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นิพนธ์ต้นฉบับ
ORIGINAL ARTICLE

ฤทธิ์ต้านเชื้อวัณโรค *Mycobacterium tuberculosis* H37Ra ของสารสกัดจากพืชสมุนไพรไทยที่มีสรรพคุณใช้ในการรักษาวัณโรคและโรคที่มีอาการคล้ายวัณโรค

กมลชนก รักเสรี[1]* ธาดา จูฑะโยธิน[2] ภมรี บิลมาศ[2] อรุณี ทองอ่อน[1] เฉลิมพล เกิดมณี[2] และประสิทธิ์ ผลิตผลการพิมพ์[2,3]

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บทคัดย่อ

งานวิจัยนี้ได้รวบรวมพืชสมุนไพรไทยที่มีสรรพคุณใช้ในการรักษาวัณโรคและโรคที่มีอาการคล้ายวัณโรค จำนวน 60 ชนิด ส่วนต่างๆ ของพืชสมุนไพรเหล่านี้นำมาสกัดด้วยไดคลอโรมีเทน ได้สารสกัดหยาบจำนวนทั้งหมด 120 ตัวอย่าง จากนั้นนำมาทำการทดสอบฤทธิ์ต้านเชื้อวัณโรคชนิด *Mycobacterium tuberculosis* H37Ra ด้วยวิธี Microplate Alamar Blue Assay (MABA) ผลการทดสอบ พบว่า สารสกัดมีฤทธิ์ต้านเชื้อวัณโรคที่ค่าความเข้มข้นของสารต่ำสุด (MIC) อยู่ในช่วงตั้งแต่ 0.1 จนถึง 200 ไมโครกรัมต่อมิลลิลิตร สารสกัดที่มีฤทธิ์ต้านเชื้อได้ดีอย่างมีนัยสำคัญมีค่า MIC น้อยกว่าหรือเท่ากับ 25 ไมโครกรัมต่อมิลลิลิตร มีจำนวนร้อยละ 15.8 ของสารสกัดทั้งหมดที่ทดสอบ ซึ่งมาจากพืชจำนวน 16 ชนิด หรือร้อยละ 26.7 ของพืชทั้งหมดที่ทำการศึกษา งานวิจัยนี้ได้แสดงให้เห็นว่า ฤทธิ์ต้านเชื้อวัณโรคที่ทดสอบมีความสอดคล้องและยืนยันข้อมูลทางด้านสรรพคุณที่มีบันทึกใช้มาในอดีต พืชเหล่านี้น่าจะมีศักยภาพเป็นแหล่งสำหรับการศึกษาหาสารสารสำคัญที่ออกฤทธิ์ต้านเชื้อวัณโรคเพื่อพัฒนาเป็นยารักษาวัณโรคในอนาคต

คำสำคัญ: ฤทธิ์ต้านเชื้อมัยโคแบคทีเรีย, พืชสมุนไพรไทย, ความเข้มข้นที่ต่ำที่สุดในการยับยั้งเชื้อจุลินทรีย์, เชื้อวัณโรค, วิธี Microplate Alamar Blue Assay

[1]กลุ่มวิชาวิทยาศาสตร์และอักษรศาสตร์ โครงการจัดตั้งวิทยาเขตอำนาจเจริญ มหาวิทยาลัยมหิดล อำนาจเจริญ 37000
[2]ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ ปทุมธานี 12120
[3]ภาควิชาจุลชีววิทยา คณะวิทยาศาสตร์ มหาวิทยาลัยมหิดล กรุงเทพมหานคร 10400
*ผู้รับผิดชอบบทความ

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ORIGINAL ARTICLE

Evaluation of sixty Thai medicinal plants used for treatment of TB and TB-related symptoms for in vitro inhibitory activity against *Mycobacterium tuberculosis* H37Ra

Kamolchanok Rukseree[1]*, Tada Juthayothin[2], Pamaree Billamas[2], Arunee Thong-on[1], Chalermpol Kirdmanee[2] and Prasit Palittapongarnpim[2,3]

Abstract

A total of 60 Thai medicinal plants documented as previously used for treatments of TB and TB-related symptoms were collected. A total of 120 dichloromethane extracts of various parts of these plants were prepared and evaluated for their activities against *Mycobacterium tuberculosis* H37Ra strain using the Microplate Alamar Blue Assay (MABA). For each extract, the minimum inhibitory concentration (MIC) was determined. The extracts had weak to significant activity, with MICs ranging from 0.1 to 200 µg/ml. Approximately 26.7 % of the collected plants (15.8 % of all plant extracts) showed antimycobacterial activity, with significant MICs ≤ 25 µg/ml. These results supported the use of ethnomedicinal records for anti-TB drug discovery. Parts of some plants may serve as candidate sources for identifying chemical constituents that have potential of being developed into anti-TB drugs.

