe with such mortality rates were due to numerous risk factors, with HIV infection as one of the major risk factors (Pai *et al.*, 2016). In general, risk factors such as poverty, malnutrition, lack of Bacillus Calmette–Guérin (BCG) vaccine, urban residence and increased age are known to escalate the rate of TB infection among people globally (Hargreaves *et al.*, 2011). In addition, TB

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