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New Finding of an Anti-TB Compound in the Genus *Marsypopetalum* (Annonaceae) from a Traditional Herbal Remedy of Laos

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Abstract

Ethnopharmacological relevance—There is widespread use of traditional herbal remedies in the Lao PDR (Laos). It is common practice to treat many diseases with local plants. This research project documented and analysed some of these traditional remedies used to treat symptoms of tuberculosis (TB).

Materials and methods—This research was executed by interviewing healers about plants used traditionally to treat the symptoms of TB. Samples of some of the plants were collected, and extracts of 77 species were submitted to various *in vitro* assays in order to determine the amount of growth inhibition of virulent *Mycobacterium tuberculosis* H37Rv (Mtb), as opposed to other microbes and mammalian Vero cells.

Results—Interviews took place with 58 contemporary healers in 5 different provinces about plants currently used, giving a list of 341 plants. Bioassay-guided fractionation was performed on *Marsypopetalum modestum* (Pierre) B. Xue & R.M.K. Saunders (Annonaceae), leading to the isolation of dipyrithione, an anti-mycobacterial compound isolated for the first time from the genus *Marsypopetalum* through this research.

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Conclusions—This research has helped to increase awareness of Laos' rich diversity of medicinal plants and will hopefully provide incentive to preserve the undeveloped forested areas that remain, which still hold a wealth of medical information for future discoveries.

Keywords

antimycobacteria; botany; chromatography; cytotoxicity; phytochemistry; Traditional medicine Asia & Oceania

Additional keywords

medical ethnobotany; Laos; *Marsypopetalum*; Annonaceae

1. Introduction

Traditional medicine is the backbone of primary health care in Laos, with herbal preparations representing the substantial portion of medications. Many of these herbal remedies are used to treat tuberculosis (TB), reflecting the fact that TB is prevalent in the country. This project began under an International Cooperative Biodiversity Group (ICBG) grant, in which different traditional herbs were analyzed for their medical potential to treat TB, cancer, HIV/AIDS, and malaria (Soejarto et al., 2012; Soejarto et al., 1999; Soejarto et al., 2006). This project focused specifically on TB. The purpose of this paper is to communicate the results of our study.

2. Background

2.1. Medicinal plants of Laos

Traditional herbal remedies have been used frequently in the Lao People's Democratic Republic (Lao PDR, or Laos) for centuries. Traditional knowledge about the use of these plants has been passed down and is held by many healers today.

In addition to its wealth of traditional herbal knowledge, Laos contains immense areas of undeveloped forests. These forests hold a wealth of information, medical and otherwise. It is thought by some scientists to be one of the "most botanically unexplored countries in Asia" (Thompson and Thompson, 2008; WWF, 2012). However, deforestation is destroying Laos' unique plant diversity at an alarming rate (Stibig et al., 2007). There are links between environmental harm and poverty (MOIC, 2012; UNDP, 2012). Giving practical value to forested areas may bring needed encouragement for conservation and sustainable utilization to take place as outside demands call to clear forests.

In order to explore the medical potential of plants, there has been some debate over whether or not traditional medicinal plants are any more likely than randomly chosen plants to contain compounds that act against microbial targets (Balick, 1990; Cragg et al., 1994; Gyllenhaal et al., 2012; Lewis and Elvin-Lewis, 1995; Saslis-Lagoudakis et al., 2012). In a review by Gyllenhaal et al. (2012), it was shown that plants that were used traditionally to treat symptoms of tuberculosis in Laos were significantly more likely to yield active test results against mycobacteria than plants chosen at random.

2.2. Tuberculosis (TB)

The focus of this research was TB, a disease that is currently ravaging the Asian continent. In 2010, 8.8 million new cases of TB were diagnosed and attributed to 1.4 million deaths (WHO, 2012a). Current predictive models estimate that one-third of the world's population

is infected with latent TB, waiting for the victims' immune systems to be compromised. While TB is a curable disease, it is also a disease that primarily affects people who can't afford the treatments. More than 95% of TB deaths happen in lower income countries (WHO, 2012b). As such, among people with HIV/AIDS, especially in developing countries, TB is a leading cause of death (WHO, 2011, 2012b). In Laos, a human female with tuberculosis was buried in NE Thailand in the Iron Age (Tayles and Buckley, 2004), signaling its presence in the region for thousands of years. In 2010, more than 3,800 new cases of TB were identified in the country. These events infer that TB was a problem of the past and is currently still a problem that many people of Laos are confronted with today. It follows that people have most likely been searching herbal remedies for something to ease the symptoms of TB, and that if something works, they have continued to use it into the present.

2.3. International collaborative research

There is understandable concern about traditional medical knowledge from developing countries being used to generate revenue for foreign pharmaceutical companies, while the communities who provided the information are weakly compensated or not compensated at all. While the Convention on Biological Diversity (CBD: <http://www.cbd.int/convention/text/>) and the Nagoya Protocol (<http://www.cbd.int/abs/text/>) have increased awareness about ownership of genetic materials and intellectual property, specific guidelines about addressing these issues have remained vague. With a goal to fairly acknowledge and compensate contributions to traditional medicine knowledge of Laos used in this project, this project was also designed to follow the guidelines set down more recently by the International Society of Ethnobiology (ISE, 2006).