Keywords: Antimycobacterial activity, Thai medicinal plant, Minimum inhibitory concentration (MIC), *Mycobacterium tuberculosis*, Microplate Alamar Blue Assay

[1] Department of Sciences and Arts, Mahidol University Amnatcharoen Campus, Mahidol University, Amnatcharoen 37000

[2] National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Pathumthani 12120

[3] Department of Microbiology, Faculty of Science, Mahidol University, Bangkok 10400

* Corresponding author: (e-mail: kamolchanok.ruk@mahidol.ac.th)

Introduction

Mycobacterium tuberculosis (*Mtb*) is the causative agent of tuberculosis (TB), one of the most common infectious diseases and leading causes of death worldwide. In 2018, the World Health Organization (WHO) estimated that approximately one-quarter of the world's population is infected with *Mtb*. It is estimated that 10 million (range: 9.0–11.1 million) people fell ill with TB and approximately 1.4 million died of the disease. Additionally, there were approximately half a million (range: 417,000–556,000) new cases of rifampicin-resistant TB (RR-TB)[1]. The increasing number of multidrug-resistant (MDR) and extensively drug-resistant (XDR) TB strains have rendered the current mode of treatment difficult and in many cases completely ineffective; leading to death of a third of infected people. The large number of TB cases in combination with the advent of drug-resistant TB strains have resulted in an urgent need for the development of new anti-TB drugs.

For several decades, many studies stated that 80% of the population in developing countries rely on traditional medicine (TM) for their primary health care[2]. In modern Western medicine, approximately 25% of the drugs prescribed worldwide are estimated to be derived from traditionally-used medicinal plants[3]. In 2016, Oyebode et al. reported that the use of TM in six middle-income countries was lower than previously stated[4]. However, an increasing amount of scientific evidence has demonstrated that many traditional plants contain beneficial bioactive compounds directly related to their ethnomedicinal uses. Furthermore, in 2019, the WHO continued to emphasize the importance of traditional and complementary medicine (T&CM) by reporting valuable information to be used as guidance by Member States for their national health[5]. Thus, medicinal plants can obviously serve as promising sources of harmless but effective anti-TB agent discovery.

Thailand is rich in natural resources owing to her geographic location. Thai TM is based on a unique blend of knowledge attained through centuries of practice together with knowledge adopted from other systems of medicine, mainly Indian, Chinese, and Khmer medicinal systems. In each region of Thailand, Thais have developed their own unique style of indigenous medicines, ranging from the simple use of medicinal plants as ingredients in food and drinks to sophisticated compound drugs formulated to treat certain ailments. The accumulated knowledge of the curative properties of medicinal plants has been handed down through generations within families, but not many written documents exist. In the 1870s, texts were collected, revised, and published in Samut Khoi (a type of folding-book manuscript), which serves as the basis for texts currently used[6]. In the 1990s, collected information on Thai traditional plants was compiled and published. This textbook, entitled Thai Traditional Plants, is comprised of five volumes that describe hundreds of plants, with over 600 pictures[7]. The information in these books was collected from data sources including Plant Resources of South-East Asia (PROSEA), the NAPRALERT database, and the Pharm database (Medicinal Plant Information Center, Mahidol University). These books include botanical descriptions, the plant parts used, chemical compounds found in the plant, Thai traditional medicinal uses, and toxicity information for safe use. These books provide comprehensive information for scientists

that are searching for new drug candidates because the curative properties were collected as a single plant use and not from the complex recipe.

In each region of Thailand, tuberculosis is known by different names, mostly according to the symptoms. Information of many plant species and medical recipes used to cure tuberculosis-related symptoms and tuberculosis has been recorded. Therefore, these plants possibly contain substances that can kill or inhibit *Mtb*. Numerous investigations demonstrated that several medicinal plant extracts exhibit biological activities directly related to their traditional uses. Therefore, we searched the aforementioned five books and several other Thai traditional medicine books for common terms that possibly indicate TB; namely phthisis, consumption, wasting fever, intra-stomach abscess, haemoptysis, chronic productive cough, pulmonary affections, and others. Consequently, a list of 182 Thai medicinal plants was compiled based on their curative uses for TB and TB-related symptoms.

In this study, we evaluated the potential antituberculosis properties of the selected Thai medicinal plants. Specifically, 60 species of the selected medicinal plants were collected, extracted, and tested for inhibitory activities against *Mtb* strain H37Ra using the Microplate Alamar Blue Assay (MABA).

Materials and Methods

Bacterial strain, chemicals, and media

M. tuberculosis strain H37Ra (ATCC 25177) was obtained from the American Type Culture Collection (ATCC; Rockville, MD). Middlebrook 7H9 broth, casitone, and Middlebrook OADC (oleic acid, albumin dextrose, catalase) growth supplement were purchased from Difco. Glycerol, Tween 80, dimethyl sulfoxide (DMSO), rifampin, isoniazid, and kanamycin were purchased from Sigma. Alamar blue solution was purchased from SeroTec Ltd.

