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Research Paper

Identification of anti-tubercular Lipophilic natural products from Malaysian *Streptomyces* species

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ABSTRACT

Extracts from actinomycete strains isolated from Sarawak, East Malaysia were screened against *Mycobacterium smegmatis* (*Msm*), a surrogate for *Mycobacterium tuberculosis* (*Mtb*). Two *Streptomyces* spp. extracts had potent whole cell activity. Long chain fatty acids identified as oleic acid and methyl myristic acid (methyl tetradecanoic acid) were isolated and identified. Oleic acid had whole cell inhibitory activity against *Mtb* and *Msm* with an IC_{50} of ~18µM. Methyl myristic acid had whole cell inhibitory activity against *Mtb* with an IC_{50} of ~35µM and *Msm* with an IC_{50} of ~20µM.

Key words: Fatty acid; *Mycobacterium tuberculosis; Streptomyces;* East Malaysia.

INTRODUCTION

WHO estimated that about 8.7 million new cases of tuberculosis (TB) were reported worldwide in 2011 with 1.4 million deaths from the disease (Watts, 2012). Since the control measures for TB such as Bacillus Calmette-Guérin (BCG) vaccination and chemoprophylaxis appear to be unsatisfactory, treatment with anti-tubercular (anti-TB) multi drugs becomes the only option available (*du* Toit et al., 2006). However, inability to detect drug resistance and failure to provide effective treatments led to *Mycobacterium tuberculosis* (*Mtb*) high mutation rate and now threatens to become a major pandemic disease again (McGarth et al., 2014). New antimycobacterial drugs are needed to combat the spread of multidrug resistant (MDR) and extensively drug resistant (XDR) TB in the twenty first century (Uplekar and Lonnroth, 2007).

Here, we used extracts from actinomycete strains isolated in Sarawak for anti-tuberculosis screening. Actinomycetes are Gram positive filamentous bacteria that

have continued to be the source of novel natural products till today (Waksman et al., 1946; Tiwari and Gupta, 2012). Extracts were screened using whole cell assays and those with significant activity were purified by bioactivity guided fractionation leading to the identification of fatty acid natural product inhibitors. Whole cell assays ensure that the compounds in the collection exhibiting poor cell permeability are removed from the priority list early in the screening process. Cytotoxic compounds can be easily identified and eliminated by performing secondary bioactivity assays with eukaryotic cells like yeast or mammalian cell lines (Cooper and Goldstein, 1976).

MATERIALS AND METHODS

***Collection of Microorganism Material*:** In general, collected soil samples were air-dried at room temperature, ground and sieved. Using a stamping method, a sterile sponge (0.5cm x 1cm) was used to transfer the soil sample onto the isolation media (Hunter-Cervera and Belt, 1992). The plates were then incubated at 28°C for up to 6 weeks. In conjunction with these methods, modified Bennett's original agar (per liter: soluble starch 5g, glucose 5g, meat extract 1 g, yeast extract 1 g, NZ-Case 2g, sodium chloride 2g, calcium carbonate 0.5g, agar 15.0g, and antifoam B emulsion 10 drops) (Iwasa et al., 1970) was used as the purification medium.

Both *Streptomyces* strains were isolated from soil samples collected in Sarawak, East Malaysia as part of the Sarawak Biodiversity Centre (SBC) bioprospecting program (Yeo et al., 2014). Strains a434 009 (extract 032022) was collected from a soil sample by a waterfall at Mount Gading National Park in 2006. This soil sample was brown in color, acidic (pH 4) and had low moisture content (14%). The processed soil sample was inoculated onto tap water agar (per liter: agar 15.0g, tap water 1L) (Lechevalier, 1964) supplemented with nalidixic acid and nystatin (each at 20 ppm) for isolation.

Strain b713 006 (extract 067037) was also isolated from a soil sample, collected beneath a rock near a Yam plant, at Semadang Village in 2008. This soil sample was brown in color, acidic (pH 5) and had high moisture content (40%). The processed soil sample was inoculated onto glycerol yeast extract agar, GYE (per liter: glycerol 5ml, yeast extract 2g, dipotassium phosphate 1.0 g, antifoam B emulsion 10 drops, and agar 15.0g) (Waksman, 1961) supplemented with nalidixic acid and nystatin (each at 20 ppm).

