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Preliminary Phytochemical and Antimycobacterial Investigation of Some Selected Medicinal Plants of Endau Rompin, Johor, Malaysia

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Abstract: Tuberculosis (TB), the primary cause of morbidity and mortality globally is a great public health challenge especially in developing countries of Africa and Asia. Existing TB treatment involves multiple therapies and requires long duration leading to poor patient compliance. The local people of Kampung Peta, Endau Rompin claimed that local preparations of some plants are used in a TB symptoms treatment. Hence, there is need to validate the claim scientifically. Thus, the present study was designed to investigate the *in vitro* anti-mycobacterial properties and to screen the phytochemicals present in the extracts qualitatively. The medicinal plants were extracted using decoction and successive maceration. The disc diffusion assay was used to evaluate the anti-mycobacterial activity, and the extracts were subjected to qualitative phytochemical screening using standard chemical tests. The findings revealed that at 100 mg/ml concentration, the methanol extract of *Nepenthes ampularia* displayed largest inhibition zone (DIZ=18.67 ± 0.58), followed by ethyl acetate extract of *N. ampularia* (17.67 ± 1.15) and ethyl acetate extract of *Musa gracilis* (17.00 ± 1.00). The phytochemical investigation of these extracts showed the existence of tannins, flavonoids, alkaloids, terpenoids, saponins, and steroids. The pronounced anti-mycobacterial properties displayed by the screened medicinal plants scientifically proved the claim by traditional people of Endau Rompin Johor. It is suggested that the extracts may be considered for further evaluation.

Keyword: Antimycobacteria; Phytochemical; Disc diffusion; Endau Rompin.

1. Introduction

Tuberculosis (TB) is a contagious ailment caused by an aerobic pathogenic bacterium called *Mycobacterium tuberculosis*. This ancient disease is among the world's most deadly epidemics and can happen to anyone, irrespective of sex, age, and nationality [1], [2]. In 2015, World Health Organization (WHO) reported that there were 10.4 million occurrences of TB around the world. About 1.4 million deaths due to TB among HIV-negative persons were documented during 2015. TB accounted for one out of ten causes of mortality globally which more when compared to deadly HIV/AIDS in 2015 [3]. More than half of the global TB cases occur in Asia region (58%), followed by Africa continent (27%). The smaller percentages happen in the East Mediterranean (8%), European region (4%), and America (3%) [4]. Like in any other developing country, TB is still a trait to public health in Malaysia. The trend of TB in the year 2010 in Malaysia showed a total of 18,517 people have been infected, which is an increase of 6% when compared to the previous year (17,341 cases in the year 2009) [5]. Furthermore, the prevalence of TB was reported as 101 cases per 100 000 population in 2011 [6]. Modern therapy including isoniazid, rifampicin, ethambutol, pyrazinamide, and streptomycin are used in the treated of TB. However, these agents have shortcomings of causing side effects, and the TB-causing bacterium can quickly gain resistance to these drugs [7]. The increase in multi-drug resistant TB and extensively drug-resistant TB strains prevalence in the world is worrisome, and for over 30 years there was no TB medicine introduced into the market [8]. Thus, it is crucial to search for a novel antimycobacterial agent. Due to their chemical variety and important role in the anti-infective agent's development, medicinal plants proffer great hope to overcome these need. For long, plant-based medicines have been used globally in the treatment of different ailments. About 75%

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of the world's populace depend mainly on plant medicines for their primary health care [1]. Jakun is a tribe from a subgroup of Proto-Malay that inhabit Kampung Peta, with the population of around 220 people consisting 67 family units, representing about 2% of the entire Orang Asli in Johor [9]. Jakun tribe living in Kampung Peta depends on their medicinal plants for primary healthcare to treat different diseases traditionally. There was claim by Jakun community in Kampung Peta that 23 species of medicinal plants are used in the treatment of TB and its symptoms [10]. Hence, there need to verify the claim scientifically. Thus, the present study was designed to investigate the *in vitro* anti-mycobacterial properties and to screen the phytochemicals present in the extracts qualitatively.

2. Materials and Methods

2.1 Study area

The Endau Romping rainforest, situated border to the north-east of Endau, Johor Darul Takzim and south to Romping, Pahan. The forest (2º25'12.94"N, 103º15'40.94"E) (Fig. 1) is one of those few virgin lowland rainforests remain in southern Peninsular Malaysia. The state government of Johor in 1993 gazetted 870 km^2 of the forest of Taman Negara Johor Endau Rompin (TNJER) as a national park. A village close to the park called Kampung Peta becomes the major entrance to the National Park. Inside the forest lie different species of plant that offer significant sources of shelters, food, medicines, etc. to the neighboring civilization [10], [11].