This research was performed under a Memorandum of Agreement (MOA) established between the Institute of Traditional Medicine (ITM) and UIC, detailing the objectives, responsibilities and benefits of the involved parties. Based on the MOA for the ICBG project, the core components address intellectual property rights, prior informed consent, and a benefit-sharing plan. These components are separated into eleven parts, covering academic exchanges; joint research; UIC, ITM, and joint responsibilities; intellectual property rights; biological material transfer; dispute resolution; and renewal and amendments, among other things. The research protocol, which involved interviewing healers in Laos, was approved by the UIC Institutional Review Board (UIC-IRB protocol #2007-0396).

3. Materials and methods

3.1. Healer interviews

The process of working with the indigenous traditional knowledge of contemporary healers in Laos started at the government level. The Institute of Traditional Medicine (ITM) contacted the provincial level Traditional Medicine Stations (TMS) prior to a field trip, and the TMS would in turn contact village chiefs, and/or abbots to ask about healer availability and willingness to be interviewed. The field interviews for this research were conducted in Bokeo, Bolikhamxay, Champasak, Luang Prabang, and Vientiane provinces. All healers were provided with a Prior Informed Consent (PIC) sheet in the Lao language, describing the research and what the healer's part would be if he/she consented to the interview. The interviewer(s) would then ask the healer questions following a semi-structured interview guide. Under a permit granted by the Ministry of Agriculture and Forestry of Laos, plant samples intended for bioassay were collected, voucher herbarium specimens were prepared, and the plants were photographed.

3.2. Plant collection and taxonomic identification

The plants were collected under a permit granted by the Ministry of Agriculture and Forestry. Plant samples and their voucher herbarium specimens were collected following the WHO Guidelines on Good Agricultural and Collection Practices for Medicinal Plants (WHO, 2003) with attention to the conservation of the species. Standard collection information and field notes were recorded following guidelines described by Alexiades (1996). Collections of 77 different plant species were made for this research, of which 19 species were recollected.

Plant taxonomic identification was carried out by comparing voucher herbarium specimens to a previously identified specimen in deposit in a herbarium, as well as with taxonomic circumscriptions and illustrations in standard floristic treatises (Ho, 1993; Inthakoun and Delang, 2011; Vidal, 1959). In the case of *Tin Tang Tia* (*Marsypopetalum modestum* (Pierre) B. Xue & R.M.K. Saunders), DNA sequencing of the chloroplast gene *rbcL* and the chloroplast intergenic spacer *trnL-trnF* were generated in the Pritzker laboratory at the Field Museum of Natural History, Chicago, using the primers and protocols described in Xue et al. (2011) and Su et al. (2008).

3.3. Sample collection and processing

For primary evaluation, 50 to 250 g of the plant part used by the healer was collected. Samples were selected from clean, non-diseased plants, and care was exercised in the collection process in order to minimize the risk of contamination with foreign matter. In collecting leaves, twigs, stems, and branches of woody plants (shrubs, trees), the desired part was cut with a clean machete. For root samples, a small piece was cut some distance from the base of the stem. The lower part of the stem and the root system were left intact, so the plants remained alive to regenerate new roots, stems and/or branches. Samples were then dried by placement on a clean concrete platform in a well-ventilated area, according to a protocol designed by Soejarto (2002).

Samples of select plants yielding extracts that exhibited high percent inhibition of *Mycobacterium tuberculosis* H37Rv (Mtb) were recollected. After the primary collection and taxonomic identification, a literature search was conducted to ensure that recollection would not pose a threat to the species population. A search for the risk status of each species was conducted online utilizing the CITES list (<http://www.cites.org/eng/app/appendices.php>) and the Red List of Threatened Species™ (<http://www.iucnredlist.org/>).

Sample extraction for primary screening was performed at the pharmacognosy laboratories of the ITM in Vientiane. Samples were extracted into 90% ethanol (EtOH) and repeated twice. The extracts were then condensed using a Heidolph Laborota 4000 rotary evaporator (rotavapor).

3.4. Biological assays

All biological assays were conducted in the laboratories of the Institute for Tuberculosis Research (ITR) at UIC. Primary evaluation was conducted in order to determine if an extract inhibited virulent *Mycobacterium tuberculosis* H37Rv (Mtb) specifically, or if the extract was a general cytotoxin. This primary screening was conducted against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Mycobacterium smegmatis*, in addition to Mtb. *E. coli* and *S. aureus* were tested according to a modified protocol described by NCCLS documents M7-A2 and M100-S3 (NCCLS, 1990; NCCLS, 1991) in cation-adjusted Mueller Hinton (CAMH) media, with the absorbance read at 570 nm at 20 hours. *C. albicans* was tested using a modified protocol described by NCCLS document M27-A2 (NCCLS, 2002) with RPMI 1640 media and absorbance reading at 48 hours at 570 nm. Rifampin, one of the

most widely used TB medications, was used as a positive control. The primary screening also included testing for potential toxicity to human cells through the use of Vero cells (Cantrell et al., 1996). The testing protocol used in this research followed the methods used by Falzari et al. (2005).

Testing against mycobacteria specifically (*M. smegmatis* and virulent Mtb) entailed the use of the microplate Alamar Blue assay (MABA) (Collins and Franzblau, 1997; Franzblau et al., 1998). In order to determine if the active components possibly target non-replicating persistent *Mycobacterium tuberculosis* (NRP Mtb), this research utilized the Low-Oxygen-Recovery Assay (LORA) (Cho et al., 2007).

