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Anitimycobacterial activity and Potential mechanism of action of *Campnosperma auriculatum* shoot extract

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**Abstract.** Tuberculosis (TB) is a great public health challenge and a number one cause of morbidity and mortality worldwide. Around one-third of the global populations are latently infected with TB. *C. auriculatum* is medicinal plant used by the Jakun tribe of Endau Romping, Johor, Malaysia in the treatment of coughing with blood (hemoptysis). *C. auriculatum* shoot was collected from Taman Negara Johor Endau Rompin, Mersing, Johor, and extracted with hexane, ethyl acetate, methanol, and water. The antmycobacterial activity of the *C. auriculatum* shoot extracts was tested against *Mycobacterium smegmatis* mc² 155 (ATCC 700084) using broth microdilution assay technique to determine the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC). The cells of *M. smegmatis* were exposed to most active extract, and then time-kill assay and scanning electron microscopy were carried out to investigate the effect on cell growth and morphology. Gas Chromatography-Mass Spectrometry (GC-MS) was used to investigate the compound present extract. The methanol extract exhibited the best antmycobacterial activity against *M. smegmatis* with the MIC and MBC values of 1.56 and 3.13 mg/mL respectively. The methanol extract resulted in a significant reduction of colony counts. Furthermore, the appeared elongated, filamentous, swollen with lesions, and there was the appearance of small globular debris. Five compounds were identified with 1-Methyl-trans-decahydroquinol-5(equat)-ol (80.32%) been dominant. The results demonstrated that *C. auriculatum* have a great potential in TB-drug research if further investigated.

**Keywords:** Antimycobacterial; Campnosperma auriculatum; medicinal plant; Broth microdilution, Time-kill assay.

**INTRODUCTION**

Tuberculosis (TB) can be considered a global public health threat due to the fact that it is a leading killer of adults in the world today. TB incidence is aggravated by the HIV co-infection and emergence of drug-resistant strains such as multi drug resistance (MDR) and extremely drug resistance (XDR) tuberculosis [1], [2]. In 2012, 450,000 individuals developed MDR-TB globally, and 170,000 deaths were reported from it [3], [4]. Consequently, the development of new anti-TB agents active through novel modes of action is crucial. A possible approach is the assessment of natural products from plants traditionally used for the treatment of TB related ailments. These plants may contain various compounds possibly active against the Mycobacterial species or may contain active anti-bacterial compounds acting together synergistically in a multi-target fashion [5], [6].
**Campnosperma auriculatum** known as habong by Orang Asli belong to the family of Anacardiaceae. It is commonly found growing in Thailand, Malaysia, and Indonesia. The tree is an evergreen with an open, flat-topped crown; it can grow up to 39 meters tall. The bole can be up to 100cm in diameter with spreading buttresses up to 3 meters high. Oil, known as 'terentang oil', is obtained in large quantity from the sap. No uses are mentioned for the oil in Malaysia, but it is reported to cause skin irritation. The only available literature on the medicinal properties of *C. auriculatum* reported that the species is used by the native people of Endau Romping, Johor, Malaysia in the treatment of coughing with blood (hemoptysis) [7]. In addition, no information is available with regard to the pharmacological and phytochemical study of this species. Thus, this study is aimed to determine the antimycobacterial activity, investigate the potential mechanism of action of *C. auriculatum* shoot extract, and to investigate the phytochemicals present in the extract.

**METHODS**

**Plant Collection and Preparation**

The plant sample was collected from Taman Negara Johor Endau Rompin (TNJER). After collection, it was then cleaned and dried in a hot oven at 40°C for 48h. The dried sample was pulverized into powder and passed through a sieve with a mesh size of 2 mm.

**Extraction**

The successive maceration was used to extract the plant material by soaking 100 g of powder sample sequentially into 500 ml of n-hexane, ethyl acetate and methanol in an enclosed flask with occasional shaking at room temperature for 24 h. The extraction was repeated three times until complete extraction. Thereafter, the mixture was then filtered through a Whatman No. 1 filter paper and then evaporated under reduced pressure in a rotary evaporator set at 40°C in a water bath.

