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Phytochemical Profiling, Antimycobacterial Activity, Time-Kill Assessment and Mode of Action of the Extract of *Musa gracilis* Holttum

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ABSTRACT

Tuberculosis (TB) is still a global emergency, thus, there is need for new approaches to discovery more effective therapeutic agents. Natural products suggest a fascinating alternative in the search for novel antimycobacterial compounds. The indigenous people of Endau Rompin, Johor, Malaysia used the sap from *Musa gracilis* to treat cough. The present study was carried out to evaluate the antimycobacterial activity of *M. gracilis* extracts and to investigate its effects on cell growth, as well as its mode of action on *Mycobacterium smegmatis*. *M. gracilis* pseudostem was sequentially macerated in hexane, ethyl acetate, methanol, and water. Furthermore, micro broth dilution assay was used to evaluate the antimycobacterial activity of the crude extracts. Time-kill analysis and electron microscopy were carried out to assess the effect on cell growth and potential mode of action. Gas Chromatography-Mass Spectrometry (GC-MS) was used to identify the phyto-constituents. This study revealed that ethyl acetate extract had the best Minimum Inhibitory Concentration (MIC) value of 0.39 mg/mL and Minimum Bactericidal Concentration (MBC) values of 3.13 mg/mL. At just 8 hours after treatment with 3X MIC of the ethyl acetate extract, the viable cells count was reduced by about 99.9%. The images from the scanning electron microscopy revealed that the extract caused a disruption of *M. smegmatis* cells. Some phyto-compounds including phytol acetate, Vitamin E, and hexadecanoic acid assumed to be responsible for the biological activity were identified.

Keywords: Antimycobacterial, Field emission-scanning electron microscopy, Time-kill, Gas chromatography-mass spectrometry, *Musa gracilis*.

Introduction

Tuberculosis (TB) is common but dangerous infectious disease that is caused by a pathogenic bacterium called *Mycobacterium tuberculosis*. The disease is said to be endemic in almost all the countries in the world, and it is considered as the number one cause of death due to bacterial diseases.[1] Around 2 billion individuals (about one-third) of the world populace are reported to be infected with TB.[2] It has been projected that around 9 million contract TB and nearly 2 million die from it yearly.[3] In 1993, World Health Organization (WHO) made an unprecedented decision to announce TB as a global emergency in public health,[4] which was the first disease ever to be declared by WHO as a global emergency.[5] Modern drugs such as streptomycin, isoniazid, pyrazinamide, rifampicin, and ethambutol are used as a treatment to fight TB. However, the aforementioned drugs have shortcomings of causing side effects including hearing loss, gastrointestinal discomfort and hepatitis.[6] Furthermore, incomplete treatment of TB always leads to a more severe situation where the etiological agent gains resistance to the drugs.[1] Consequently, the potential efficacy of folk medicines has inspired scientists especially in Africa to look into conventional medicine for thetreatment of many illnesses including TB.

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Due to their chemical diversity and history in drug development, medicinal floras offers great hope in order to overcome these needs.[7] The phytochemical research of some medicinal plants has produced a good number of natural active products, however, very few species were expansively explored for their therapeutic activities. Thus far, few species have been tested against mycobacteria, and not many exhibited anti-TB activity. *Musa gracilis* Holttum from the family of Musaceae is a native to Peninsular Malaysia andis the only wild banana species in Johore, Malaysia.[8] This plant species grows to 7 ft (< 2 meters) tall with upright pinkish-purple bud which produces slim bananas that have green and magenta stripes (Figure 1).[9]

Figure 1: Fruits, flowers and leaves of *Musa gracilis* Holttum[9]