3.5. Isolation and structure elucidation

Primary fractionation was usually achieved through the use of solid phase extraction (SPE) cartridges (Bond Elut C18, 500 mg, 6 ml cartridges from Agilent). A small amount of each extract was loaded and washed with 20% MeOH, 40% MeOH, 60% MeOH, 80% MeOH, 100% MeOH, and then 100% CHCl₃. A Waters Delta 600 high pressure liquid chromatography (HPLC) system equipped with a Waters 996 photodiode array detector and semi-preparative column was used for final fractionation. At a flow rate of 2 mL/min, a linear gradient running from 95% H₂O: 5% MeOH to 100% MeOH over 30 min followed by 15 min of 100% MeOH was used.

High-resolution mass spectrometry was performed with a Shimadzu Prominence XR HPLC system coupled to a Shimadzu Ion Trap – Time-of-Flight (Shimadzu IT-TOF) mass spectrometer. The NMR data were obtained and recorded on Bruker AVANCE 600 and/or 900 NMR spectrometers at 600 and 900 MHz, respectively. Samples were run in D₂O and deuterated MeOH (CD₃OD) for comparison with previously published data. NMR data was analyzed using MestReNova Version 6.1.0-6224 and PERCH NMR tools version 2010.1.

4. Results

4.1. Biological assays

Bioassay results are presented in Table 1 and Table 2. The tables report the findings of all of the plants in entirety. Table 1 lists the primary plant collections with the results for bioassays involving a spectrum of microbes. This was done to predict specificity of the plant extract to mycobacteria as opposed to other pathogens. A subset of Table 1 was previously published (Elkington et al., 2009). After finding the results, some of the plants were recollected. Table 2 lists results from recollected plants against three types of mycobacteria (virulent *Mycobacterium tuberculosis* H₃₇Rv (Mtb), non-replicating persistent Mtb (NRP Mtb), *Mycobacterium smegmatis*), and Vero cells.

From the 77 total species evaluated, 12 exhibited above 90% inhibition against *Mycobacterium tuberculosis* H₃₇Rv (Mtb) at 100 µg/mL in the first evaluation. MIC values from these plants ranged from 0.05 to 96.6 µg/mL (Table 3).

4.2. Analysis of *Tin Tang Tia*

This plant was reported by a healer as a component in three different formulations consisting of up to 32 different plant species. Traditionally, the stem or root is dried and rubbed on a stone to produce a powder, which is then mixed with water and powder from the other plants and given to the patient to drink. The healer said that it can also be boiled with the other plants, rather than making into a powder.

The first sample was collected in August 2007 and submitted to the primary bioassays. After confirming that it exhibited a very low MIC against Mtb, recollections were carried out in different seasons. Voucher herbarium specimens were prepared for all collections, which were used as basis for taxonomic identification as well as for biological evaluation.

When other healers were asked about *Tin Tang Tia*, they indicated three separate taxonomic species, *Marsypopetalum modestum*, *Anomianthus dulcis*, and *Uvaria rufa*, all members of the Annonaceae family. Alternatively, when shown a photo of *Marsypopetalum modestum* and asked for the common name, other healers often gave the name of *Pii Pouan*. Given the many potential routes for confusion around the name, considerable effort was made to sort out the taxonomic identity. Through DNA sequencing from herbarium specimen collections bge253 and bge255 and a comparison with BLAST (<http://blast.ncbi.nlm.nih.gov>), the DNA sequences were found to be most similar (>97% identical) to existing sequences of *M. pallidum* and *M. crassum*. Based both on the genetic and phenotypic similarities, the plant was determined to be *Marsypopetalum modestum* (Pierre) B. Xue & R.M.K. Saunders.

4.2.1. Marsypopetalum modestum—*M. modestum* are small trees, which have been found in peninsular Southeast Asia, growing 3 to 4 m in height. Leaves are simple, alternate, acuminate, 3 to 8 cm in width by 8 to 25 cm in length, on 0.5 cm petioles. They bear the characteristics of other *Marsypopetalum* leaves, with straight secondary veins and prominent arcuate loops (Xue et al., 2011). Flowers are approximately 1 cm in diameter, with numerous stamens and greenish fleshy petals. The apocarpous fruit consists of a group of umbelliform fruitlets, disposed in extra-axillary clusters, glabrous, ellipsoid, one-seeded, and turning from green to bright red, as shown in Fig. 1.

4.2.2. Bioassays—EtOH extractions of bge080, bge113, and bge115 were performed at the ITM's pharmacognosy laboratories. At UIC, 5 g of dried stem material from bge253 was extracted three times into 18 mL EtOH. The resulting extract was condensed by rotavapor and redissolved in DMSO for testing. A H₂O extraction of bge253 was also performed at UIC, by boiling approximately 5 g of dried stem in 200 mL water. The results of the primary biological evaluation are presented in Table 4 below.

The values for cytotoxicity (IC₅₀) are calculated and compared to the MIC values through calculation of a Selectivity Index (SI) for each extract through the following formula: SI=IC₅₀/MIC, seen in the far right columns. A higher value indicates a higher degree of selectivity to Mtb than to mammalian cells.

4.2.3. Isolation and structure elucidation from the primary collection—Based on the high SI value for selectivity for Mtb, bge080 was fractionated with an SPE cartridge. The fractions were then resubmitted to the bioassays. Further fractionation continued with the use of HPLC. Five fractions were obtained based on peaks and time. The five HPLC fractions (A–E) were then submitted to bioassays, with the results given in Table 5.