The *Streptomyces* strains were characterized through (i) morphology (ii) molecular (16S rDNA) and (iii) bioactivity (antimicrobial and cytotoxic screening) properties. Strains a434 009 and b713 006 were cultivated in liquid media for screening and storage purposes. The strains were kept in media containing 20% glycerol (v/v) in liquid nitrogen and -80°C for long term storage.

Strains were identified based on morphological and molecular techniques. Morphology was observed on yeast extract-malt extract agar, ISP 2 (Shirling and Gottlieb, 1966), and sporulation on soil extract agar (Hamaki et al., 2005). Sporulation patterns were observed directly using a 50x long distance objective (Olympus LMPLFLN; Olympus, Tokyo, Japan). Spore chain structures and colonial morphology were recorded and photo-documented. Molecular identification was made based on the amplification of the 16S rDNA gene using primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-

3') was amplified using following parameters: 5 min at 96°C, 30 cycles of 45 sec at 96°C, 2 min at 55°C, 4 min at 72°C and the final annealing for 7 min at 72°C. Amplified products were purified (GFX PCR DNA GE Healthcare) and sequenced using *BigDye*® Terminator v3.1 Cycle Sequencing Kits based on Sanger's dideoxy sequencing method.

Dried butanol extracts of the strains were screened for antimicrobial activity against *Aspergillus niger, Trichoderma* spp., *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus, Escherichia coli, Pseudomonas aeruginosa, Saccharomyces cerevisiae* and *Rhodotorula glutinis*.

***Fermentation and Compound Isolation*:** Cultivation in liquid began with preparation of a pre-culture by inoculating 0.1% storage culture into the seed medium (per liter: soluble starch 20.0g, D-glucose 10.0g, NZ-Case 5.0g, yeast extract 5.0g, $CaCl_2$ 0.5g and Vitamin B 0.05g). The pre-culture was shaken at 200 rpm and 28°C for 7 days. To prepare the samples for screening, 0.5ml of pre-culture is transferred into a 125 ml Erlenmeyer flask containing 20ml production medium (per liter: corn starch 20.0g, D-glucose 10.0g, NZ-Amine A 5.0g, yeast extract 5.0g, $CaCO_3$ 1.0g). Cultures were shaken at 200 rpm and 28°C for 7 days. At the end of fermentation, 20 ml of solvent (100% 1-butanol) was added into the liquid culture and shaken on the reciprocating shaker for 1 hour at 200rpm. Solvent, water and cell layers were then separated by centrifugation at 4,000rpm for 10 min at 4°C. The solvent layer was transferred and dried using a centrifugal evaporator.

The extract was dissolved in a minimum amount of methanol and injected onto a preparative reverse phase HPLC column, Atlantis T3® (Waters), 10 X 250 mm, 100° pore size, 5µm particle size. Several rounds of bioactivity guided fractionation were carried out using a gradient between water and acetonitrile. 0.1 % formic acid was uniformly added to all solvents. Fractions were collected directly into test tubes using a Waters fraction collector. An aliquot of each fraction was tested for anti-mycobacterial activity against *Mtb* and *Msm* to identify bioactive fractions. For the final round of purification of a bioactive fraction a 200µl aliquot of the methanol solution was injected onto a reverse phase HPLC column, Atlantis T3® (Waters), 4.6 X 250mm, 100° pore size, 5µm particle size. The final HPLC gradient system used for the separation of the bioactive natural product was as follows: Solvent A – water + 0.1% formic acid; Solvent B – acetonitrile + 0.1% formic acid, flow rate – 1 ml/min, gradient system; 100% A to 70% B and 30% A in 10 minutes, then 70% B and 30 % A to 100% B in 50 minutes, then 100% B continued for 10 minutes. The fractions were collected directly into the wells of a 96 well block. 50µl of each fraction were aliquoted into another 96 well plate, the solvent was evaporated with a centrifugal evaporator and the fractions reconstituted with 10µl DMSO. 1µl of this stock was used for the whole cell anti-mycobacterial assay. The remaining volume of the fractions was used for the structural characterization of the bioactive natural product.