Plant materials and preparation of extracts

Plant materials were purchased from public sources commonly used by Thai ethnomedicine practices. Confirmation of the species of the selected plants was based on morphologic characteristics of the plants, which was conducted by Dr.Weerachai Nanakorn (Queen Sirikit Botanic Garden, Thailand). The species and family names, traditional uses, and the parts of the plants that were used in the study are shown in Table 1. Only fresh plant materials were collected. Different parts of the plants were treated individually. Each part was cut into small pieces that were then air-dried. The dried materials were soaked in dichloromethane in a 1:10 ratio (w/v) in a clean glass bottle with a screw cap. The bottle was closed and placed in an unlighted hood for three days. Then, the initial supernatant was filtered through No.1 Whatman filter paper into a new sterile glass bottle. The remaining plant materials were re-extracted by soaking in fresh dichloromethane as described above. The second filtered-supernatant was collected into the same bottle as the initial supernatant and the lid of the bottle was left open in a hood cabinet until the supernatant volume was sufficiently minimized for transfer into a sterile 1.5-ml microtube, which was then further evaporated to dryness. Subsequently, about 10-20 mg of each of each air-dried extract was weighed into a new 1.5-ml microtube and dissolved in DMSO to give a 20 mg/ml stock extract for use in

subsequent antimycobacterial activity assays. The remaining air-dried extracts and stock solutions were stored at -20 ^{o}C until use.

Preparation of *Mycobacterium tuberculosis* strain H37Ra

The seed inocula were prepared according to Collins and Franzblau[8]. Briefly, *Mtb* H37Ra was grown at 37 ^{o}C on a rotary shaker in Middlebrook 7H9 broth supplemented with 0.2% glycerol, 1.0 g/l casitone, 10% OADC, and 0.05% Tween 80 (7H9GC-Tween) until the culture density reached an optical density of 0.4-0.5 at 550 nm. Bacteria were pelleted, washed twice, resuspended in Dulbecco's phosphate-buffered saline, and filtered through an 8-µm membrane. Then, 200 µl of filtered bacteria were aliquoted into sterile 1.5-ml microtubes and maintained as seed inocula stock at -80 ^{o}C. The next day, one seed stock tube was thawed and diluted across at least six concentrations to determine viable cell concentration (CFU/ml) and the most suitable dilution to use in subsequent antimycobacterial activity testing.

Antimycobacterial assay using the Microplate Alamar Blue Assay in 96-well plate format

The Microplate Alamar Blue Assay (MABA) was performed in a 96-well microplate (Corning, NY, USA), as previously described by Collins and Franzblau[8] with a slight modification in which outer perimeter wells were filled with sterile water to prevent dehydration of the test wells. Crude extracts were initially diluted with DMSO and then were diluted to a concentration of 400 µg/ml in 7H9GC (no Tween80). The wells corresponding to rows B to G of columns 2, 4, 5, 6, 8, 9, 10 of the microplate were filled with 100 µl of 7H9GC. The wells in column 11 were inoculated with 200 µl of the medium that served as media controls (M). Bacteria only controls (B) were set-up in column 10. One hundred microliters of each crude extract solution (400 µg/ml) were added to three wells in one row of columns 2 (or 6), 3 (or 7), and 4 (or 8). One hundred microliters were transferred from column 4 (or 8) to column 5 (or 9), the contents of the wells in column 5 (or 9) were mixed and then 100 µl of mixed medium were discarded. The wells in columns 2 and 6 served as test sample controls at the highest concentration tested. Frozen bacterial inocula were diluted 1:200 in 7H9GC medium. One hundred microliters of the bacteria were added to the wells in rows B to G in columns 3 (or 7), 4 (or 8), 5 (or 9) and 10, resulting in final bacterial concentrations of approximately 5×10^{5} CFU/ml.

The wells in column 10 served as bacteria (only) controls (B). For primary screening, final concentrations of each extract were 200, 100, and 50 µg/ml in columns 3 (or 7), 4 (or 8), and 5 (or 9), respectively. The plates were sealed with parafilm and were incubated at 37 ^{o}C for 5 days. At day 6 of incubation, 20 µl of Alamar Blue and 12.5 µl of 20% Tween 80 were added to one B well and one M well and the plates were incubated at 37 ^{o}C. Wells were observed at 24 hours for a color change from blue to pink. If the B wells turned pink by 24 hours, Alamar Blue was added to the entire plate. If the well remained blue, the additional M and B wells were tested daily until a color change occurred at which time reagents were added to the other remaining wells. The microplates were then resealed with Parafilm and were then incubated at 37 ^{o}C. The results were recorded 24 hours after adding reagent. All experiments were run in duplicate and were

repeated if the results were not equal. However, the higher concentration value is reported if the results of each extract were not in agreement.