Isolate D exhibited the lowest MIC and was selected for further investigation. The structure was elucidated by HRMS² and NMR analysis and confirmation with reference standards. The positive mode electrospray HRMS analysis provided a monoisotopic molecular weight of 252.0019 from the protonated molecule ([M+H]⁺, 253.0092). A sodiated adduct ([M+Na]⁺, 274.996) and a potassiated adduct ([M+K]⁺, 290.965) were also observed. The tandem MS spectrum of the protonated molecule showed a major product ion at *m/z* 141.99 and minor product ions at *m/z* 237.12, 205.04, 126.00, and 111.02. The neutral loss of 48 amu (S₁O₁), giving the minor product ion at *m/z* 205.04 with the accompanying mass defect increase, suggested the presence of sulfur. This led to a probable molecular formula of C₁₀H₈N₂O₂S₂ (with a calculated exact mass of 252.0027). With the analysis of the NMR

data, the compound was proposed to be 2,2'-dithiobis(pyridine N-oxide), also known as dipyrithione. The structure was confirmed by comparison with a reference standard of dipyrithione purchased from AK Scientific, Inc., Lot # LC26013. Comparative IR analysis was also performed with a Nicolet 6700 FT-IR Spectrometer, giving peaks at $\nu_{\max}$ 3385, 1600, 1465, 1422, 1221, 838, and 763 cm^{-1} in agreement with previously reported values (Nicholas et al., 2001; O'Donnell et al., 2009). NMR data (^1H and ^{13}C) and IR data are available upon request.

4.2.4. Comparison with collection from a cultivated source—Because the MIC of *Mtb* growth from a crude plant extract is very rarely as low as that seen in the *M. modestum* collection, a question of contamination arose, possibly due to pesticides or pollutants. In order to confirm whether the source of activity came from the plant, an additional source of the plant material was sought. Another plant in the wild could not be found, so a recollection was carried out from a cultivated source.

Similarly to the process for the original collection, the crude EtOH extract was fractionated with a SPE cartridge to yield 6 fractions. Further fractionation with reversed-phase HPLC afforded five fractions. From this, another isolate, called isolate 3, exhibited similar bioactivity to isolate D. Samples were prepared at 0.1 mg/mL concentrations in MeOH and the HRMS² was repeated. The major component in isolate 3 ($[\text{M}+\text{H}]^+$, 253.010) showed HRMS and tandem MS spectra consistent with dipyrithione. The formula and tandem MS pattern of the minor component ($[\text{M}+\text{H}]^+$, 237.016) was consistent with dipyrithione with one less oxygen atom. HRMS² data are available upon request.

In order to confirm that the two isolates were the same compound, they were compared with the purchased reference standard. A comparison of biological activity is shown in Table 6.

In addition, the NMR spectra in CD_3OD matched for the two isolates and the dipyrithione standard, as shown in Fig. 2 and Fig. 3.

5. Discussion

Healers reported 223 different common names of plants. While the importance of surveying both males and females has been demonstrated (Pfeiffer and Butz, 2005), this study made no preference as to the gender of the healer, and the majority of the healers chosen by the TMS and heads of each village happened to be male. A major constraint for plant collection was inconsistency of the common names. Many plants known under a single common name have represented multiple taxonomic species. In cases where one common name referred to more than one plant, efforts were made to collect all of the plants. An online search for previous research involving *Mycobacteria* was conducted with NAPRALERT®, PubMed, Embase, and Scifinder®, and no entries were found about previous testing of *M. modestum* against *Mtb*.

Dipyrithione, the active constituent that was isolated, is currently used as a pesticide and fungicide. It has previously been isolated from other natural products (Nicholas et al., 2001; O'Donnell et al., 2009), and similar compounds have been reported from the closely related species, *Trivalvaria costata* (Hook. f. & Thomson) I.M. Turner (Lu et al., 2010). In addition, other plants that were collected from the same area as bge080 (bge079, bge081 through bge084, bge114) did not exhibit the same activity in the assays. This species was also collected from two different locations, one of which was in the wild, and both collections exhibited similar activity and contained this compound, as demonstrated by NMR and LC-MS.

6. Conclusions

The results of primary evaluation of all samples are presented in Table 1 and Table 2. Of all of the collected species, 10% of the plants named by healers (8 of 77) were active (defined as exhibiting greater than 90% inhibition in the MABA or LORA). However, this research only examined *in vitro* inhibition of Mtb and Vero cells. The plants and formulations studied may well have other healing properties for respiratory ailments that were beyond the scope of this research, such as analgesic, antitussive, or immune system boosters.

While not all healing systems and techniques are translatable through hard scientific terms at this point in time, this research has taken on as a goal to encourage retention and passing of medicinal plant traditions from one generation of healers to the next through the translation of some traditional treatments into biomedical terms. It is anticipated that this and similar types of research will increase awareness of Laos' rich medicinal plants and plant diversity and provide incentive to preserve the undeveloped forested areas that remain, which still hold a wealth of information for future discoveries.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. *Tin Tang Tia* (bge255)
Fruit and flowers.

Fig. 2. ^1H NMR spectra comparison

Proton NMR spectra (900 MHz, CD_3OD) were obtained with a Bruker AVANCE 900 NMR spectrometer and analyzed using MestReNova Version 6.1.0-6224.

Fig. 3. ^{13}C NMR spectra comparison

Carbon-13 NMR spectra (DEPTQ-135, 225 MHz, CD_3OD) were recorded on a Bruker AVANCE 900 NMR spectrometer and analyzed using MestReNova Version 6.1.0-6224.

Table 1

Primary evaluation of ethanolic extracts of collections bge43 to bge108.