***Bioactivity Screening*:** A total of 3,990 actinomycetes extracts were tested for growth inhibition activity using a *Mycobacterium smegmatis* (Mc^2155) assay with a fluorescent read-out for metabolic activity (Collins and Franzblau, 1997). The final concentration of extract tested was 0.01 X broth equivalents in 2% DMSO. The bioactivity tests were performed in a 384 well format. Rifampicin, at concentrations ranging from 100µM to 0.01µM was used as a minimum growth control for *Msm* and *Mtb* whole cells. Cell growth in the presence of media and absence of any inhibitor or

fractions was considered as the maximum growth control. The 384 well plates containing the cells were incubated at 37°C for 24 hours for *Msm* cells and 6 days for *Mtb* cells. After this period, 1.25µl of resazurin dye was added to the wells at concentration of 0.2mg/ml and left overnight in the incubator at 37°C. The fluorescence intensity was then measured spectrophotometrically at 544nm and 590 nm. Wells with cell growth are able to reduce the resazurin dye which then turns pink (formation of resorufin). The resazurin dye in the wells with no cell growth remained blue. 148 extracts with initial activity were re-tested against *Msm* and *Mtb*. Of the initial hits, two lipophilic constituents were prioritized for further study.

An aliquot of each fraction was used for the *Msm* and *Mtb* whole cell bioassays as described above. Whole cell bioactivity against a human cancer cell line was used to determine general cytotoxicity. Since the concentrations of the compounds in each fraction were unknown, an arbitrary value of 50X was assigned to the active compound in each original extract dissolved in 150µl solvent. Based on this arbitrary value and taking into account the dilution factors during the fractionation, evaporation of the fractions, resolubilization of the individual fractions, and preparation of stock solutions from the fractions, the fractions were tested at a final concentration of 0.5X.

RESULTS AND DISCUSSION

***Structure elucidation*:** The bioactive fractions from 032022 and 067037 were analyzed by a high resolution time-of-flight mass spectrometer (TOF HRMS). Bioactivity of 032022 appeared to be associated with a compound of mass m/z 281.25 $[M-H]^-$ and that of 067037 with a compound of mass m/z 241.21 $[M-H]^-$. Based on the high resolution masses of the two active compounds, Bruker SmartFormula® predicted the 032022 active compound to have a molecular formula of $C_{18}H_{33}O_2$ $[M-H]^-$ (error = 14.6 ppm and mSigma = 4.5), and the 067037 active compound gave a formula of $C_{15}H_{29}O_2$ $[M-H]^-$ (error = 1.8 ppm and mSigma = 11.7). A search in the Dictionary of Natural Products database revealed structural enantiomers of octadecenoic acids as possible matches for the 032022 active compound, and structural isomers of methyl tetradecanoic acid for the 067037 active compounds. No significant fragmentation pattern was observed for either compound, again suggesting the possibility of a long chain fatty acid like structure.

NMR spectroscopic data was obtained for the sample in deuterated dimethylsulfoxide (D_6 DMSO). The ^1H NMR and ^{13}C NMR spectra (500 MHz, D6-DMSO, Table 1) for 032022 showed the presence of a long aliphatic chain through a large signal in the region of δ_H 1.21 – 1.25. ^{13}C NMR gave a cluster of signals in the range δ_C 32 – 33. The methylene groups were confirmed by a DEPT 135° experiment, which showed the presence of 14 methylene groups. Correlation between the methylene group carbons and the associated protons were observed in the ^1H-^{13}C HSQC spectra. The terminal methyl group was observed at δ_H 0.85 (3H, t, J= 6.67 Hz) and at δ_C 14.43 in DEPT 135°. Evidence for one double bond in the aliphatic chain was visible at δ_H 5.34 (2H, t, J = 4.73 Hz). ^{13}C NMR spectra showed a signal for the double bond carbons at δ_C 133.66. ^1H-^{13}C HSQC correlation was observed between the double bond carbons at δ_C 133.66 and the associated protons at δ_H 5.34. Methylene groups of the aliphatic long chain flanking the double bond at both ends were seen at δ_H 1.95 (4H, q, J = 6.58 Hz). A heteronuclear ^1H-^{13}C HMBC