**Test Organism**

The *Mycobacterium smegmatis* strain used in the study was collected from microbiology laboratory of Universiti Tun Hussein Onn Malaysia. The pure isolates were prepared from the stock cultures and then maintained on Middlebrook 7H10 agar medium. The stock culture was then stored at 4°C until further use.

**Determination of Antimycobacterial activity**

Tetrazolium microplate assay (TEMA) using sterile 96-well microplate was employed to determine the minimum inhibitory concentration (MIC) of *M. smegmatis*. Briefly, the inoculum was prepared, and the density of the suspensions was adjusted to 0.5 McFarland standards, which was approximately 1.5 x 10^6 CFU/ml. This suspension was further diluted to 1:100 ratio to ensure a density of approximately 1 x 10^5 CFU/ml. 50 µl of broth medium was added to all the 96 wells. Then, another 50 µl of the obtained working samples were added to the first well of each row; then the two-fold dilution series were done across the column of the plate. Additionally, 50 µl of inoculum was added to the entire well except for the wells for sterility test, giving the final testing concentrations of 0.098 to 25 mg/ml. The same process was done for rifampicin (positive control) to obtain concentration of 0.098 to 50 µg/ml. Medium with extract and medium only were used as sterility controls. The growth control used are extracts free medium with strain suspensions. The microplates were tightly sealed and then incubated at 37°C for 24 hours. After that, 30 µl of tetrazolium-Tween 80 mixture (MTT) prepared by diluting 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl-tetrazolium bromide in 1 mg/ml of absolute ethanol and mixed with sterile 10% Tween 80 solution in equivalent amounts (v/v) was added to the growth control wells and reincubated for 5 hours. When the colour in control well turned purple which indicated bacterial growth, MTT was poured into all the wells and incubated again for 24 hours. The MIC was defined as the lowest concentration of extract that prevented a change in colour of the MTT from yellow to purple (visual determination) [8]. All the wells that showed inhibition of *M. smegmatis* were plated onto Middlebrook MH10 agar and streak. Thereafter, the agar plates were sealed with parafilm and then incubated at 37°C for 72 hours. The MBC was determined by the lowest concentration of samples that shows no viable cells on plates.
Time-kill Assay

The time-kill assay for determining the colony forming unit per ml (CFU/ml) was performed by a method described by Silva et al. (2011)[9]. Briefly, multiples of the MIC (1, 2 and 3) were used to detect differences in the killing. Cultures without any extract sample and with rifampicin were included as a standard and positive control in the assay. Sterile 250 ml shake flasks containing 10 ml of Middlebrook 7H9 broth medium with the appropriate crude extract concentrations were inoculated with a culture of *M. smegmatis* with a density of approximately 1.0 x 10⁵ CFU/ml. The inoculated flasks were incubated at 37°C in shaking incubator set at 150 rpm. The aliquots were harvested at time 0, 8, 24, 48 and 72 hours after inoculation and serial dilution were prepared in Middlebrook 7H9 broth medium to determine viable cell counts using drop plate methods previously described by Chen et al. (2003) with few modifications[10]. 10 μL from the diluted samples were dropped onto Middlebrook 7H10 agar plates with appropriate distance and left to dry. The plates were incubated at 37°C for 72 hours, and the total colony counts were determined thereafter. All tests were performed in triplicate. The results were expressed as mean log (CFU/mL).

CFU/mL = No of individual colonies x dilution factor (10ⁿ) / 0.1 mL (volume plated).