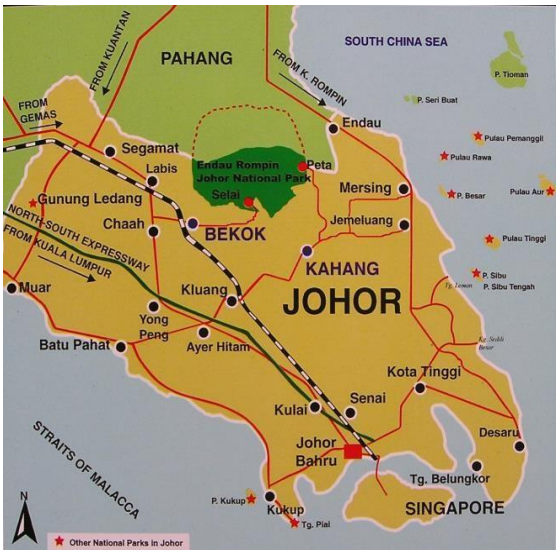

Fig. 1 The location of Kampung Peta in Johor National Park Endau-Rompin

2.2 Samples collection and preparation

The plant specimens (Table 1) were collected from Taman Negara Johor Endau Rompin, Johor, Endau Rompin and were identified using the morphometric method in which, the species are recognized based on the characteristic of their leaf, flower shape and/or branching structure. The plant materials were cleaned, chopped into smaller pieces and then dried in a hot oven at 40ºC-60ºC for 24-72 h [12]–[14]. The dried samples were then ground using a stainless steel blender into a powder which was later sieved. The sieved samples were weight and transferred to a plastic container and stored at room temperature.

Table 1 List of the medicinal plants used in the study

Scientific name	Local name	Family	Part collected	Collected from
Campnosperma auriculatum (Blume) Hook.f.	Habong	Anacardiaceae	Shoot	Bununt 49m 02º31.857'N 103º22.624'E
Musa gracilis Holttum	Pisang sum	Musaceae	Pseudostem	Sungai Samawak 42m 02º31.7491'N 103º23.807'E
Macaranga gigantea (Rchb.f &Zoll.)M.A.	Tudung	Euphorbiaceae	Stem	Pantai Burung 30m 02º31.864'N 102º24.604'E
Nepenthes ampularia Jack	Sentoyot	Nepenthaceae	Root	Lubuk Bong 43m 02º31.542'N 103º21.706'E

Scaphium macropodum (Miq.) Beum´ ee ex.Heyne	Kembang semangkok	Sterculiaceae	Stem bark	Ethnobatanical garden 62m 02º31.7491'N 103º24.845'E

2.3 Extraction

2.3.1 Decoction

The aqueous extract was prepared by immersing the plant materials in a specified volume of distilled water in 1:4 ratios. The extraction mixture was gently heated to the temperature of 60ºC in a water bath until the volume of the water was brought down to one-fourth its original volume [15]. Then, the mixture was cooled and strained (filtered) through the Whatman no. 1 filter paper and the filtrate was frozen at -80ºC in a freezer and then freeze-dried at -44ºC using a freeze drier. The weights of the dried crude extracts were determined.

2.3.2 Successive maceration

The successive maceration method was used for solvent extraction. The powdered plant materials were sequentially macerated with the specified volume of n-hexane, ethyl acetate, and methanol in order of increasing polarity of the solvents in 1:5 ratios in an enclosed flask with occasional shaking. The mixture was kept at room temperature for 24 h. This extraction procedure was repeated three times until complete extraction. The mixture was then strained through a No. 1 Whatman filter paper. The filtrate was later evaporated to a minimum volume a rotary evaporator set at 40ºC in a water bath. The weights of the dried crude extracts were determined.

2.4 Phytochemicals investigation

The plant crude extracts were screened for the presence of phytochemicals including flavonoids, tannins, alkaloids, saponins, terpenoids, and steroids using the standard procedures previously described by Bargah (2015), Abdulkadir et al. (2015), and Amabye & Tadesse (2016) [16]–[18].

2.5 Test organism and preparation of inoculums

Mycobacterium smegmatis used in the study were obtained from microbiology laboratory of Universiti Tun Hussein Onn Malaysia. The pure isolate was prepared from the stock culture of *Mycobacterium smegmatis* and then preserved on Middlebrook 7H10 agar medium. The pure culture was then stored at 4℃ until further use. From the pure cultures, the inoculum was prepared by subculturing onto Middlebrook 7H9 broth medium. The bacterial suspension density was pre-adjusted to 0.5 McFarland standards.