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Traditionally, indigenous people from Goiás, Brazil use different parts of banana to treat illnesses. The juice obtained from the pseudostem is administrated orally in the treatment of diarrhoea; Infusion of the flowers in water is used to treat eyes and pulmonary problems.[10]The leaves, fruits, peels, stalks, and root of *Musa. paradisiaca* L. have been orally admistered as a traditional treatment of intestinal colitis, dysentery and diarrhoea.[11] It has been reported that Arabs utilized *Musa*spp against heat in the lungs, the bladder, and the chest.[12] Sabran *et al.* reported that the indigenous people of Endau Rompin, Johor, Malaysia used sap from the pseudostem of *M. gracilis* in the treatment of a cough.[13] A good number of studies have previously reported the biological activity of *Musa* species including antibacterial activity,[14] anti-hyperglycemic activity,[11] antifungal activity,[15] antidiarrhoeal activity,[16] antioxidant activity,[17] anti-allergic activity[18] and antimalarial activity.[19] However, the literature review showed that no study was published on pharmacological activities of *M. gracilis*. The extremely infectious characteristic of *M. tuberculosis* limits its usage for large scale screening of possible drug candidates. Compared to *M. tuberculosis* (disease causing strain)*, M. smegmatis* is non-infectious and fast growing. It has been reported that the *M. smegmatis* exhibit a drug susceptibility feature closest to that of *M. tuberculosis*[20] and as such, screenings against it can be employed to shortlist natural product with antimycobacterial activity. The present study was carried out to evaluate the antimycobacterial activity of *Musa gracilis* extract and to investigate its effects on cell growth, as well as its mode of action on *M. smegmatis*.

Materials and Methods

Study area

The Taman Negara Johor, Endau Rompin forest is located at the border to North-East of Endau, Johor state and South to Rompin, Pahang state. The rainforest (2°25'12.94"N, 103°15'40.94"E) (Figure 2) is one of those rare remaining areas of virgin lowland rainforest in Peninsular of Malaysia. A Jakun ethnic group is a subgroup of Malay, which is a dominant aborigine (Orang Asli) tribe in Johor state. In Kampung Peta (a village situated in the forest), there are approximately 220 occupants with 67 households that solely relies on the natural resources around them.[13]

Sample collection

The pseudostems of *M. gracilis* were collected from Endau Rompin, Johor, Malaysia on 20th July, 2016. The sample was authenticated by a botanist in biodiversity laboratory, Faculty of Applied Sciences and Technology (FAST), Universiti Tun Hussein Onn Malaysia (UTHM). The plant sample after collection was cleaned by running tap water, then cut into pieces and then oven-dried at 40°C for 72 h. The dried sample was pulverised using a stainless-steel blender and sieved through a mechanical sieve shaker (mesh size = 2 mm). The sieved material was weighed using an analytical scale. It was then stored in plastic container at room temperature until further use.

Extracts preparation

One hundred grams of powdered sample was sequentially macerated with 500 mL of hexane, ethyl acetate, and methanol for 24 h each at room temperature after which it was strained through a filter paper (Whatman No. 1). The filtrate was then evaporated in a rotary evaporator at 40°C. On the other hand, the aqueous extract was prepared by dissolving the powdered sample in sterile distilled water and was steadily heated in a water bath at 60°C until the volume of the water was reduced to one-fourth of its initial volume.[21] Thereafter, the cooled mixture was strained through a filter paper (Whatman No. 1) and the filtrate obtained was then freeze-dried at −44°C using a freeze dryer. All the crude extracts were kept at −20°C till use.

Preparation of test organism

Mycobacterium smegmatis MC2 155 (ATCC 700084) used in the research was maintained on Middlebrook 7H10 Agar at 37°C. The bacterial inoculum was prepared by dissolving few colonies of *M. smegmatis* in Middlebrook 7H9 broth supplemented with 10% OADC. The inoculum density was standardized to attained 0.5 McFarland standard (1.5×10^8 CFU/mL) which was diluted further to 1:100 (approximately 1×10^6 CFU/mL).