Collection number ^d and scientific name	Common name	Percent inhibition ^b				
		Mth ^c	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
bge043 (Asteraceae) <i>Elephantopus scaber</i> L.	Khii Fai Nok Koum	46	-181	-8	30	-24
bge044 (Rubiaceae) <i>Benkara sinensis</i> (Lour.) Ridsdale	Kheuah Khai Khao	5	-151	14	32	-9
bge045 (Rhamnaceae) <i>Colubrina pubescens</i> Kurz	Khan Toum	39	-159	1	31	-21
bge046 (Bignoniaceae) <i>Millingtonia hortensis</i> L. f.	Kang Khong	69	-103	20	31	-18
bge047 Fruit (Burseraceae) <i>Canarium cf. hirsutum</i> Willd.	Kohk Keuam	10	-23	22	41	93 (24.7) ^d
bge047 Stem (Burseraceae) <i>Canarium cf. hirsutum</i> Willd.	Kohk Keuam	-1	-230	9	38	63
bge048 (Araliaceae) <i>Heteropanax fragrans</i> Seem.	Oy Xang	-7	-19	-14	26	38
bge049 (Araliaceae) <i>Schefflera</i> sp.	Tin Nohk	-10	-34	-10	26	53
bge050 (Bignoniaceae) <i>Oroxylum indicum</i> (L.) Kurz	Lin Mai (mak)	74	2	58	27	94
bge051 (Bignoniaceae) <i>Fernandoa cf. adenophylla</i> (Wall. Ex G. Don) Steenis	Khae Pa	98 (83.3) ^d	-85	66	28	105
bge052 (Celastraceae) <i>Salacia chinensis</i> L.	Tah Kai	-18	-153	31	23	79
bge053 (Stemonaceae) <i>Stemona cochinchinensis</i> Gagnep.	Sam Sip (hua)	53	-237	15	31	-5
bge054 (Arecaceae) <i>Caryota mitis</i> Lour.	Tao Hang	-18	-63	17	37	-4
bge055 (Fabaceae) <i>Milletia</i> sp.	Hang Yen	30	-50	13	30	-8
bge056 (Moraceae) <i>Ficus hispida</i> L. f.	Deua Pong	-11	-86	7	31	38
bge057 (Lygodiaceae) <i>Lygodium microphyllum</i> (L.) Sw.	Koot Ngong	-9	-12	6	31	-21
bge058 (Rubiaceae) <i>Mirrargyna rotundifolia</i> (Roxb.) Kuntze	Tohm Phai	63	-4	19	22	-37
bge059 (Araceae) <i>Lasia spinosa</i> (L.) Thwaites	Bo Nam / Pak Nam	-5	-231	49	30	66
bge060 (Rubiaceae) <i>Psychotria</i> sp.	Kuk Mohk	18	-45	6	23	-1
bge061 (Rutaceae) <i>Melicope pteleifolia</i> (Champ. Ex Benth.) T.G. Hartley	Khom Lah Wan Joh	-1	-42	78	27	30
bge062 (Lygodiaceae) <i>Lygodium flexuosum</i> (L.) Sw.	Koot Ngong / Koot Khee Pa	26	-12	-6	25	-26
bge063 (Rubiaceae) <i>Ixora</i> sp.	Khai Nao (Noy)	13	-52	11	30	3
bge064 (Irvingiaceae) <i>Irvingia malayana</i> Oliver ex Bennett	Bohk	19	-100	31	38	98 (11.4) ^d
bge065 (Solanaceae) <i>Solanum melongena</i> L.	Mak Kheuah Kheum (hak)	9	-19	-13	27	-15