three-bond correlation was observed between protons on these methylene groups and the SP^2 hybridized carbon atoms of the double bond at δ_C 133.66. The carbonyl carbon atom of the acidic group was observed at δ_C 178. The acidic proton however was not visible in the NMR spectrum. The methylene group alpha to the carbonyl carbon appeared at δ_H 2.19 (2H, t, J = 7.32 Hz) and at δ_C 37.73 in the ^{13}C NMR spectra. A heteronuclear ^{1}H-^{13}C HMBC three-bond correlation was observed between the protons on α carbon atom at δ_H 2.19 and the carbonyl carbon at δ_C 178. The methylene group at β position appeared at δ_H 1.44 (2H, t, J = 7.02 Hz) and at δ_C 26.10 in the ^{13}C NMR spectra. ^{1}H-^{1}H COSY correlations were observed between the methylene groups of the aliphatic chain and the methylene group at the β position. ^{1}H-^{1}H COSY correlations were also observed between the protons on the β carbon atom and the protons on the α carbon atom. Based on the spectroscopic and mass spectrometry data, the active compound in extract 032022 is proposed to have the chemical structure of oleic acid **(Figure-1).**

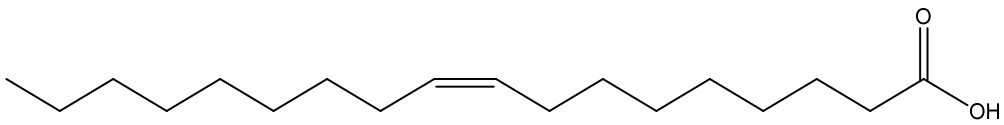

Figure-1: Oleic acid

The ^{1}H NMR spectra and ^{13}C NMR spectra (400 MHz, D6-DMSO, **Table-1**) for 067037 also showed the presence of a long aliphatic chain through a signal at δ_H 1.25.

Table-1: ^{1}H and ^{13}C NMR spectroscopic data for the active compounds from the extracts 032022 and 067037.

Position	Oleic acid (extract 032022)		Methyl myristic acid (extract 067037)	
	δ_C, type	δ_H (*J* in Hz)	δ_C, type	δ_H (*J* in Hz)
1	178, C		176, C	
1 - OH		Not visible		Not visible
2	37.7, CH_2	2.2, t (7.3)	34.1, CH_2	2.2, t (7.3)
3	28.5, CH_2	1.4, t (7.0)	25.0, CH_2	1.5, m (6.7)
4	30.6, CH_2	1.2, s	38.9, CH_2	1.2, m (6.6)
5	33.1, CH_2	1.2, s	27.8, CH	1.5, m (6.7)
– CH_3			23.0, CH_3	0.9, d (6.5)
6	33.1, CH_2	1.2, s	38.9, CH_2	1.2, m (6.6)
7	32.9, CH_2	1.2, s	29.8, CH_2	1.3, s
8	32.6, CH_2	1.9, q (6.6)	29.7, CH_2	1.3, s
9	133.7, CH	5.3, S	29.4, CH_2	1.3, s
10	133.7, CH	5.3, S	29.3, CH_2	1.3, s
11	32.5, CH_2	1.9, q (6.6)	29.2, CH_2	1.3, s
12	32.8, CH_2	1.2, s	29.0, CH_2	1.3, s
13	32.7, CH_2	1.2, s	29.0, CH_2	1.3, s
14	32.7, CH_2	1.2, s	11.7, CH_3	0.8, t (6.7)
15	32.7, CH_2	1.2, s		
16	35.2, CH_2	1.2, s		
17	26.0, CH_2	1.2, s		
18	17.9, CH_3	0.9, t (6.7)		