Fluorescence units (FU) were measured using a Cytofluor II microplate fluorometer (PerSeptive Biosystems, Framingham, Mass.) in bottom-reading mode with excitation of 530 nm and emission of 590 nm. Background subtraction of the mean of triplicate M wells was performed on all wells. Percent inhibition was defined as 1 - (test well FU/mean FU of triplicate B wells) x 100. If any crude extract was active (≥ 90% inhibition) at 50 µg/ml, it was retested with higher dilutions to derive the minimum inhibitory concentration (MIC) value, which is defined as the lowest concentration with an inhibition of ≥ 90%. MICs of standard drugs; namely rifampin, isoniazid, and kanamycin were determined in duplicate in an additional plate as control tests. The MIC of these acceptable standard drugs were 0.0047-0.0095, 0.05, and 2.5 µg/ml, respectively.

Results and discussion

By using the terms indicating TB and TB-related symptoms, 182 plant names were compiled. Of these, 157 plant species (86%) were from the 5-volume traditional plant textbook and the other 25 medicinal plants (14%) are from other several traditional Thai medicine books. One third of the listed plants was searched for and collected for crude extraction and evaluation in the antimycobacterial activity assay. In this study, 60 medicinal plants belonging to 29 families were evaluated for antimycobacterial activity: Acanthaceae (3), Amaranthaceae (1), Apocynaceae (1), Araceae (2), Asparagaceae (1), Basellaceae (1), Cannaceae (1), Capparaceae (1), Caricaceae (1), Clusiaceae (1), Combretaceae (1), Compositae (2), Convolvulaceae (1), Cucurbitaceae (4), Euphobiaceae (1), Labiatae (1), Leguminosae (3), Liliaceae (1), Malvaceae (1), Myrtaceae (1), Oxalidaceae (1), Punicaceae (1), Rutaceae (4), Sapotaceae (1), Simarubaceae (1), Solanaceae (2), Umbelliferae (1), Verbenaceae (2), and Zingiberaceae (15). Each part of each plant was individually extracted with dichloromethane (CH2Cl2), dried, dissolved in DMSO, and then tested against Mtb H37Ra using the MABA. In total, 120 extracts from various plant parts were prepared as described above. The overall proportion of plant part extracts is shown in Figure 1. Each crude extract was tested at concentrations of 50, 100, and 200 µg/ml in duplicate. At the lowest concentration (50 µg/ml), if the extracts showed ≥ 90% inhibition of Mtb H37Ra growth, additional two-fold serial dilutions were tested to determine their MICs. We found that 84.2%, 51.7%, and 25.8% of all crude extracts exhibited antimycobacterial activity at ≤ 200, ≤ 100 and ≤ 50 µg/ml, respectively.

In previous work, antimicrobial activities of natural product extracts were generally reported based on the concentrations tested; for example, 100 µg/ml, 500 µg/ml, 1 mg/ml, and 20 mg/ml. After Kuete's publication in 2010, many publications of antimicrobial activity have stated that the activity of plant extracts should be classified as either significant (MIC < 100 µg/ml), moderate (100 < MIC ≤ 625 µg/ml), or weak (MIC > 625 µg/ml)[9]. Therefore, recent reports have considered only those extracts with MICs lower than 100 µg/ml as potential sources of new antimicrobial agents and a couple of studies mentioned that a significant MIC should be lower than 50 µg/ml. Several investigations of antimycobacterial activity still used a variety of cut-off values to define an MIC value as being

active when the MIC is ≤100-200 µg/ml. In this study, we defined extracts with an MIC value of ≤ 200 µg/ml as being active and considered extracts with an MIC value < 50 µg/ml as being significant activity that is worthy of subsequent isolation and purification.