Collection number ^d and scientific name	Common name	Percent inhibition ^b				
		Mtb ^c	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
bge066 (Rutaceae) <i>Feroniella lucida</i> Teijsm. & Binn.	<i>Ka Sung (mak / kohk)</i>	86 (91.5) ^d	-11	4	27	23
bge067 (Chrysobalanaceae) <i>Purinari</i> sp.	<i>Polk</i>	6	-107	40	25	73
bge068 (Acanthaceae) <i>Justicia adhatoda</i> cf. L.	<i>Hou Ha (kohk)</i>	86	-127	6	29	46
bge069 (Meliaceae) <i>Sandoricum koetjape</i> (Burm. F.) Merr.	<i>Kho Phou</i>	11	-29	49	23	67
bge070 (Tiliaceae) <i>Microcos paniculata</i> L.	<i>Khom Som</i>	26	-29	40	31	76
bge071 (Melastomataceae) <i>Melastoma malabathricum</i> L.	<i>Ben Ah / En Ah</i>	-4	-19	17	35	99 (6.0) ^d
bge072 (Lauraceae) <i>Litsea cubeba</i> (Lour.) Pers.	<i>Sii Khai Tone</i>	80	-126	35	26	-15
bge073 (Euphorbiaceae) <i>Jatropha curcas</i> L.	<i>Niao Khao (mak)</i>	12	-64	7	28	-7
bge074 (Euphorbiaceae) <i>Jatropha gossypifolia</i> L.	<i>Niao Deng (mak)</i>	18	-26	13	26	-10
bge075 (Rhamnaceae) <i>Ziziphus oenoplia</i> (L.) Mill.	<i>Nam Lep Mayoh</i>	17	-33	17	25	-18
bge076 (Euphorbiaceae) <i>Anitidesma diandrum</i> (Roxb.) Roth	<i>Mao (mak)</i>	-23	-56	23	27	97
bge077 (Verbenaceae) <i>Clerodendrum palmatolobatum</i> Dop	<i>Phouang Phii Deng</i>	32	-89	13	28	32
bge078 (Rutaceae) <i>Micromelum</i> cf. <i>falcatum</i> (Lour.) Tanaka	<i>Sa Mat</i>	60	-43	23	25	80
bge079 (Rutaceae) <i>Glycosmis cochinchinensis</i> (Lour.) Pierre	<i>Xom Xeun</i>	26	-59	7	22	7
bge080 (Annonaceae) <i>Marsypopetalum modestum</i> (Pierre) B.Xue & R.M.K.Saunders	<i>Tin Tang Tia</i>	99 (0.72) ^d	84 (93.3) ^d	86 (11.9) ^d	94 (24.2) ^d	96 (<0.4) ^d
bge081 (Apocynaceae) <i>Myriopterion extensum</i> (Wight & Arn.) K. Schum.	<i>Oy Sam Souan</i>	67	-61	20	20	43
bge082 (Myrsinaceae) <i>Ardisia</i> sp.	<i>Tin Cham Kholn</i>	-6	57	-6	27	97 (20.7) ^d
bge083 (Rutaceae) <i>Micromelum minutum</i> Wight & Arn.	<i>Summat Khao</i>	46	-8	-6	24	-23
bge084 (Apocynaceae) <i>Tabernaemontana bufalina</i> Lour.	<i>Phet Pa (mak)</i>	77	-10	-16	25	-23
bge085 (Loganiaceae) <i>Strychnos nux-blanda</i> A.W. Hill	<i>Toum Kah Khao</i>	14	-12	-11	26	40
bge086 (Sapindaceae) <i>Dimocarpus longan</i> Lour.	<i>Kha Leen</i>	12	-23	30	25	25
bge087 (Verbenaceae) <i>Vitex trifolia</i> L.	<i>Phii Sena</i>	89 (81.0) ^d	-64	-5	24	-13
bge088 (Capparaceae) <i>Capparis</i> cf. <i>micrantha</i> A. Rich.	<i>Kheuah Khao Mohk</i>	-9	-69	0	36	-19
bge089 (Fabaceae) <i>Cassia tora</i> L.	<i>Nya Lap Meun</i>	55	-127	21	24	39
bge090 (Meliaceae) <i>Aglaita</i> sp.	<i>Phii Mob</i>	2	-66	25	23	-17
bge091 (Capparaceae) <i>Capparis micrantha</i> A. Rich.	<i>Xai Xou Tonh (hak)</i>	18	-88	0	19	14

Collection number ^a and scientific name	Common name	Percent inhibition ^b				
		Mtb ^c	<i>M. smegmatis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>C. albicans</i>
bge092 (Euphorbiaceae) <i>Chaetocarpus castanocarpus</i> (Roxb.) Thwaites	Bolih Khai	11	24	4	27	94 (97.1) ^d
bge093F (Rutaceae) <i>Aegle marmelos</i> (L.) Corrêa	Mak Toum	2	-101	25	28	9
bge093S (Rutaceae) <i>Aegle marmelos</i> (L.) Corrêa	Mak Toum	97 (54.9) ^d	-77	18	27	42
bge094 (Fabaceae- Papil) <i>Mucuna pruriens</i> (L.) DC.	Tam Yay	15	-11	4	28	-22
bge098 (Fabaceae) <i>Dalbergia cf. ramosa</i> Roxb.	Padong Khor	-12	-49	-2	27	-18
bge099 (Euphorbiaceae) <i>Sauropus androgynous</i> (L.) Merr.	Wan Ban (hak)	28	-41	0	28	-35
bge100 (Solanaceae) <i>Solanum lasiocarpum</i> Dunal	Mak Euk / Mak Kheuah Euk	71	-70	18	31	-14
bge101 (Moringaceae) <i>Moringa oleifera</i> Lam.	Ii Houm (hak)	14	-39	16	27	-15
bge102 (Amaranthaceae) <i>Amaranthus spinosus</i> L.	Phak Hom (hak)	-7	-78	3	26	-26
bge103 (Meliaceae) <i>Azadirachta indica</i> A. Juss.	Khom Kat Dao (Khom Kadao)	71	-115	23	30	2
bge104 (Annonaceae) <i>Rollinia mucosa</i> (Jacq.) Baill.	Khanthaloht (peuk)	97 (49.2) ^d	13	-24	31	66
bge105 (Anacardiaceae) <i>Spondias cf. pinnata</i> (L. f.) Kurz	Kohk (mak / peuk)	34	0	-13	28	96 (6.0) ^d
bge106 (Moraceae) <i>Ficus glomerata</i> Roxb.	Deuah Kieng	20	-10	21	30	58
bge107 (Polypodiaceae) <i>Drynaria quercifolia</i> (L.) J. Sm.	Koot Hohk	2	33	19	33	-20
bge108 (Poaceae) <i>Saccharum officinarum</i> L.	Oy Dam	34	-63	-4	29	42

^a The collection numbers in parentheses represent the voucher herbarium specimen numbers.

^b All of the extracts were tested at 100 µg/mL.

^c Mtb stands for virulent *Mycobacterium tuberculosis* H37Rv (Mtb), NRP Mtb stands for non-replicating persistent Mtb

^d Values in parentheses represent the Minimum Inhibitory Concentration (MIC) in µg/mL, or the smallest concentration of the extract required to inhibit 90% of the Mtb growth.

Table 2
Results of bioassay evaluation for ethanolic or aqueous extracts of collections bge110 to bge256.