Signals for the methylene groups of the hydrocarbon chain were seen between δ_C 29.0 and δ_C 30.0 in the ^{13}C NMR spectra and were confirmed by the DEPT 135° experiment. The terminal methyl group at the non-polar end was observed at δ_H 0.82 (3H, t, J = 6.67 Hz) and at δ_C 11.7 in the corresponding ^{13}C NMR spectra. ^{1}H-^{13}C HSQC spectra confirmed the correlation between the carbon and protons of the terminal methyl group. Evidence for an additional methyl group on the aliphatic chain was obtained from the overlapping signal in the ^{1}H NMR spectra at δ_H 0.85 (3H, d, J = 6.54 Hz). This methyl group was observed at δ_C 22.98 in the ^{13}C NMR spectra along with corresponding correlation between the carbon atom and the protons of the methyl group observed in the ^{1}H-^{13}C HSQC spectra as well as appearing in the DEPT 135° spectra. Evidence for a methylene group adjacent to a tertiary carbon atom was seen at δ_H 1.15 (2H, m, J = 6.6 Hz). A signal for the tertiary methine group on the aliphatic long chain was observed at δ_H 1.50 (1H, m, J = 6.65 Hz). From the ^{1}H-^{13}C HMBC spectra and DEPT 135° spectra the signal for tertiary carbon of the methine was found at δ_C 27.8. Heteronuclear ^{1}H-^{13}C HMBC three-bond correlation was observed between the protons on the branched methyl group and the tertiary carbon atom. The ^{1}H-^{13}C HMBC spectra also suggested a correlation between the proton observed at δ_H 1.50 and a signal at δ_H 24.97 corresponding to a methylene group (DEPT 135°). The methylene group at the position α to the carbonyl carbon was observed at δ_H 2.19 (2H, t, J = 7.27 Hz) with a corresponding signal at δ_C 34.1 in the ^{13}C NMR spectra which could be confirmed through the ^{1}H-^{13}C HSQC correlation spectra. Heteronuclear ^{1}H-^{13}C HMBC three-bond correlation was observed between the protons on α methylene group and the carbonyl carbon atom. The solvent and water peaks were observed at δ_H 2.5 and δ_H 3.33 respectively. The acidic proton of the carboxylic acid group was missing. Based on the NMR spectroscopic and mass spectrometry data for the 067037 active, the compound is proposed as methyl tetradecanoic acid. While there is evidence for the presence of a branched methyl group, its position cannot be conclusively determined from the NMR data. The methyl group can be speculated to be present between positions 4 and 12 on the aliphatic carbon chain (**Figure-2).**

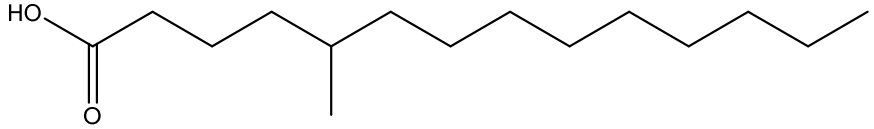

Figure- 2: Methyl myristic acid (with methyl group putatively at position 5)

***Cytotoxicity and Antimicrobial activity*:** Whole cell bioactivity assays were performed on *Msm* and *Mtb*. Through the process of bioactivity guided fractionation the crude extract was purified and the potential source of bioactivity was isolated. The crude extract of 032022 was shown to have no antimicrobial activity towards *A. niger, Trichoderma* spp., *B. subtilis, S. aureus, M. luteus, E. coli, P. aeruginosa, S. cerevisiae* and *R. glutinis*. Extract of the 067037 culture was shown to have positive antimicrobial activity against *M. luteus* and *P. aeruginosa* in earlier studies, no inhibitory activity towards *A. niger, B. subtilis, E. coli* and *S. cerevisiae*.

The 032022 active compound killed both *Msm* and *Mtb* cells at an IC_{50} of ~18 µM. The 067037 active killed *Msm* cells at an IC_{50} of ~20µM and *Mtb* cells at an IC_{50} of ~35µM. When tested against human cancer cell lines both crude extracts showed

no cytotoxicity indicating that the bioactivity was specific to mycobacteria. The LC-MS chromatogram of the isolated bioactive compound from extract 032022 was compared to the LC-MS chromatogram of an oleic acid standard under identical chromatographic conditions. The retention times of the oleic acid standard and the bioactive compound from 032022 (extracted for the exact mass of oleic acid from the total ion chromatogram) were identical and overlapped at 5.5 min (**Figures-3, 4).** The results provide further confirmation that the identified bioactive compound from extract 032022 is oleic acid.