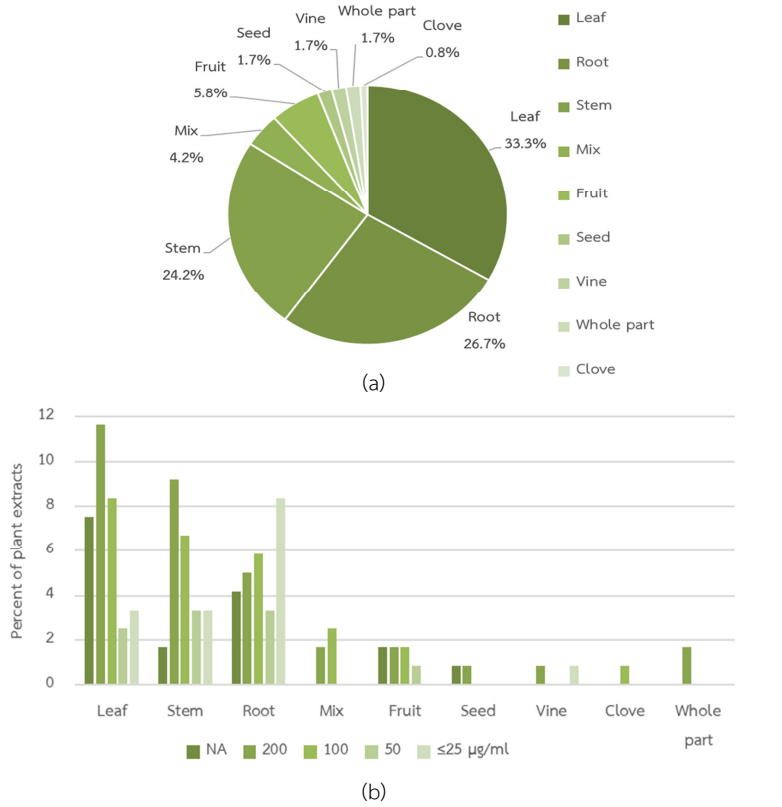

Figure 1 (a) Proportion of all extracted plant parts. (b) Percentage of plant part extracts exhibiting various minimum inhibitory concentrations.

The results showed that low levels of inhibition against *Mtb* H37Ra were common in the collected medicinal plants, as 59 of 60 plant species (98.3%) exhibited antimycobacterial activity, at least at the concentration of 200 µg/ml. The following 16 of 60 plants (26.7%) (half in the Zingiberaceae family) showed significant activity against *Mtb* H37Ra, with MICs ≤ 25 µg/ml: *A. galangal*, *A. nigra*, *A. purpurata*, *B. hispida*, *C. asiatica*, *C. aurantifolia*, *C. hystix*, *C. papaya*, *C. zedoaria*, *E. elatior*, *E. longifolia*, *H. coronarium*, *K. parviflora*, *S. trilobatum*, *T. bracteata* and *Z. officinalis*. The most active crude extracts were derived from the rhizomes of *A. nigra* and *A. galanga,* with MICs of 0.1 µg/ml. Hexane- or aqueous-crude extracts of both rhizomes were investigated to determine whether higher antimycobacterial activity could be obtained. Dichloromethane- and hexane-crude extracts of both Alpinia rhizomes had comparable MICs in the range of 0.05-0.1 µg/ml, while aqueous crude extracts had MICs in the range of 6.25-12.5 µg/ml, indicating 125 to 250 times less active. These results are in agreement with other studies that showed that most aqueous extracts had little or no antimycobacterial activity[10][11]. Subsequently, 1'-s-acetoxychavicol acetate (ACA), the major component of rhizomes, was shown to have an MIC of 0.1 µg/ml[12]. All MICs of the medicinal plant extracts are shown in Table 1.

Table 1 Antimycobacterial activity minimum inhibitory concentrations of Thai medicinal plant extracts

Species	Family	Traditional uses (associated with TB and TB-related symptoms)	Plant part used	MIC (μg/ml)
Abutilon graveolens W.&A.	Malvaceae	R/ ni: wasting fever, consumption	Fl	NA
			L	200
			S	50
Acanthus ebracteatus Vahl	Acanthaceae	S: intraabdominal abscess	L+S+R	100
Acorus calamus Linn.	Araceae	ni: chest disease, intrastomach abscess	L+R	200
Allium sativum Linn.	Liliaceae	Bl/ Cl: chest disease, chronic chest disease, tuberculosis	Cl	100
Alpinia galanga (Linn.) Sw.	Zingiberaceae	Oil: antituberculosis	R	0.1
Alpinia nigra (Gaertn.) B.L.Burtt	Zingiberaceae	Oil: antituberculosis	L	1.56
		R: wasting fever	S	3.12
		ni: deep cough	R	0.1
Alpinia purpurata	Zingiberaceae	R: wasting fever	S	25
Alpinia spp.	Zingiberaceae	R: wasting fever	S	100
Amaranthus viridis Linn.	Amaranthaceae	L: productive cough	L	NA
			Sd	NA
			S	NA
			R	NA
Andrographis paniculata (Burm. F.) Nees	Acanthaceae	W: pneumonia, tuberculosis	W	200
Asparagus racemosus Willd.	Asparagaceae	R: liver and lung rehabilitation	L	200
			S	200
Averrhoa carambola Linn.	Oxalidaceae	Fr: haematemesis	L	200
			S	100
Baccaurea ramiflora Lour.	Euphobiaceae	R: internal abscess, tuberculosis	L	200
Basella alba Linn.	Basellaceae	W: abscesses	L	100
Benincasa hispida (Thunb.) Cogn.	Cucurbitaceae	V: pneumonia	L	200
		Sd: productive cough, tuberculosis	V	200
			R	25
		Fr: haematemesis		
Boesenbergia rotunda (L.) Mansf.	Zingiberaceae	R: chronic cough, intraabdominal abscess	L	200
			R	50

