A comparative study of antituberculosis activities of *Tetracera macrophylla* Wall. Ex Hook. f. &Thoms. stem fractions using different chromatographic stationary phases

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**Abstract.** Mempelas (*Tetracera macrophylla* Wall. Ex Hook. f. & Thoms) is a climbing liana that has been used by several ethnics in Malaysia for its medicinal values. In regards to tuberculosis (TB) therapy, *T. macrophylla* has ethnombotanically demonstrated promising antit-B activities. Current research aimed to determine the antit-B activities of *T. macrophylla* stem fractions eluted from two different stationary phases via column chromatographic technique. *T. macrophylla* stems were extracted using a semi-polar solvent via maceration method. Two portions of the extract were fractionated through column chromatography using Silica (Si) gel 60 and Mitsubishi Chemical Ion (MCI®) gel. Eluted fractions were monitored via pre-coated Si gel 60 F254 aluminium plates as thin-layer chromatography (TLC). Anti-TB bioassay were conducted via the employment of Tetrazolium Microplate Assay (TEMA) procedure. Fractionation of the extract has resulted in the elution of 12 and 16 combined fractions from MCI® gel and Si gel 60, respectively. TEMA result has revealed that none of the combined fractions eluted from MCI® gel inhibited the test organism, *Mycobacterium tuberculosis* H37Ra. On contrary, 7 of 16 fractions as eluted from Si gel 60 inhibited *M. tuberculosis* H37Ra at Minimum Inhibition Concentration (MIC) ranging from 400 to 800 µg/ml. The results from this study has led to the conclusion that Si gel 60 is a more suitable stationary phase to be used in fractionating plant extracts for TB research.

1. Introduction

Since 2007, tuberculosis (TB) has been the leading cause of death from a single infectious agent and it ranked just above HIV/AIDS [1]. Being caused by *Mycobacterium tuberculosis*, World Health Organization (WHO) has declared TB as a global public emergency in 1993 [2]. Despite relentless efforts from WHO and governments, TB persists in claiming millions of lives around the globe, annually. With more than 95% cure rates, currently-used anti-TB chemotherapy were proven highly efficacious [3]. However, the existing anti-TB drugs are challenged by the arising side-effects, compliance issues by TB patients and pharmacokinetic drug-drug interaction conflicts [4]. In addition, the emergence of resistance strains of TB has worsen this global health concern [5, 6]. Therefore,
alternative anti-TB drug discovery efforts from natural products, particularly medicinal plants, is seen as a promising way forward in tuberculosis study.

*Tetracera macrophylla*, a climbing woody vine locally known as “Mempelas”, belongs to Dilleniaceae family which comprised of 10-14 genera with approximately 500 species [7]. Largely found across South-east Asian countries including Malaysia, Indonesia, Brunei and Thailand, genus *Tetracera* consists of 45 species which includes *T. macrophylla* [8]. Ethnobotanically, *T. macrophylla* has been used by several ethnic in Malaysia for traditional medicinal purposes. Decoction of stem of *T. macrophylla* is used by Temuan people in Kampung Tering, Negeri Sembilan, Peninsular Malaysia, to treat physical weak [9]. Apart from that, the leaves of *T. macrophylla* are used by the Jah Hut people in Kampung Pos Penderas, Pahang, Peninsular Malaysia as a part of healing ceremony of treating various ailments [10]. In the most recent study by Sabran, Mohamed and Abu Bakar [11], the Jakun community from Kampung Peta, Johor south of Peninsular Malaysia, drunk sap from stems of the plant to treat TB and its related symptoms.

Plants are usable for medicinal purposes including for treating TB, owing to the arsenal of secondary metabolites within them [12]. Plants secondary metabolites, also referred to as phytochemicals, are nature-derived chemicals that present in plants. Unlike primary metabolites, these chemicals do not directly take part in the growth, reproduction and development of plants [13]. However, phytochemicals play a pivotal role as the plant defense mechanism against both biotic and abiotic stresses [14]. In the efforts of finding medicinal-worthy phytochemicals, conducting the extraction and fractionation process properly, is extremely crucial.

Extraction of plant materials involved the use of various types of chemical solvents, which in turn, were precisely selected based on texture and water content of the plant material and on the type of compounds that is intended to be isolated [15]. Crude extracts obtained generally encompassed numerous, unspecified mix of compounds, of which, some of them may exhibit bioactivity while others may not. Therefore, the extract will then need to go further separation and purification steps, referred to as fractionation and isolation. The purification of natural constituents mainly incorporates one or another, or a combination of several chromatographic techniques such as column chromatography (CC), thin-layer chromatography (TLC), gas chromatography (GC), liquid chromatography and gas liquid chromatography (GLC) [16].