2.6 Determination of Anti-mycobacterial and Antibacterial Activity

Disc diffusion assay previously described by Rafael *et al.* (2011) with few modifications was used to determine the antimycobacterial activity of the extract. In brief, a filter paper of 5 mm was prepared and sterilized in an autoclave for 15 minutes at 121°C. The filter papers were impregnated with 20 µL of extract at of 12.5, 25, 50 and 100 mg/ml concentrations, and 20 µL of rifampicin (12.5, 25, 50 and 100 µg/mL). The prepared disks were aseptically and carefully transferred onto the Middlebrook 7H10 agar plates inoculated with the cell suspension. The inoculated petri dishes were sealed with parafilm and then incubated at 37°C for 72 hours, after which the diameters of inhibition zone were measured and recorded. This test was performed in triplicate [19].

3. Results and Discussions

The inhibitory effect of hexane, ethyl acetate, methanol, and water extracts of *C. auriculatum, M. gigantea, M. gracilis N. ampularia,* and *S. macropodum* using agar disk diffusion assay against *M. smegmatis* at 100, 50, 25, and 12.5 mg/mL concentration are presented in Table 2. It has been shown that at 100 mg/mL concentration, methanolic extract of *N. ampularia* displayed the largest DIZ (DIZ = 18.67 ± 0.58 mm). On the other hand, at 50 mg/mL, ethyl acetate extract of *N. ampularia* exhibited the largest DIZ (DIZ = 13.67 ± 3.06 mm). Likewise, ethyl acetate extract of *M. gracilis* was found to have largest DIZ (DIZ = 11.33 ± 0.58 mm) at the 25 mg/mL concentration. Again, the largest DIZ (DIZ = 9.00 ± 0.00 mm) at 12.5 mg/mL was displayed by ethyl acetate extract of *M.*

gracilis. Interesting, except for hexane extract of *N. ampularia*, all the hexane extracts were not active in agar disk diffusion assay even at 100 mg/mL concentration. Thus, 13 out of 20 representing 65% of crude extracts were active against the tested bacteria in *vitro* agar disk diffusion assay. The findings were supported by Fyhrquist *et al.* (2014) which stated that the diameter of inhibition zone of > 6.00 mm is an indication of antimycobacterial activity against *M. smegmatis* [20]. The present study showed that methanol and ethyl acetate extracts exhibited the largest diameter of inhibition zone. This is in line with the studies from previous researchers whose proved that ethyl acetate and methanol extracts were active against *M. smegmatis* [21]–[25]. A literature search showed that the study of the antimycobacterial activity of *C. auriculatum*, *M. gigantea*, *M. gracilis N. ampularia*, and *S. macropodum* extracts against *M. smegmatis* were not previously reported.

The phytochemical screening result found that the extracts contain vast arrays of phytochemicals (Table 3). The phytochemicals including, alkaloids, flavonoids, tannins, saponins, terpenoids, and steroids were found in most of the extracts screened. Arya (2011) stated that wide range of phytochemicals such as were responsible for antimycobacterial activity alkaloids, flavonoids, tannins, saponins, terpenoids, and steroids [26]. Solsodomine A, a pyrrole alkaloid isolated from *Solanum sodomaeum* exhibited antimycobacterial activity against *M. intracellulare* with 10 µg/mL MIC value [27]. Two flavonoids linaroside and lantanoside were isolated from *Lantana camara* exhibited antimycobacterial activity against *M.*

Table 2 Diameter of inhibitory zone (DIZ) of plant crude extracts against *M. smegmatis*

Plant species	Solvent used	100 mg/mL	50 mg/mL	25 mg/mL	12.5 mg/mL
C. auriculatum	Hexane	NA	NA	NA	NA
	Ethyl acetate	NA	NA	NA	NA
	Methanol	8.67 ± 0.58	7.33 ± 0.58	NA	NA
	Water	10.67 ± 0.58	8 ± 0.00	NA	NA
M. gigantea	Hexane	NA	NA	NA	NA
	Ethyl acetate	NA	NA	NA	NA
	Methanol	14.67 ± 0.58	12.33 ± 0.58	11.00 ± 0.00	8.67 ± 0.58
	Water	15.33 ± 0.58	NA	NA	NA
M. gracilis	Hexane	NA	NA	NA	NA
	Ethyl acetate	17.00 ± 1.00	13.00 ± 0.00	11.33 ± 0.58	9.00 ± 0.00
	Methanol	13.00 ± 0.00	10.33 ± 0.58	NA	NA
	Water	NA	NA	NA	NA
N. ampularia	Hexane	9.00 ± 0.00	NA	NA	NA
	Ethyl acetate	17.67 ± 1.15	13.67 ± 306	10.67 ± 3.06	NA
	Methanol	18.67 ± 0.58	11.67 ± 1.53	8.00 ± 1.00	7.33 ± 0.58
	Water	12.00 ± 1.00	10.33 ± 0.58	8.333 ± 0.58	7.33 ± 0.58