Species	Family	Traditional uses (associated with TB and TB-related symptoms)	Plant part used	MIC (µg/ml)
Canna generalis L.H.Bailey	Cannaceae	R: lung strengthening, haemoptysis, haematemesis, cough, tuberculosis, lung strengthening	L	50
			R	200
Capparis micracantha DC.	Capparaceae	R, B: intraabdominal abscess	Fl	NA
			L	200
			R	100
Capsicum frutescens Linn.	Solanaceae	antimycobacterium	L	100
			Sd	200
			S	200
Carica papaya Linn.	Caricaceae	antimycobacterium	L	NA
			S	50
			R	12.5
Cassia tora Linn.	Leguminosae	L: chronic cough	L	100
Centella asiatica (Linn.) Urban	Umbelliferae	W: tuberculosis	L	NA
			R	25
Citrus aurantifolia (Christm & Panz) Swingle	Rutaceae	Fr (Juice): cleaning lung and blood L: intraabdominal abscess	L	12.5
			S	100
Citrus hystix DC.	Rutaceae	R: detoxification, internal abscesses, productive cough	L	25
			S	200
Codariocalyx motorius Ohashi	Leguminosae	S/ L/ ni: intraabdominal abscess, internal and intraabdominal abscesses	L	NA
			S	NA
			R	100
Combretum quadrangulare Kurz.	Combretaceae	ni: chest abscesses	L	50
Croton tiglium Linn.	Euphorbiaceae	R: expectorants ni: clearing sputum, antimycobacterium	L+S+R	100
Curcuma aeruginosa Roxb.	Zingiberaceae	ni: intraabdominal abscess	R	100
Curcuma longa Linn.	Zingiberaceae	R: antituberculosis	R	50
Curcuma xanthorrhiza Roxb.	Zingiberaceae	R: intraabdominal abscess, antimycboacterium	R	100
Curcuma zedoaria Roscoe	Zingiberaceae	ni: intraabdominal abscess	R	25
Drypetes roxbughii Wall.	Euphorbiaceae	Five parts: tuberculosis	L	200
			S	200
			R	200

Species	Family	Traditional uses (associated with TB and TB-related symptoms)	Plant part used	MIC (µg/ml)
Eclipta prostata Linn.	Compositae	antimycboacterium	L	NA
			S	200
			R	100
Elephantopus scaber Linn.	Compositae	R: chronic cough	L	100
			S	100
			R	NA
Erythrina suberosa Roxb.	Leguminosae	HW/ SW: intraabdominal	L	NA
		abscess	S	100
		B: haemoptysis		
Etlingera elatior (Jack) R.M. Smith	Zingiberaceae	antimycboacterium	L	100
			S	25
			R	12.5
Eurycoma longifolia Jack	Simarubaceae	R: tuberculosis	R	12.5
Hedychium coronarium J.Koenig	Zingiberaceae	S: throat infection	Fl	100
			L	25
Homalomena aromatica Schott	Araceae	ni: liver and lung diseases	L	200
Ipomoea aquatica Forsk.	Convolvulaceae	R: chronic cough	L	100
			S	200
			R	50
Kaempferia parviflora Wall. ex Baker	Zingiberaceae	ni: intraabdominal abscess	R	25
Lantana camara Linn.	Verbenaceae	Fl: tuberculosis,	L	200
		haematemesis	S	100
			R	100
Mammea siamensis Kosterm	Clusiaceae	ni: liver disease, lung	L	100
		strengthening	S	50
Mimusops elengi Linn.	Sapotaceae	Fl: tonic, lung strengthening,	L	200
		expectorants	S	200
			R	NA
Momordica charantia Linn.	Cucurbitaceae	L: chronic cough	W	200
		Fr: Leprosy		
		antimycobacterium		
Momordica cochinchinensis (Lour.) Spreng.	Cucurbitaceae	Sd: cough,	L	50
		tuberculosis,	Fr-sd	100
		intrapulmonary abscess	Fr-pulp and skin	50