Determination of antimycobacterial activity

Tetrazolium Microplate Assay (TEMA) by means of 96-well microtitre plates were used to evaluate the antimycobacterial activity of the test extracts. Fresh solutions were prepared by dissolving organic solvent extracts in dimethyl sulfoxide (DMSO) and aqueous extract in sterile distilled water. Briefly, 50 µL of 7H9 Middlebrook broth was dropped to the entire 96 wells. Then, an equal amount of prepared extract solutions was added and serial dilutions (2-fold) were made across the column giving the final concentration range of 0.098–25 mg/mL. The same procedure was followed for positive control (RIF) to obtain the concentration range of 0.098–50 µg/mL. Thereafter, 50 µL of diluted bacterial inoculum (1×10^6 CFU/mL) was added to the entire wells except for sterility test. The broth medium only and broth medium mixed with the extracts were used as sterility controls. Broth medium with bacterial inoculum was used as growth control, while, DMSO was employed as negative control. The prepared 96-well plates were incubated at 37°C overnight. Thereafter, the entire wells were supplied with 30 µL of tetrazolium-Tween 80 (MTT) and incubated again at 37°C overnight. The lowest concentration of crude extract at which no color change of the MTT was observed (from yellow to purple) was interpreted as the minimum inhibitory concentration (MIC).[22]To determine minimum bactericidal concentration (MBC), all the microtitre wells that showed growth inhibition were spread on Middlebrook MH10 agar and incubated at 37°C for 72 h. The MBC was interpreted as the lowest concentration of sample that shows no visible mycobacterial colony on the Middlebrook MH10 agar plate.

Time-kill studies

The time-kill assessment of the crude extract was done using a technique previously demonstrated by Silva *et al.* with slight modifications. A suspension of *M. smegmatis* (approximately 1.0×10^5 CFU/mL) was inoculated into 10 mL of Middlebrook 7H9 broth mixed with the ethyl acetate extract of *M. gracilis*/RIF at MIC, 2X MIC, and 3X MIC concentrations. The extract or RIF free culture was used as a standard. The prepared flasks were then incubated in shaking incubator (150 rpm) at 37°C. The aliquots were harvested after 0, 8, 24, 48 and 72 h inoculation and dilutions were prepared in series using Middlebrook 7H9 broth to determine viable cell counts by method (drop plate) described previously by Chen *et al.*From the diluted samples, 10 µL were dropped on the agar plate of Middlebrook 7H10 with appropriate distance and kept to dry. The prepared plates were incubated for 72 hours at 37°C, and the total colony counts were evaluated after that.[23,24] The experiments were carried out in triplicate and the mean log (CFU/mL) was calculated using the formula.[2]

$$\text{CFU/mL} = \frac{No.of\ colonies \times dilution\,factor\,(10^n)}{0.1mL\,(volume\,plated)}$$

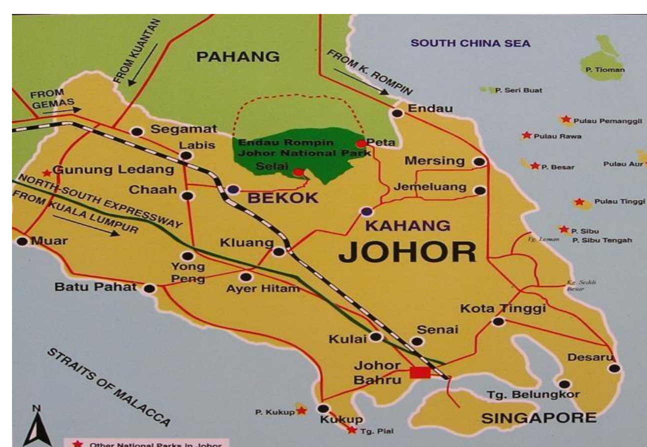

Figure 2: Location of Endau Rompin, Johor, Malaysia[13]

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Field Emission-Scanning Electron Microscopy (Fe-Sem) Evaluation

Field Emission - Scanning Electron Microscopy (FE-SEM) of the extract-treated and untreated *M. smegmatis* were performed to evaluate the mode of action of the test extract on mycobacterial cell as described by Piroeva*et al.*[25] The mycobacterial cells were prepared and treated asdemonstratedin the time-kill assessment above. The treated *M. smegmatis* cells were harvested (8, 24, 48, and 72 h after exposure) by centrifugation for 5 minutes (1 mL) at 600 rpm. Afterward, the supernatants were discarded, and this was repeated twice. The pellets were then placed on the sterilised coverslips coated with 0.8% agar and kept at room temperature for 30 minutes. The preparations were later dehydrated for 12 hours at the temperature of 37°C. The attached mycobacterial cells were further dehydrated in ethanol for 30 minutes in every concentration (10, 25, 50, 75, 96 and then 99.99%). The prepared slides were left in an oven for 1 hour at 37°C to dry. Thereafter, they were coated with gold and examined using a Field Emission Scanning Electron Microscope, Japan (JEOL JSM-7600F).