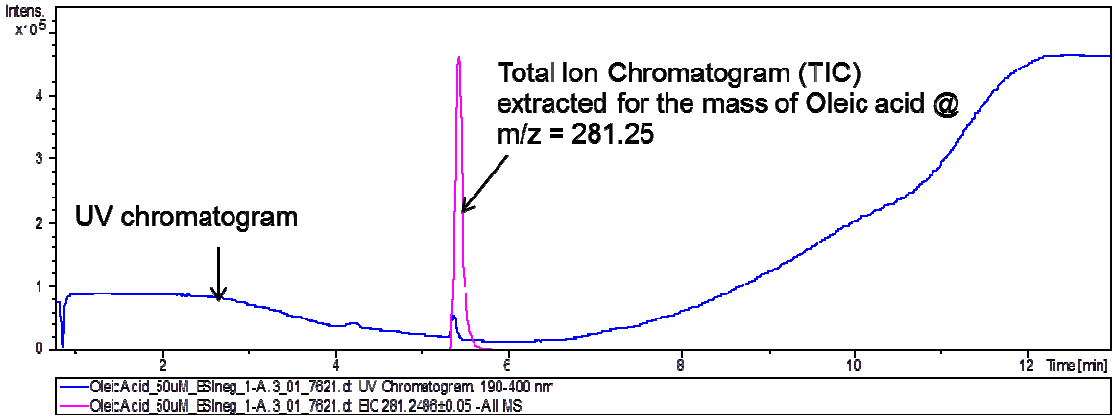

Figure-3: LC-MS profile of 50 µM oleic acid with the mass of m/z 281.25 extracted from the total ion chromatogram (TIC).

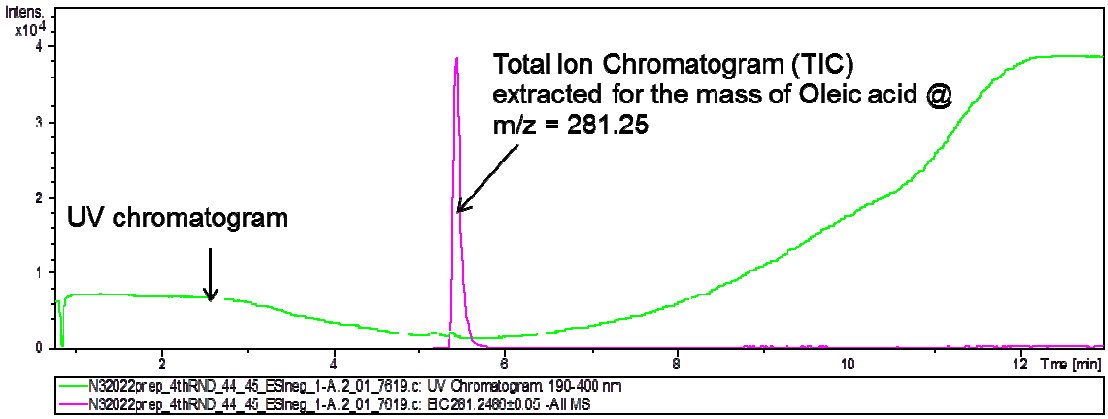

Figure-4: LC-MS profile of 50 µM bioactive compound from the 032022 extract with the mass of m/z 281.25 extracted from the total ion chromatogram (TIC).

In order to investigate the bioactivity of known naturally occurring fatty acids on *Mtb* and *Msm*, eight different fatty acids were selected and tested for whole cell bioactivity against both mycobacteria in identically performed whole-cell assays at concentrations ranging from 20µM to 0.15µM. Interestingly, the simple fatty acids showed whole cell inhibitory activity against the mycobacteria. Palmitic acid and stearic acid inhibited *Mtb* whole-cells with an IC_{50} of > 15µM (**Figure 5**) and oleic acid and linoleic acid inhibited *Msm* whole-cells with an IC_{50} of > 5µM (**Figure 6).**