Species	Family	Traditional uses (associated with TB and TB-related symptoms)	Plant part used	MIC (µg/ml)
Murraya paniculata (L.) Jack	Rutaceae	Fl: chronic cough	L	100
			S	200
			R	200
Murraya siamensis Craib	Rutaceae	R: tuberculosis, chronic lung disease	L	NA
			S	50
			R	200
Nyctanthes arbor-tristis Linn.	Verbenaceae	Fr: wasting fever	L	200
			S	100
			R	NA
Ocimum tenuiflorum Linn.	Labiatae	L: chronic cough	L	NA
			S	200
			R	200
Plumeria rubra L.	Apocynaceae	HW: Detoxification, intraabdominal abscess	L+S	100
Psidium guajava Linn.	Myrtaceae	antimycobacterium	L	200
			S	200
Punica granatum Linn.	Punicaceae	antimycobacterium	L	100
			S	200
			R	NA
Rhinacanthus nasutus Kurz.	Acanthaceae	ni: tuberculosis	L+S+R	200
Sapium indicum Willd.	Euphorbiaceae	ni: intraabdominal abscess	L	100
			S	100
			R	200
Solanum trilobatum Linn.	Solanaceae	R/ L: tuberculosis	L	200
			S	25
Trichosanthes bracteata Voigt.	Cucurbitaceae	Fr: chronic liver and lung diseases	Fr-pulp and skin	200
			Fr-sd	200
			V	25
Zingiber montanum (Koen.) Theilade	Zingiberaceae	R/ Fl: haematemesis	L	NA
		R: Bleeding through nose and mouth	R	100
Zingiber officinalis Roscoe	Zingiberaceae	Fl: wasting fever	R	25
		ni: deep cough,		
		R: chest disease		
Zingiber zerumbet (Linn.) Smith.	Zingiberaceae	Fl: wasting fever	R	50

Used part: B: bark; Bl: bulb; Cl: clove; Fl: flower; Fr: fruit; HW: heartwood; L: leaf; S: stem/stalk; Sd: seed; SW: sapwood; R: root/rhizome; V: vine; W: whole plant; Five parts; flower, fruit, leaf, root and stem; ni: not indicated.

NA: not active at the highest concentration (200 µg/ml) tested.

Other plant crude extracts of the *Zingiberaceae* family that showed significant activity against *Mtb* H37Ra with MIC values less than 50 µg/ml should be further investigated to identify the chemical constituents responsible for antimycobacterial activity. Rhizome extracts of *A. purpurata*, *C. zedoaria*, *H. coronarium*, *K. Parviflora,* and *Z. Officinalis* had MIC values of 25 µg/ml, while rhizome and stem extracts of *E. elatior* had MIC values of 12.5 and 25 µg/ml, respectively. These results are in agreement with other reports of antimycobacterial activity. For example, fatty alcohols from *A. purpurata* inhibited *Mtb* H37Rv, with an MIC of 64 µg/ml[13]. 5,7,4'-trimethoxyflavone and 3,5,7,4' -tetramethoxyflavone from *K. parviflora* inhibited *Mtb* H37Ra, with MICs of 50 and 200 µg/ml, respectively[14]. 10-gingerol from *Z. officinalis* rhizomes inhibited *M. avium* and *Mtb* H37Rv, with $MIC_{99\%}$ values of 25 and 50-100 µg/ml, respectively[15]. Crude alcoholic extracts of *E. elatior* inhibited the growth of *Mtb* H37Rv[16]. Although a variety of biological activities of *C. zedoaria* essential oils are known, its antimycobacterial activity has never been reported, to the best of our knowledge. However, *C. longa*, a closely related species of *C. zedoaria*, contains curcuminoid constituents that possess antimycobacterial activity at approximately 100 µg/ml, which, after modification, its derivatives containing mono-*O*-methylcurcumin isoxazole showed MICs of 0.09 and 0.195–3.125 µg/ml against drug sensitive and drug resistant clinical *Mtb* isolates, respectively[17]. (+)-coronarin A and coronarin D methyl ether from *H. Coronarium* rhizomes exhibited antitubercular activities against *Mtb* H37Rv, with MICs of 80 and 50 µg/ml, respectively[18].

Leaf extracts of *C. aurantifolia* and *C. hystrix* (Rutabeae family) exhibited strong antimycobacterial activity, with MICs ranging between 12.5 to 50 µg/ml. The study published by Camacho-Corona Mdel et al. (2008) of extracts of the citrus species *C. aurantifolia* and *C. sinensis* demonstrated that both extracts were active against both drug susceptible and drug resistant strains of *Mtb* H37Rv, with MICs of 25-100 µg/ml [19][20].

S. trilobatum has been documented in Thai traditional medicine as a cure for tuberculosis. Our results show that stem and leaf extracts exhibited MIC values of 25 and 200 µg/ml, respectively. This plant possesses a broad spectrum of antibiotic, antibacterial, antifungal, and anticancer activities[21] [22] mediated by several compounds including sobatum, β-solamarine, solaine, solasodine, glycoalkaloid, diosogenin, and tomatidine[23]. Leaf extracts of this plant appear to be responsible for immunomodulatory effects, as previously reported[24]. However, further investigation of stem extracts may help to clarify the identity of the active constituents responsible for antimycobacterial activity.