S. macropodum	Hexane	NA	NA	NA	NA
	Ethyl acetate	12.00 ± 1.00	9.33 ± 0.58	8.00 ± 1.00	7.33 ± 0.58
	Methanol	9.33 ± 0.58	7.67 ± 0.58	NA	NA
	Water	10.00 ± 1.00	7.33 ± 0.58	7.00 ± 0.00	NA
RIF (µg/mL)	n/a	15.67 ± 0.58	10.00 ± 0.00	9.33 ± 1.15	7.33 ± 0.58
DMSO	n/a	NA	NA	NA	NA

Notes: n/a= not applicable; NA= not active; RIF= Rifampicin; DMSO= Dimethyl sulfoxide. The values were expressed as the mean ± SD perform in triplicate

tuberculosis H37Rv by inhibiting 30, 37% of the growth, respectively at 6.25 µg/mL concentration [28]. Tannins, ellagitannin and punicalagin obtained from *Combretum molle* stem bark were active against *M. tuberculosis* typus humanus [29]. Triterpenoid, friedelin was isolated from *Terminalia avicennioides*, which is medicinal plant used by Nupes people of North Central Nigeria in TB treatment. When tested against Bacillus Calmette Guerin (BCG), the compound exhibited antimycobacterial activity with 4.9 µg/mL MIC value [30]. Elsohly *et al.* (1999) isolated new saponin, jujubogenin 3-*O*-α-L-arabinofuranosyl (1→2)-[3-*O*-(trans)-*p*-coumaroyl-*β*-D-glucopyranosyl (1→3)]-α-L-arabinopyranoside from the stems of *Colubrina retusa*. The compound exhibited antimycobacterial activity against *M. intracellulare* with the MIC value of 10 µg/mL [31]. From aerial part of *Ruprechtia triflora*, 5α,8α-epidioxyergosta-6,22-dien-3*β*-yl stearate which a novel sterol was isolated and exhibited antimycobacterial activity with the MICs of 2-128 µg/mL [32]. Those previous findings indicated that the presence of active compounds was considerably responsible for their antimycobacterial activity.

Table 3 Phytochemical class of crude extracts of selected medicinal plants by chemical analysis

Plant species	Solvents	Alkaloids	Flavonoids	Tannins	Saponins	Terpenoids	Steroids
Campnosperma auriculatum	Hexane	+	+	+	(-)	+	+
	Ethyl acetate	+	+	+	+	+	+
	Methanol	+	+	+	+	+	+
	Water	+	+	(-)	+	(-)	(-)
Musa gracilis	Hexane	+	+	(-)	+	+	+
	Ethyl acetate	+	+	+	+	+	+
	Methanol	+	+	+	+	+	+
	Water	(-)	+	(-)	+	+	(-)
Macaranga gigantea	Hexane	+	(-)	+	+	+	+
	Ethyl acetate	+	+	+	+	+	+
	Methanol	+	+	+	+	+	+
	Water	+	+	+	+	(-)	+
Nepenthes ampularia	Hexane	+	+	+	+	+	+
	Ethyl acetate	(-)	+	+	+	+	+
	Methanol	+	+	+	+	+	+
	Water	+	+	+	+	+	(-)
Scaphium macropodum	Hexane	+	+	+	(-)	+	+
	Ethyl acetate	+	+	+	+	(-)	+
	Methanol	+	+	+	+	+	+
	Water	+	+	+	+	+	+

Notes:
+ : denotes present; (-) : denotes not present

4. Conclusion

The pronounced antimycobacterial properties displayed by the screened medicinal plants scientifically proved the claim by traditional people of Endau Rompin Johor. It is suggested that the extracts may be refined and standardized to be used as an alternative or complementary medicine and further studies should be carried out to isolate the bioactive compounds which could be potential anti-TB drug leads.

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