Gas Chromatography-Mass Spectrometry (GC- MS) Analysis

Gas Chromatography-Mass Spectrometry (GC-MS-2010 Plus-Shimadzu) was used to analyzed the ethyl acetate extract of *M. gracilis*in order to identify the bioactive compounds present. The system column (0.25 µm thickness, 30.0 m length, 0.25mm ID) temperature was set at 50°C for 4 min, which was subsequently increased to 300°C at 3°C/min rate. The volume of the sample injected was 0.1 L and 250°C was set as the temperature of the injector. The bioactive components of the extract were identified upon comparing the mass spectra with the available data in the database of library of National Institute Standard and Technology.

Statistical analysis

All tests in this research were done in triplicates. The statistical software, IBM SPSS Version 20.0 was used. The time-kill assay data were statistically analysed by One-way analysis of variance (ANOVA) where $p < 0.05$ was used as a significance level.

Results and Discussion

The indigenous people of Endau Rompin, Johor, Malaysia used sap from the pseudostem of *M. gracilis* in the treatment of severe cough.[13] In this study, the antimycobacterial activity of *M. gracilis* was explored using *M. smegmatis*.

The result of antimycobacterial evaluation of *M. gracilis* extracts is demonstrated as MIC and MBC in Table 1. It has shown that ethyl acetate extract of *M. gracilis* demonstrated the lowest MIC (0.39 mg/mL) followed by the hexane extract (MIC = 3.13 mg/mL), while aqueous extract exhibited no antimycobacterial activity. Similarly, the research revealed that the ethyl acetate extract exhibited the best MBC (3.13 mg/mL) compared to hexane and methanol extracts with the MBC value of 6.25 mg/mL each. This shows that the extracts possesses antimycobacterial activity against *M. smegmatis* with ethyl acetate extract exhebiting the most active while the aqueous extract less active. This could be due to the fact that the low polarity compounds from the extract of ethyl acetate could possibly cross the lipid barrier of mycobacterial cell wall.[26] The aqueous extract obtained by means of decoction in this study either displayed weak antimycobacterial activity or inactivity, which is contrary to the anticipation. However, owing to the inability of water to extract nonpolar compounds, water preparation is usually not suitable in antimicrobial discovery.[27] Another reason could be as a result of the better solubility of the active compounds in organic solvents.[28]

The time kill assessment was carried out to evaluate the killing effect of the selected crude extracts in the time and concentration dependent manner. Even though the values of MIC and MBC were used to foretell the possible antimycobacterial activity of the extract, however it has limitations in that it does not consider the exposure and action time-related to antimycobacterial effects, like the rate of killing. Unlike the MIC/MBC assay, in time-kill assessment, the rapidity of bactericidal effect of the extract can be determined.[29] In the present study, the time kill assay was carried out to determine the killing potential of the ethyl acetate extract of *M. gracilis* in a time and concentration-dependent manner. The ethyl acetate extract was selected based on the fact that it demonstrated the outstanding antimycobacterial activities. Different concentrations were made out of this extract including 1X MIC, 2X MIC, and 3X MIC (Figure 3). Our findings showed that the *M. smegmatis* growth was suppressed by ethyl acetate extract of *M. gracilis* at 1X MIC just 8 hours after the commencement of the treatment, however, regrowth occurred after 24 hours. This corroborates the previous study by Olajuyigbe & Afolayan which assessed the time-kill effect of ethanolic stembark extract of *Erythrina caffra* against some bacteria in diarrhea.[30] A possible explanation for this regrowth is that the plant extracts degraded and the cell culture was able to re-proliferate.[31]At 3X MIC,the ethyl acetate extract of *M. gracilis* significantly decreased about 99.9% of the viable cells of *M. smegmatis* compared to the growth control ($p<0.05$) just 8 hours after exposure. Patience *et al.* previously reported that a compound can only be considered as an antitubercular agent when it causes the reduction of ≥90% of viable cell counts compared with the untreated growth control.[32] Generally, this research showed that the tested extract displayed *in vitro* killing dynamics in both time and dose-dependent manner against the growth of *M. smegmatis*. Similar, Nyambuya *et al.* revealed that the time-kill kinetics of *Combretum zeyheri* extracts against *M. smegmatis* demonstrated both a concentration and time-dependent bactericidal activity.[33] They found that the increase in extract concentration with the longer treatment duration is directly proportional to inhibition of growth and efficacy of the *C. zeyheri* extract.