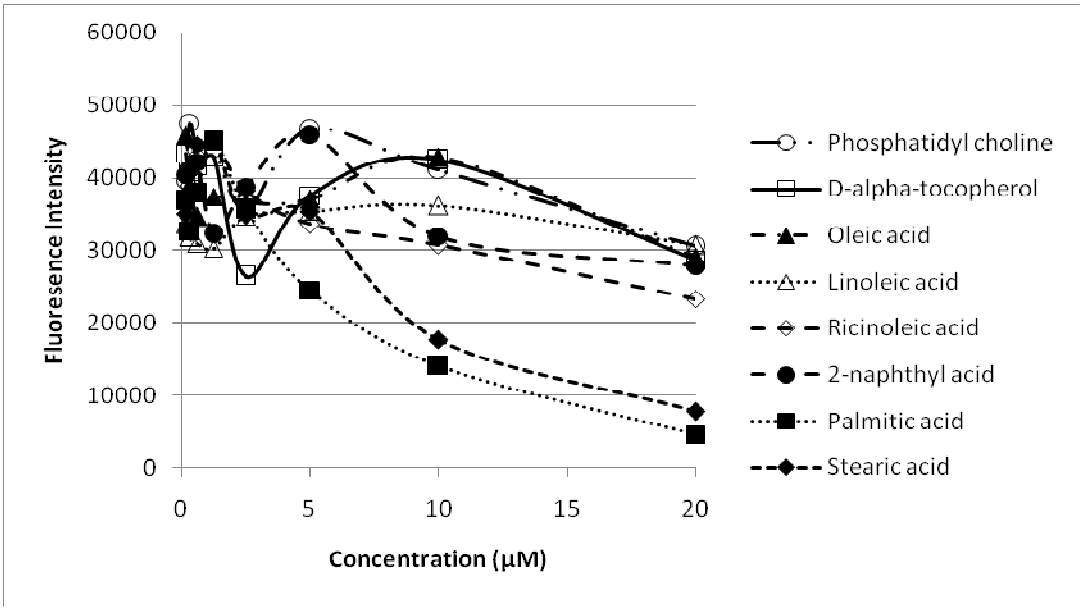

Figure-5. Whole-cell bioactivity assay of naturally occurring fatty acids against *Mtb*. Palmitic acid and stearic acid inhibited whole-cell growth of *Mtb* with an IC_{50} of > 15 µM.

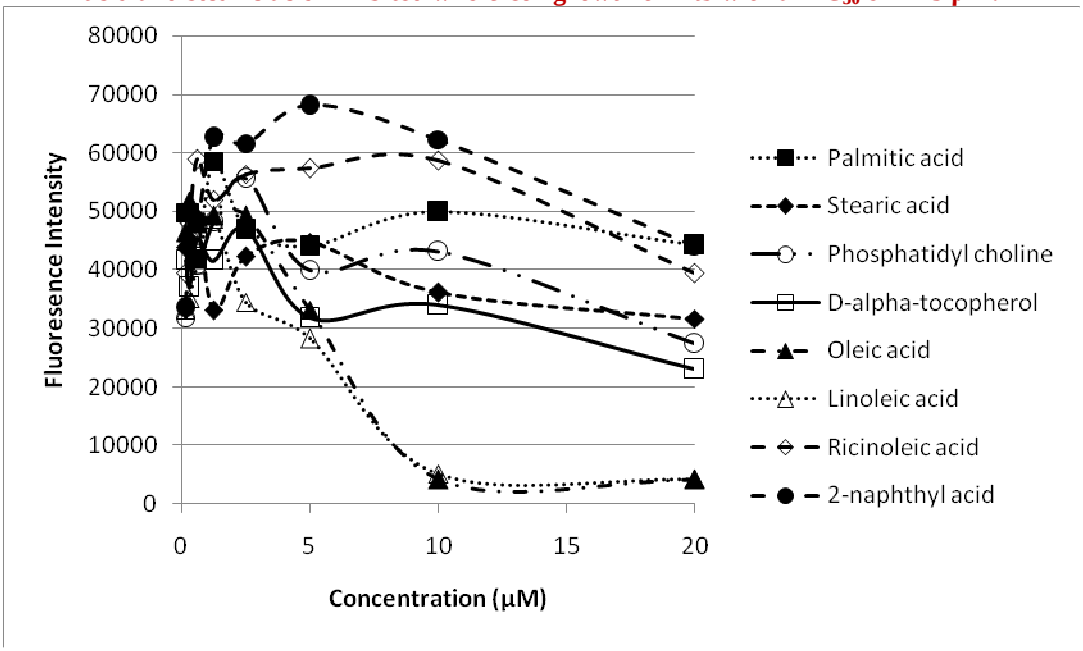

Figure-6. Whole-cell bioactivity assay of naturally occurring fatty acids against *Msm*. Oleic acid and linoleic acid inhibited whole-cell growth of *Msm* with an IC_{50} of > 5 µM.