Collection number ^a and scientific name	Common name	MIC (µg/mL) ^b			IC ₅₀ (µg/mL)
		Mtb	NRP Mtb	M. smegmatis	Vero
bge110 (Rutaceae) <i>Feroniella lucida</i> Swingle	<i>Sung (mak / kohk)</i>	>100	>100	>100	>100
bge111 Root (Solanaceae) <i>Solanum cyanocarphum</i> Blume	<i>Mak Kheuah Kheun (hak)</i>	>100	>100	>100	97.2
bge111 Stem (Solanaceae) <i>Solanum cyanocarphum</i> Blume		>100	>100	>100	82.7
bge112 (Bignoniaceae) <i>Millingtonia hortiensis</i> L. f.	<i>Kang Khong</i>	>100	>100	>100	>100
bge113 (Annonaceae) <i>Marsypopetalum modestum</i> (Pierre) B.Xue & R.M.K.Saunders	<i>Tin Tang Tia</i>	11.4 to 13.9	1.47 to 22.9	29.8	60.9 to >100
bge114 (Rutaceae) <i>Micromelum minutum</i> Wight & Arn.	<i>Sa Mat Khao</i>	>100	>100	>100	>100
bge115 (Annonaceae) <i>Marsypopetalum modestum</i> (Pierre) B.Xue & R.M.K.Saunders	<i>Tin Tang Tia</i>	5.5 to 6.6	8.5 to 39.5	>100	47.5 to 76.2
bge116 (Fabaceae) <i>Mucuna pruriens</i> (L.) DC.	<i>Tam Yay</i>	>100	>100	>100	>100
bge117 (Bignoniaceae) <i>Fernandoa adenophylla</i> (Wall. Ex G.Don) Steenis	<i>Khue Pa</i>	>100	>100	>100	>100
bge118 (Rutaceae) <i>Aegle marmelos</i> (L.) Corrêa	<i>Mak Tuum</i>	>100	>100	>100	>100
bge119 (Polygalaceae) <i>Securidaca inappendiculata</i> Hassk.	<i>Kheuah Khao Mwak</i>	>100	>100	>100	63.4
bge120 (Rubiaceae) <i>Benkara sinensis</i> (Lour.) Tirveng.	<i>Kheuah Khat Khao</i>	>100	>100	>100	>100
bge122 (Menispermaceae) <i>Tinospora crispa</i> (L.) Hook. f. & Thomson (aqueous extract made from herbarium specimen)	<i>Kheuah Khao Ho</i>	>100	>100	>100	11.14
bge122 (Menispermaceae) <i>Tinospora crispa</i> (L.) Hook. f. & Thomson (EtOH extract made from herbarium specimen)		59.7	28.7	>100	20.78
bge137 (Annonaceae) <i>Uvaria rufa</i> Blume	<i>Tin Tang Tia</i>	33.1	93.6	>100	>100
bge239 (Bignoniaceae) <i>Oroxylum indicum</i> (L.) Kurz	<i>Lin Mai</i>	>100	>100	>100	88.5
bge240 (Menispermaceae) <i>Tinospora crispa</i> (L.) Hook. f. & Thomson	<i>Kheuah Khao Ho</i>	>100	>100	>100	>100
bge241 (Annonaceae) <i>Uvaria</i> cf. <i>microcarpa</i> Champ. ex Benth.	<i>Phii Phouan</i>	43.2 to >100	>100	>100	>100
bge242 (Moraceae) <i>Sireblus asper</i> Lour.	<i>Som Phor</i>	>100	>100	>100	>100
bge243 (Verbenaceae) <i>Vitex trifolia</i> L.	<i>Phii Seua</i>	>100	>100	>100	>100
bge244 (Menispermaceae) <i>Tinospora crispa</i> (L.) Hook. f. & Thomson	<i>Kheuah Khao Ho</i>	96.3	>100	>100	>100
bge245 (Moraceae) <i>Sireblus asper</i> Lour.	<i>Som Phor</i>	>100	>100	>100	>100
bge246 (Euphorbiaceae) <i>Sauropus androgynus</i> (L.) Merr.	<i>Phak Wan Ban</i>	>100	>100	>100	>100

Collection number ^a and scientific name	Common name	MIC (µg/mL) ^b			IC ₅₀ (µg/mL)
		Mtb	NRP Mtb	<i>M. smegmatis</i>	Vero
bge247 (Fabaceae) <i>Crotalaria pallida</i> Aiton	Hing Hai	>100	>100	>100	>100
bge248 (Rutaceae) <i>Glycosmis pentaphylla</i> (Retz.) DC.	Xom Xuuan	>100	93.5 to >100	>100	>100
bge249 (Rutaceae) <i>Melicope</i> cf. <i>pteletifolia</i> (Champ. ex Benth.) T.G. Hartley	Khom Lah Wan Joh	>100	>100	>100	23.7 to 71.3
bge250 (Lygodiaceae) <i>Lygodium microphyllum</i> (Cav.) R. Br.	Koot Ngong	>100	>100	>100	>100
bge251 (Simaroubaceae) <i>Irvingia malayana</i> Oliver ex Bennett	Bohk	>100	>100	>100	>100
bge252 (Rubiaceae) <i>Mitragyna hirsuta</i> Havil.	Tohm Phai	>100	>100	>100	>100
bge253 (Annonaceae) <i>Marsypopetalum modestum</i> (Pierre) B. Xue & R.M.K. Saunders	Tin Tang Tia	5.9 to 23.5	2.5 to 5.9	38.2 to >100	5.9 to 14.5
bge254 (Bignoniaceae) <i>Millingtonia hortensis</i> L. f. (aqueous extract)	Kang Khong	>100	>100	>100	>100
bge256 (Rutaceae) <i>Clausena harmandiana</i> (Pierre) Guillaumin (aqueous extract – highest test concentration 15 µg/mL)	Song Fa	83.1	>100	>100	>100
		>15	>15	>15	>15

Unless specified, all plants were extracted into EtOH, dried, and redissolved in DMSO for testing at 100 µg/mL.