Table 1: Anti-mycobacterial activity of *M. gracilis* extracts

Sample	MIC (mg/mL)	MBC (mg/mL)
Hexane	3.13	6.25
Ethyl acetate	0.39	3.13
Methanol	6.25	6.25
Water	NA	NA
Rifampicin (µg/mL)	3.13	6.25

NA: Not Active

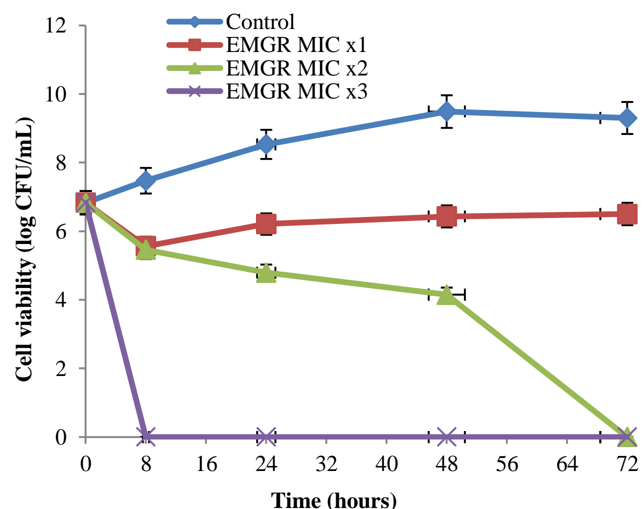

Figure 3: Time-kill assessment displaying the effects of the ethyl acetate extract of *M. gracilis* on the *M. smegmatis* growth.

The effect of *M. gracilis* ethyl acetate extract on cellular morphology of *M. smegmatis* was investigated using FE-SEM. Plate 1a, shows the appearance of healthy untreated control cells of *M. smegmatis* with slender and normal rod-like shape structure, typical of mycobacterial cells, having a smooth and intact surface, with the size of approximately 0.4 µm width x 4.0 µm length. However, 24 and 48

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hours after treatment with the ethyl acetate extract of *M. gracilis*, the mycobacterium cell appeared filamentous, elongated with coarse and wrinkled surface (Plate 1b and c). The cell appeared shrunken, large lesions produced and there is presence of ghost cell. The cell surface appeared irregularly wrinkled, coarse, and cracked. Prolong exposure of the cells to the extract for 72 hours showed harsher morphological changes (Plate 1d). The cells were shrunken, cracked, large lesions produced and there was presence of ghost cell.