Electron Microscopy study

Field Emission Scanning Electron Microscopy of the untreated as well as the extract-treated *M. smegmatis* were carried out in order to investigate the changes in cellular morphology according to Piroeva et al. (2013) was followed [11]. Briefly, the *M. smegmatis* preparation and it treatment was performed as described in time-kill assay above. The mycobacterial cells were harvested (after 24, 48, and 72 hours) by centrifugation at 600 rpm for 5 minutes (1mL). Thereafter, the supernatant was discarded and the procedure was repeated twice by re-suspending the pellet in dH20. The resulted pellets were suspended in 20 μL dH20 and homogenized. The cells were fixed on to sterilized cover slips (18x18mm) coated by 0.8% agar. The homogenized cells were placed on the coated cover slips and left for 30 minutes at room temperature to allow the cell samples to be embedded in the agar layer. The agar was dehydrated in an oven at 37°C for 12 hours. After that, the fixed cells were dehydrated in ethanol by increasing the concentrations (10, 25, 50, 75, 96 and absolute 99.99%) for 30 minutes in each ethanol concentration. The samples were then subjected to drying at 37°C for 1 hour in an oven. The prepared samples were coated for 30 minutes with a thin gold film sputtering. The cells were then observed under JEOL JSM-7600F Field Emission Scanning Electron Microscope, Tokyo, Japan.

Gas Chromatography-Mass Spectrometry (GC-MS) analysis

Gas chromatography equipped with mass spectrometry (GC-MS-2010 Plus- Shimadzu) was used to analyze the methanol extract in order to identify the phytoconstituents present. Briefly, the column (30.0 m length, 0.25mm ID, 0.25 μm thickness) temperature was set for 4 min at the temperature of 50°C, which was then increased to 300°C at the rate of 3°C/min, and then sustained for 10 min. The temperature of the injector was set at 250°C and the volume is 0.1 L. The identification of the compounds was done by matching their mass spectra with the available library data in the National Institute Standard and Technology (NIST) database.

RESULTS

The antimycobacterial activity of extracts of *C. auriculatum* is shown in Table 1. The methanol extract of *C. auriculatum* has exhibited the lowest MIC and MBC with the values of 1.56 and 3.13 mg/mL respectively.

<table>
<thead>
<tr>
<th>Sample</th>
<th>MIC (mg/mL)</th>
<th>MBC (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane extract</td>
<td>6.25</td>
<td>12.5</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>6.25</td>
<td>6.25</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.56</td>
<td>3.13</td>
</tr>
<tr>
<td>Water</td>
<td>25.00</td>
<td>NA</td>
</tr>
<tr>
<td>Rifampicin (μg/mL)</td>
<td>3.13</td>
<td>6.25</td>
</tr>
</tbody>
</table>
The untreated control as shown in Figure 1 demonstrated a progressive increase in colony counts over the period of 72 hours.

![Figure 1](image1)

**FIGURE 1.** Time-kill analysis showing the effects of the methanol extract of *C. auriculatum* on the growth *M. smegmatis*

Although, slight reduction was observed just 8 hours after treatment with methanol extract of *C. auriculatum* at MIC and 2x MIC, but this eventually increased after 24 hours. Interestingly, 24 hours after treatment with 3x MIC, there was 99.9% reduction of the colony counts.

The effect of the methanol extract of *C. auriculatum* on the morphology of *M. smegmatis* cells was investigated using FE-SEM. As displayed in Figure 2A, the features of the untreated *M. smegmatis* cells were slender, rod-shaped with smooth and intact surface. However, after 24 hours of exposure to the methanol extract of *C. auriculatum*, the cells appeared elongated and filamentous (Figure 2B). After 48 hours of exposure to the extract, the cells appeared swollen with small lesions (Figure 2C). Figure 2D showed that after 72 hours of exposure, the cells appeared elongated, stick to one another, and there was the presence of tiny globular debris which might be as a result of the release of cytoplasmic materials from the injured cells.