^aThe collection numbers in parentheses represent the voucher herbarium specimen numbers.

^bValues represent the Minimum Inhibitory Concentration (MIC), or the smallest concentration of the extract required to inhibit 90% of the Mtb growth.

Table 3

Crude plant ethanolic or aqueous extracts exhibiting greater than 90% inhibition of Mtb.

Scientific name (collection number)	Common name	MIC (µg/mL) Mtb
(Annonaceae) <i>Marsypopetalum modestum</i> (Pierre) B.Xue & R.M.K.Saunders (bge080, 113, 115, 253)	<i>Tin Tang Tia</i>	0.05 to 11.9
(Annonaceae) <i>Rollinia mucosa</i> (Jacq.) Baill. (bge104)	<i>Khanthaloht</i>	43.9 to 75.2
(Annonaceae) <i>Uvaria cf. microcarpa</i> Champ. ex Benth. (bge241)	<i>Phii Phouan</i>	43.2 to >100
(Annonaceae) <i>Uvaria rufa</i> Blume (bge137)	<i>Mak Phii Phouan / Tin Tang Tia</i>	33.1 to >100
(Bignoniaceae) <i>Fernandoa cf. adenophylla</i> (Wall. Ex G. Don) Steenis (bge051, 117) ^a	<i>Khae Pa</i>	79.7 to >100
(Menispermaceae) <i>Tinospora crispa</i> (L.) Hook. F. & Thomson (bge122, 240, 244)	<i>Kheuah Khao Ho</i>	2.43 to 96.2
(Rutaceae) <i>Aegle marmelos</i> (L.) Corrêa (bge093, 118) ^a	<i>Mak Toum</i>	47.8 to >100
(Rutaceae) <i>Clausena harmandiana</i> (Pierre) Guillaumin (bge256)	<i>Song Fa</i>	83.1 to >100
(Rutaceae) <i>Feroniella lucida</i> Swingle (bge066, 110) ^a	<i>Kohk Sung</i>	90.4 to >100
(Rutaceae) <i>Glycosmis pentaphylla</i> (Retz.) DC. (bge248)	<i>Xom Xeuan</i>	93.5 to >100
(Rutaceae) <i>Micromelum minutum</i> Wight & Arn. (bge83, 114) ^a	<i>Sa Mat Khao</i>	45.7 to >100
(Verbenaceae) <i>Vitex trifolia</i> L. (bge087, 243) ^a	<i>Phii Seua</i>	77.6 to >100

The table is in alphabetical order by taxonomic family of each species. The collection numbers in parentheses represent the voucher herbarium specimen numbers. All extracts were tested at 100 µg/mL.

^aRecollections of these species did not exhibit activity in the bioassays.

Table 4

Tin Tang Tia (Marsypopetalum modestum) bioassay data.

Collection Number	Plant Part	Extraction Solvent	MIC (µg/mL)			IC ₅₀ (µg/mL)	SI	
			Mtb	NRP Mtb			Mtb	NRP Mtb
bge080	stem	EtOH	1.33	5.85		51.49	38.71	8.80
bge113	stem	EtOH	13.93	22.87		60.95	4.38	2.66
bge115	stem + root	EtOH	6.58	8.50		47.52	7.22	5.59
bge253	stem	EtOH	5.97	2.50		<6.25	<1.0	<2.5
bge253	stem	H ₂ O	23.52	5.95		>100 (81%)	>4.25	>16.81

The collection number represents the voucher herbarium specimen number. Extracts were tested at 100 µg/mL.

Table 5

Bioassay data after preparative HPLC fractionation of the 20% MeOH SPE fraction from bge080.

Fraction ^a	Fraction weight (mg)	MIC (µg/mL) ^a		IC ₅₀ (µg/mL) ^a	SI	
		Mtb	NRP Mtb		Mtb	NRP Mtb
A	91.74	> 10 (2%)	> 10 (11%)	> 10 (13%)	NA	NA
B	0.29	> 10 (0%)	> 10 (35%)	> 10 (0%)	NA	NA
C	5.21	1.18	0.49	> 10 (36%)	> 8.47	> 20.41
D	1.22	0.06	< 0.039	1.44	24.00	36.92
E	4.44	4.06	1.49	8.66	2.13	5.81

^a All preparative HPLC fractions (codified A to E) were tested at 10 µg/mL.

Table 6

Comparison of biological activity.

Fraction/Isolate	MIC (µg/mL)		IC ₅₀ (µg/mL)	SI	
	Mtb	NRP Mtb		Mtb	NRP Mtb
Crude bge080	11.94	4.94	16.89	1.41	3.42
bge080 isolate D	0.06	<0.039	1.44	24.00	36.92
Crude bge115	9.46	39.53	>10	>1.06	>0.25
bge115 isolate 3	4.46	1.12	>10	>2.24	>8.93
Dipyrrithione	<0.039	<0.039	7.46	>191.3	>191.3

Bioassay results for the crude extracts are given for comparison.