The finding is consistent with the study by Dziadek *et al.* which stated that some elongated and filamentous cells were produced when *M. smegmatis* subjected to a condition that accelerate the production of FtsZ, which leads to eventual lyse of the cells.[34] Similarly, some filamentous shapes were observed in a study done by Sieniawska *et al.* when *M. tuberculosis* H_{37}Ra strain were exposed to essential oil extracted from *Mutellina purpurea* L.[35] These filamentous mycobacteria were considered to be sick, overstressed and dying.[35] Additionally, the treated cells in this study were wrinkled, cracked and ruptured, this in line with the previous study by Lai *et al.* which revealed that after exposing the *M. smegmatis* cell to LysA and LysB, some remarkable morphological changes including wrinkling, cracking and collapse of were observed.[36] The lesions and ghost cells were observed in the treated *M. smegmatis* cells,this was supported by Kang *et al.* which stated that 72 hours after treating *M. bovis*with neutrophil β-defensins 5, the cell wall perforated at the outer membrane of the cell which results in the disruption of the cell wall.[37]Likewise, Rastogi, Goh, & David reported the presence of the plasmolysed bacilli, with the appearance of symmetric cytoplasmic membranes and disappearance of ribosomes. It is then followed by the clearing of the cytoplasmic contents and ultimately, led to ghost cells formation whose cellular contents have leaked outside.[38] It has been suggested that the ethyl acetate extract of *M. gracilis* in this study kill *M. smegmatis* cell by disrupting the mycobacterial cell wall. If an extract can interrupt synthesis of cell wall, it may be possible drug candidate.[39] The mechanism for this is that, the extracts could positively bind to the negatively charged groups of the mycobacterial membrane which result in the increased permeability of the cellular membrane.

The GC-MS result of *M. gracilis* ethyl acetate extract showed 51 peaks. Thirty (30) compounds were identified representing 58.92% (Table 2). The major phyto-constituents were Formic acid, dodecyl ester (5.22%), (3-Methoxyphenyl) methanol, 2-methylpropyl ether (5.22%), 1-Tetradecene (4.84%), 3-Ethoxyphenylhydrazine (4.55%), Pentadecanol<n-> (3.65), 25-Nor-9,19-cyclolanostan-24-one, 3-acetoxy-24-phenyl- (3.53%), Heptadecanol<n-> (3.01%), Cumidine (2.78%), Ketone, methyl 2-methyl-1,3-oxothiolan-2-yl (2.67%), Hexadecanoic acid <n-> (2.41%), Phytol (2.17%), Cholest-1-eno[2,1-a]naphthalene, 3',4'-dihydro- (2.14%), n-Heptadecylcyclohexane (1.89%), 1-Eicosene (1.83%), 7-Oxo-5-cholesten-3beta-yl benzoate (1.29%), 1-Docosene (1.14%) 1-Dodecene (1.07%) and Vitamin E (1.03%).Chemical constituents in the extract were reported to possess many therapeutic values. The literature search revealed that some of the phytochemicals identified from the extract including phytol acetate,[40] pentadecanol,[41] Vitamin E,[42] hexadecanoic acid[43,44] were reported by different researchers to exhibited antimycobacterial activities against different strains of mycobacterial species. Thus, the effective antimycobacterial activity demonstrated by the ethyl acetate extract of *M. gracilis* in the present study might be attributedto the effect of these compounds.