Therefore, the present study was performed to evaluate the antimycobacterial activities of *T. macrophylla* stem fractions eluted from two different stationary phases via column chromatographic technique: Silica (Si) gel and Mitsubishi Chemical Ion (MCI®) gel.

2. Methodology

2.1. Collection and Preparation of Plant Materials

Fresh stems of *T. macrophylla* were collected from Taman Negara Johor Endau-Rompin. On-field identification of the collected plant materials was made using key genera as provided by Hoogland [17]. Voucher specimens of each samples were prepared for further species verification by botanists from Forest Research Institute of Malaysia (FRIM).

All of the collected plant stems were air-dried and oven-dried at 40°C for six days, respectively. The dried plant stems were cleaned to remove their dirt and bark. The total dry weight of the collected samples was 8.052 kg. All of the plant materials were grounded to powder using an industrial grinder in Jabatan Pertanian, Serdang.

2.2. Extraction of Plant Materials

In general, the finely ground powder obtained were macerated using semi-polar solvent with a slight modification [18]. It is generally known that organic extracts with medium polarity have the probability of containing phytochemicals including phenolic compounds, alkaloids and terpenoids with antimicrobial activities [19]. In a ratio of 1:5 (sample: solvent), a total of 3 kg of plant sample in powder form were soaked repetitively in ethyl acetate for 72 hours for 3 cycles at room temperature. The samples
were filtered using Whatman® qualitative filter paper No. 1 (pore size 11 µm). The filtrates obtained were combined and fully concentrated to dryness under reduced pressure using rotary evaporator at 40 to 45 °C. The fully dried and concentrated crude extract were then stored in suitable flasks in the refrigerator until further use.

2.3. Fractionation and Monitoring of Plant Materials using Chromatographic Techniques
Fractionation of natural compounds from T. macrophylla stem barks crude extracts were performed according to the protocol by Fomogne-Fodjo et al. [19] with a slight modification. Two parts of the crude extract (30 g and 20 g, respectively) were chromatographed on both Mitsubishi Chemical Ion (MCI®) gel and Silica gel 60 (Si, 230-400 mesh). Column chromatography using MCI® gel was eluted with a mixture of methanol (MeOH) and distilled water (dH2O) with starting ratio of 2:8, then gradually increasing the polarity of the solvent system until the final ratio was 100:0. On the other hand, column chromatography using Si gel 60 (230-400 mesh) was eluted with chloroform (CHCl3) and MeOH with starting ratio of 100:0 until 50:50. All of the eluted fractions were collected in test tubes that have previously been weighed and numbered.

Separations of all fractions were monitored using pre-coated Si gel 60 F254 aluminium plates as thin-layer chromatography (TLC). For MCI gel column chromatography, TLC plates were eluted with CHCl3, MeOH and dH2O with increasing polarity (9:1:0.1, 7:3:0.5, 6:4:1 and 5:5:1.5). On the other hand, hexane and CHCl3 with increasing polarity were used as solvent systems for the elution through Si 60 gel column chromatography. 10% H2SO4 was used as spray reagent for the TLC plates. All of the developed TLC plates were also observed under shortwave and long-wave ultra-violet (UV) lamps. Fractions with similar Rf values were combined and concentrated under reduced pressure. Concentrated fractions were stored into 14 mL vials in the refrigerator until further use.

2.4. Preparation of Bacterial Inoculum
A stock culture of M. tuberculosis H37Ra (ATCC 25177) was cultured on Middlebrook 7H10 agar (BD Difco™) supplemented with Middlebrook Oleic acid-Albumin-Dextrose-Catalase (OADC) enrichment (BD BBL™) and glycerol, at 37°C in 8% CO2 for 10 days. A few loopful of the 10-day culture of the bacteria on the Middlebrook 7H10 agar was then sub-cultured in 10 ml Middlebrook 7H9 broth (BD Difco™) supplemented with Albumin-Dextrose-Catalase (ADC) enrichment (BD BBL™) at 37°C in 8% CO2 until log phase growth is achieved (5-10 days). The inoculum was prepared by diluting the log phase growth culture with Middlebrook 7H9 broth supplemented with OADC so that its turbidity matched that of a McFarland no. 1 standard solution (approximatey 3 x 107 CFU/ml). The suspension was further diluted 1:20 in Middlebrook 7H9 broth.