Palmitic acid and stearic acid are long chain fatty acids with no unsaturations in the aliphatic carbon chain while oleic acid and linoleic acid are long chain fatty acids with one and two unsaturations in the aliphatic carbon chain respectively. Long chain and very long chain fatty acids have been used as inhibitors of cholesterol and fatty acid biosynthesis (Natali et al., 2007), and in cancer therapy (Rose and Connolly, 1990). Fatty acids included as growth media components have been shown to inhibit the growth of mycobacteria (Saito et al., 1984). Primary targets of these fatty acids have been the enzymes of the fatty acid synthase (FAS II) system in bacteria that are crucial in the construction of cell membranes (Morbidoni et al., 2006). Oleic acid and

palmitoleic acid are highly toxic to mycobacteria with reported MIC's in the range of 3.2 to 6.25µg/ml. A change in the permeability caused by the disruption of the cell membrane has been speculated to be the possible mode of inhibition (Saito et al., 1984). Synthetic derivatives of fatty acids like 2-alkynoic fatty acids and 2-methoxy fatty acids as well as polyunsaturated fatty acids have been investigated for the inhibition of mycobacteria and have shown promise (Carballeira et al., 2004). The oleic acid and methyl myristic acid identified in the Malaysian soil bacterial extracts may be speculated to inhibit *Mtb* and *Msm* through a similar mechanism of action.

***Microorganism*:** The producing actinomycete strains, a434 009 (032022) and b713 006 (067037) were both taxonomically identified as *Streptomyces* spp., however both differ in morphological and bioactivity characteristics. Colony color of strain a434 009 was reddish brown with diffusible brown pigment, and its spore chain is long and straight. Colony color of strain b713 006 was white, without diffusible pigment, having long spiral spore chains. Molecular identifications of the 16S rDNA gene regions (~1300 bp) were compared with a public database, GenBank (Benson et al., 2012) (**Figure-7**). Strain a434 009 showed sequence similarity with *Streptomyces aureus* strain NBRC 100912 and b713 006 with *S. prasinosporus* strain NRRL B-12431.

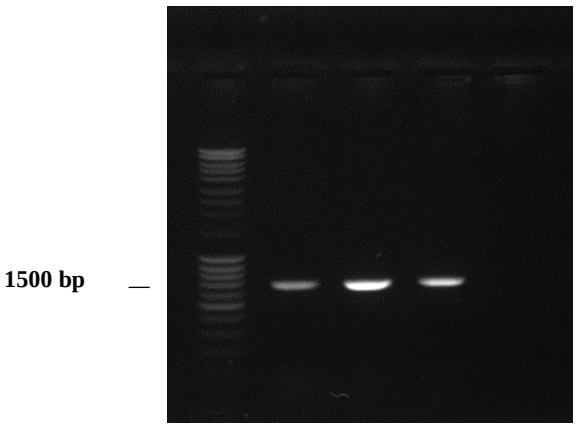

Figure-7: Results of PCR assay for DNA extracts using primers 27F and 1492R.

- Lane M: FermentasMassRuler DNA Ladder mix #SM0403, Lane 1: strain a434 009 (032022), Lane 2: b713 006 (067037), Lane 3: Positive control: *Streptomyces griseus* NBRC12875, Lane 4: negative control.

CONCLUSION

The process of screening natural product extracts for antibacterial activity is one that began with the discovery of Streptomycin back in 1944. Streptomycin was isolated from an actinomycete, which to this day continues to be the most prolific producer of antibacterial agents. The findings suggest that new avenues are available for using synthetic or natural products containing a fatty acid like core structure to find promising inhibitors of *Mtb*. The identification of antimycobacterial fatty acids from different strains of *Streptomyces* helps to reaffirm faith that 70 years after the discovery of the first naturally occurring antibiotic, natural products research still holds potential for discovering novel antibiotics against the dreaded disease of TB.

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