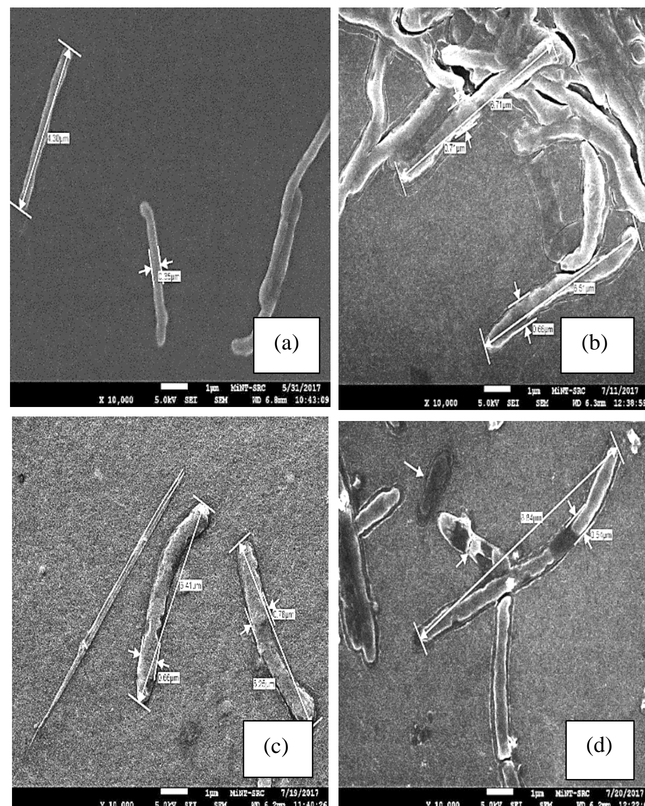

Plate 1: Images of *M. smegmatis* cells obtained by Field emission scanning electron microscope following treatment (a: 0 h; b: 24 h; c: 48 h; d: 72 h) with *M. gracilis* ethyl acetate extract.

Table 2: Phytochemical constituents identified from the ethyl acetate extract of *M. gracilis*using GC-MS

ID	RT (min)	Identified compounds	Peak area (%)	S/I
1	15.669	Ketone, methyl 2-methyl-1,3-oxothiolan-2-yl	2.67	98
2	19.363	1-Dodecene	1.07	98
3	21.576	Cumidine	2.78	99
4	22.535	1,1,2-Triacetoxyethane	0.82	98
5	28.760	1-Tetradecene	4.84	99
6	28.957	(3-Methoxyphenyl) methanol, 2-methylpropyl ether	5.22	98
7	30.106	3-Ethoxyphenylhydrazine	4.55	99
8	33.755	Vanillate<methyl->	0.56	98
9	35.399	*β*-d-Ribopyranoside, methyl, 3-acetate	0.89	97
10	37.334	Formic acid, dodecyl ester	5.22	99
13	45.061	Pentadecanol<n->	3.65	99
14	46.560	Neophytadiene	0.57	98

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16	47.965	Pentadecanoic acid	0.69	99
17	51.649	Hexadecanoic acid <n->	2.41	95
18	51.873	1-Eicosene	1.83	94
19	52.069	Heptadecanol<n->	3.01	98
20	52.212	Palmitate <ethyl->	0.80	85
23	56.004	Phytol	2.17	99
24	58.459	1-Docosene	1.14	98
25	59.120	Phytol acetate	0.87	98
26	60.808	Tricos-(9Z)-ene	0.84	97
27	63.327	n-Heptadecylcyclohexane	1.89	98
28	64.326	Docosyl trifluoroacetate	0.44	96
30	67.270	Phthalic acid, di(2,4,4-trimethylpentyl) ester	0.63	96
34	75.198	Docosa-2,6,10,14,18-pentaen-22-al, 2,6,10,15,18-pentamethyl-, all-trans	0.82	98
39	82.859	Vitamin E	1.03	97
41	85.532	Cholest-1-eno[2,1-a]naphthalene, 3',4'-dihydro-	2.14	96
44	87.423	22,23-Dibromostigmasterol acetate	0.55	91
50	90.371	25-Nor-9,19-cyclolanostan-24-one, 3-acetoxy-24-phenyl-	3.53	98
51	90.705	7-Oxo-5-cholesten-3beta-yl benzoate	1.29	95

Conclusion

The study showed that ethyl acetate extract of *M. gracilis*displayed the lowest MIC and MBC values of 0.39 and 3.13 mg/mL respectively. Likewise, the result showed that within 8 hours of treatment at 3-fold MIC, ethyl acetate extract of *M. gracilis* significantly ($p < 0.05$) reduced over 99.9% of *M. smegmatis* cells. It was further demonstrated that, the extract treated *M. smegmatis* cells were seriously damaged as observed by FE-SEM images. Some phytochemicals including phytol acetate, pentadecanol, Vitamin E and hexadecanoic acid suspected to be responsible for the antimycobacterial activity of the plant were identified.
The ethyl acetate extract of *M. gracilis* should be further investigated to isolate the novel active principles in them using bioassay-guided fractionation and the toxicity studies of the selected plants crude extracts should be carried out to determine their safety indices.

Conflict of interest

There was no declared conflict of interest by the authors.

Authors' Declaration

The authors hereby declared that, this work is original and any liability for claims regarding to the content of this article will be borne by them.

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