![Figure 2](image2)

**FIGURE 2.** Scanning electron microscopy showing the effects of the methanol extract of *C. auriculatum* on *M. smegmatis* cells (A: 0 hours; B: 24 hours; C: 48 hours; D: 72 hours).
The result of GC-MS analysis of methanol extract of *C. auriculatum* revealed 7 peaks. Five compounds representing 95.7% of the total crude extracts were identified. These compounds were 1-Methyl-trans-decahydroquinol-5(eqat)-ol (80.32%), Phthalic acid, di(2,4,4-trimethylpentyl) ester (8.50%), Eicosen-1-ol, cis-9- (3.27%), Hexadecanoic acid <n-> (2.07%), and Hexadecanoate <methyl-> (1.54%) (Table 2).

**TABLE 2.** Phytochemical compounds identified in the methanol crude extract of *C. auriculatum* using GC-MS analysis

<table>
<thead>
<tr>
<th>ID</th>
<th>RT (min)</th>
<th>Name of identified compounds</th>
<th>Peak area (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28.885</td>
<td>1-Methyl-trans-decahydroquinol-5(eqat)-ol</td>
<td>80.32</td>
</tr>
<tr>
<td>3</td>
<td>49.919</td>
<td>Hexadecanoate &lt;methyl-&gt;</td>
<td>1.54</td>
</tr>
<tr>
<td>4</td>
<td>51.267</td>
<td>Hexadecanoic acid &lt;n-&gt;</td>
<td>2.07</td>
</tr>
<tr>
<td>5</td>
<td>60.812</td>
<td>Eicosen-1-ol, cis-9-</td>
<td>3.27</td>
</tr>
<tr>
<td>7</td>
<td>68.391</td>
<td>Phthalic acid, di(2,4,4-trimethylpentyl) ester</td>
<td>8.50</td>
</tr>
</tbody>
</table>

**DISCUSSION**

In the present study, TEMA technique was used to investigate the antimycobacterial activity of the tested crude plant extracts. This method was choose because it is rapid, easy to perform, and low-cost [3]. Moreover, Mohamad et al., 2011 reported the antimycobacterial activity of some Malaysian medicinal plants against *M. tuberculosis* using TEMA [12]. Since the methanol extract demonstrated the lowest MIC and MBC, it was then selected for further investigation of its effects on *M. smegmatis* cell growth and morphological changes.

Our study revealed that the methanol extract of *C. auriculatum* shoot significantly reduced the 99.9% of the viable cells count of *M. smegmatis* compared to the control with the p<0.05. Patience et al. 2017 reported that a compound can be regarded as an antitubercular agent when it results in the reduction of ≥90% of viable cell counts in the test medium compared with the medium untreated control [13]. This result substantiated the susceptibility of *M. smegmatis* to the methanol extract of *C. auriculatum*.

The study revealed that upon exposure to the methanol extract of *C. auriculatum*, the *M. smegmatis* cells appeared elongated and filamentous. Sieniawska et al., reported that the filamentous bacteria were considered to be overstressed, sick and dying members of the population [14]. Furthermore, there was the appearance of lesions on the treated cell and presence of tiny globular debris which might be as a result of the release of cytoplasmic materials for the perforated cells. Kang et al. which reported that 72 hours upon exposing *M. bovis* to mature bovine neutrophil β-defensins (mBNBD) 5, the cell wall perforation (lesion formation) was observed at the outer membrane of the mycobacterial cell which leads to the disruption of the cell wall [15]. Although, it is not certain if this mechanism of action is similar.

The phytochemical compounds identified in the methanol extract of *C. auriculatum* including 1-Methyl-trans-decahydroquinol-5(eqat)-ol, Hexadecanoate <methyl->, Hexadecanoic acid <n->, Eicosen-1-ol, cis-9-, and Phthalic acid, di(2,4,4-trimethylpentyl) ester suspected to be responsible for the antimycobacterial properties in this study.

**CONCLUSION**

It has been proved that the extracts of *C. auriculatum* shoot demonstrated antimycobacterial activity, and this could be via the mycobacterial cell wall damage. However, the active compounds identified in this study should be isolated and purified.

**ACKNOWLEDGEMENT**

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