2.5. In-vitro Antimycobacterial Assay
Generally, antimycobacterial activities of T. macrophylla fractions were determined using a micro-broth dilution method, specifically Tetrazolium Microplate Assay (TEMA). To reduce evaporation from the plates, sterile distilled water (200 µl) was added to perimeter wells. Middlebrook 7H9 broth supplemented with OADC (100 µl) was added to wells in rows B to G in columns 3 to 11. Fraction working solution (100 µl) was then added to the wells in rows B to G in columns 2 to 3. By using a multichannel pipette, solution of fraction working solution and Middlebrook 7H9 broth (100 µl) was transferred from column 3 to column 4 and was mixed well. Identical serial two-fold dilutions was continued through column 10. Excess medium (100 µl) from the wells in column 10 was discarded. Pre-prepared inoculum of M. tuberculosis H37Ra (100 µl) was added to the wells in rows B to G in columns 2 to 10 (Final volume per well was set to 200 µl). The wells in column 11 served as negative controls to monitor the viability of the bacteria while isoniazid (INH) was used as the positive control. The plate was sealed with Parafilm and incubated at 37 °C in 8% CO2 for 5 days before pre-prepared solution of Tetrazolium and 10% Tween 80 (50 µl) was added to all wells. Plates were then sealed with Parafilm and incubated for a further 24 hour. A change in colour of Tetrazolium from yellow to purple was interpreted as growth while well with yellow colour indicated absence of bacterial growth. The
Minimum Inhibition Concentration (MIC) is defined as the lowest concentration which prevent a colour change from yellow to purple. All controls and fractions were tested in two sets with each set were performed in duplicates (total of 4n). Final desired fractions concentration in the microplate ranges from 800 to 3.125 µg/ml (800, 400, 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/ml).

Following the determination of MIC, 7-10 µL of broth suspension in the 96-well of microplates that show no visible growth (remained yellow) was inoculated to fresh Middlebrook 7H10 (supplemented with OADC) agar plates. The agar plates was incubated for at 37 ºC in 8% CO2 for 21 to 28 days. On day 21 to 28, the lowest concentration at which there is no bacterial growth was considered as the Minimum Bactericidal Concentration (MBC) value. Tests were performed in two sets with each set were also performed in duplicates.

3. Results and Discussions

3.1. Collection and Preparation of Plant Materials

Fresh mass of the samples collected from Taman Negara Johor Endau-Rompin was approximately 12.0 kg. On the other hand, the mass of the samples after being completely dried and grounded was 8.052 kg. The purpose of refinement of the samples via grinding process was to widen the surface area of the samples in order to aid in contact of the solvent with the samples during the extraction [20].

3.2. Extraction of Plant Materials

With 3.0 kg of T. macrophylla raw materials, 79.9 g of ethyl acetate crude extract was obtained through the maceration methodology. The result has also showed that the percentage yield of T. macrophylla extraction was 2.663%.

3.3. Fractionation and monitoring of Plant Materials using Chromatographic Techniques

Fractionation of 30.0g of ethyl acetate extract of T. macrophylla samples via MCI gel has afforded 132 fractions. Meanwhile, column chromatography of T. macrophylla ethyl acetate extract using Si gel 60 has eluted a total of 337 fractions.

Fractions eluted from both stationary phases were combined based on the same Rf values and similarity of the chemical content profile of the stains shown on TLC [12]. As shown in table 1, a total of 12 main fractions eluted from MCI gel, were combined (designated as M1-2, M4, M11-12, M16, M19-24).

Table 1. MCI® gel column chromatography solvent systems, fractions and combined fractions number.

| Solvent system | Fractions | Combined fractions |
|----------------|-----------|--------------------|
| MeOH (%) | dH2O (%) | 1-10 | M1 (3-4) |
| 20 | 80 | | M2 (5-8) |
| 30 | 70 | 11-30 | M4 (9-20) |
| 40 | 60 | 31-45 | M11 (21-56) |
| 50 | 50 | 46-63 | M12 (57-63) |
| 60 | 40 | 64-79 | M16 (78-90) |
| 70 | 30 | 80-94 | M19 (91-100) |
| 80 | 20 | 95-110 | M20 (101-106) |
| 90 | 10 | 111-123 | M21 (107-112) |
| 100 | 0 | 124-132 | M22 (113-118) |
| | | | M23 (119-127) |
| | | | M24 (128-132) |
On the other hand, fractions eluted via Si 60 gel were combined into 16 main fractions (designated as S1-S2, S3 and S5-S17). This is shown in the table 2 below.