B. hispida and *T. bracteata* belong to the family Cucurbitaceae. The fruit of *B. hispida* is highly recommended for TB patients in Ayurvedic medicine; however, it seems to be beneficial in numerous other diseases as well. *T. bracteata* has been widely used in several traditional systems. The fruit is used as a treatment for leprosy in Unani medicine and, in Thailand, it is used as an anti-fever remedy, an anthelmintic, a treatment for migraines[25], chronic liver and lung disease, and other ailments. Chemical constituents isolated from *T. bracteata* were previously reported[26] [27]

but have not been proven to possess antimycobacterial activity. Our study demonstrated that *B. hispida* root extract and *T. bracteata* stem extract had an inhibitory effect against *Mtb* H37Ra, with an MIC value of 25 µg/ml.

Extracts of *C. papaya* have been reported as having antifungal, antileishmaiasis, antimalarial, antiamebic, and antimicrobial activities[10]. Aqueous and methanol leave extracts of *C. papaya* were reported to have MIC values against *Mtb* H37Ra more than 100 µg/ml[28]. However, dichloromethane extracts of root and stem parts of this plant exhibited stronger MIC values of 12.5 and 50 µg/ml, respectively.

In Thailand, *C. asiatica* is thought to have some medicinal properties. The entire plant has been indicated for TB treatment and in India it is used to treat leprosy patients. Asiaticoside is a triterpene glycoside that in liposomal form was found to have better microbicidal properties against *M. leprae* and *Mtb* as compared to its free form[29]. Interestingly, our results showed that a leaf extract at 200 µg/ml did not inhibit *Mtb* H37Ra whereas the root extract was active, with an MIC value of 25 µg/ml.

E. longifolia Jack, an herb found in Southeast Asia, has been widely used in TM. It has been scientifically proven to possess antimalarial, anticancer, anti-diabetic, aphrodisiac, and antimicrobial activities. The plant parts are rich in various bioactive compounds; alkaloids and quassinoids are major components. Aqueous leaf extracts showed antibacterial activity against *Staphylococcus aureus* and *Serratia marscesens*, while root extracts had no antibacterial activity against the bacteria tested[30]. Our results evaluated its traditional use in Thai medicine, which showed a significant MIC value of 12.5 µg/ml. Further investigation of the dichloromethane extract of *E. longifolia* root should be performed to identify the active chemical that inhibits *Mtb*. The MIC results associated with *B. hispida*, *C. asiatica*, *C. aurantifolia*, *C. hystix*, *C. papaya*, *E. elatior*, *S. Trilobatum,* and *T. Bracteata* should raise the awareness of natural product researchers to collect the other parts of plants that have not been indicated in traditional uses in order to expand the chances of finding good sources of bioactive constituents.

Mtb H37Ra could be used as a reliable surrogate for the more virulent H37Rv strain for the in vitro antituberculosis activity assay, although the MICs of certain antibiotics tested were lower for H37Ra as compared to H37Rv[8] [31] [32]. This may also be beneficial, as extracts having lower antimycobacterial activity could be found. Therefore, in this study, we considered those plant extracts that had MIC values < 50 µg/ml to be significant and deserving of further investigation. In addition, the use of *Mtb* H37Ra for testing the antimycobacterial activity of natural extracts would enable those anti-TB research scientists who have access to a BSL-2 laboratory but not a BSL-3 to contribute to anti-TB drug discovery and development[32,33]. Additionally, the antimycobacerial activity assay could be made simpler and less expensive by freshly preparing the inoculum from the mycobacteria grown on solid medium and using 0.01% (w/v) resazurin instead of the commercial Alamar Blue solution[34].

Dichloromethane is the only solvent used for extraction in this study due to its relatively low boiling point and fewer manipulations needed during the extraction process. In addition, as

compared to water extraction, dichloromethane extraction potentially yields chemical mixtures having higher antimycobacterial inhibitory effects[11], likely due to concentrated non-polar compounds extracted from the plant. Previously, 270 Peruvian plant samples were extracted and screened at a 50 µg/ml concentration against *Mtb*H37Rv using the BACTEC 460 system[10]. Although half of the dichloromethane extracts inhibited the mycobacteria, only 3% gave strong inhibition (> 90%). In this study, 25.8% of all plant extracts (43.3% of the collected plants) were active at ≤ 50 µg/ml ($MIC_{90\%}$). Apparently, the selection of medicinal plants based on their traditional uses provided more chances of discovering natural sources of anti-TB constituents.

Conclusion

This study evaluated 60 medicinal plants previously documented as treatments for TB and TB-related symptoms; 26.7% of the collected plants (15.8% of all plant extracts) exhibited antimycobacterial activity, with significant MICs ≤ 25 µg/ml. Overall, our results suggest a strong positive correlation between the antimycobacterial activity and the traditional knowledge of plants used for TB and TB-related diseases in the Thai Medicinal Plants. The results herein provide initial information to researchers for prioritizing plants of interest for elucidating active compounds for development of new anti TB drugs.

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