**Table 2. Si 60 gel column chromatography solvent systems, fractions and combined fractions number.**

| Solvent system | Fractions | Combined fractions |
|----------------|-----------|--------------------|
| CHCl (% ) | MeOH (%) | 1-8 | S1 (6-7) |
| 100 | 0 | 9-37 | S2 (8-10) |
| 99 | 1 | 38-71 | S3 (11-44) |
| 98 | 2 | 72-110 | S5 (45-53) |
| 97 | 3 | 111-144 | S6 (54-74) |
| 95 | 5 | 145-177 | S7 (75-98) |
| 93 | 7 | 178-210 | S8 (99-110) |
| 90 | 10 | 211-231 | S9 (111-129) |
| 85 | 15 | 232-262 | S10 (130-152) |
| 80 | 20 | 263-299 | S11 (153-184) |
| 50 | 50 | 300-337 | S12 (185-199) |
| 20 | 80 | | S13 (200-213) |
| | | | S14 (214-248) |
| | | | S15 (249-263) |
| | | | S16 (264-276) |
| | | | S17 (277-337) |

3.4. Tetrazolium Microplate Assay (TEMA)

It is clear that the most ideal test organism in TB studies is the pathogenic *M. tuberculosis* itself. However, handling such a test organism requires extra precautionary and safety steps. Hence, non-pathogenic *M. tuberculosis* H37Ra (ATCC 25177) as used in this study, is one of the plausible alternatives to be opted for. This is because the mycobacterial strain has drug susceptibility profile and genetic composition that is close to the pathogenic *M. tuberculosis* [16]. In addition this study was made more reasonable by the inclusion of a clinically used anti-TB drug: isoniazid.

From the results in Table 3 and 4, it is revealed that *M. tuberculosis* H37Ra has been susceptible only to fractions eluted from Si 60 gel column chromatography. The anti-TB activities of the fractions eluted from ethyl acetate crude extract correlates well with a previous study [21], where the ethyl acetate extract of the roots of *Anogeissus leiocarpa* (DC.) Guill and Perr demonstrated the lowest MIC (625 µg/ml) against *Mycobacterium smegmatis*. The antimycobacterial activities may be attributable to the presence of ellagic acid derivatives and elligitannins, both are phenolic compounds in the extract [21].

Result from table 3 has showed that, out of the 16 tested Si 60 gel fractions, 7 of them inhibited the growth of *M. tuberculosis* H37Ra at MIC values range from 400 to 800 (µg/ml). In a broader point of view, results from table 3 has also revealed that the combined employment of semi-polar (CHCl3) and polar solvent (MeOH) as the mobile phases in Si 60 column chromatography has successfully eluted fractions with antimycobacterial activities. These results are in agreement with a previous report, in which the use of semi-polar (ethyl acetate) and polar solvents (MeOH) as eluents for column chromatography has led to the discovery of two plants compounds (both were 5-alkylresorcinols) with noteworthy MIC values (11.9 and 25.2 µg/ml) [22].

MCI® gel is a reversed-phase stationary gel that possess a suitable chemical stability though, with a high sample loading capacity [23]. In a reversed-phase stationary material, polar solvents is used as the eluent, hence polar compounds were eluted first. [24]. The presence of polar compounds eluted from
the solvent system with high polarity (MeOH and dH₂O) as used in this MCI⁰ gel column chromatography may cause a significant decrease or even loss of anti-TB activity [25]. This is due to the high lipophilicity properties of mycobacterial outer envelope and cell wall that requires lipophilic drug molecules for their penetration through the bacterial cells [19]. These facts may therefore explain the poor antimycobacterial activities of fractions eluted from MCI⁰ gel column chromatography as shown in Table 4.

| Table 3. Antimycobacterial activity of the fractions eluted from Si 60 gel column chromatography. |
|-----------------------------------------------|
| Fraction | MIC (µg/ml) |
| S1      | >800       |
| S2      | 800        |
| S3      | 800        |
| S5      | 400        |
| S6      | 400        |
| S7      | >800       |
| S8      | >800       |
| S9      | >800       |
| S10     | >800       |
| S11     | 800        |
| S12     | 400        |
| S13     | >800       |
| S14     | >800       |
| S15     | 800        |
| S16     | >800       |
| S17     | >800       |

| Table 4. Antimycobacterial activity of the fractions eluted from MCI gel column chromatography. |
|-----------------------------------------------|
| Fraction | MIC (µg/ml) |
| M1      | >800       |
| M2      | >800       |
| M4      | >800       |
| M11     | >800       |
| M12     | >800       |
| M16     | >800       |
| M19     | >800       |
| M20     | >800       |
| M21     | >800       |
| M22     | >800       |
| M23     | >800       |
| M24     | >800       |

4. Conclusion
For many decades, TB has remained a worldwide health problem. In the anti-TB drug discovery efforts from medicinal plants, the process of extraction, fractionation and isolation is very crucial.

Current study has compared the antimycobacterial activities of *T. macrophylla* fractions eluted from two types of stationary phases of column chromatography: Si gel 60 and MCI⁰ gel. The results from this study has revealed that only Si gel 60 fractions showed antimycobacterial activities. Therefore, it can be conclusively said that Si gel 60 is a more suitable stationary phase in column chromatography for fractionating plant samples in TB researches. Further studies to evaluate the toxicity of *T.*
macrophylla fractions and elucidate the synergistic interactions between the fractions and existing anti-TB drugs would be highly valuable.

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