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Isolation of actinomycetes with antibacterial activity against multi-drug resistant bacteria

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ABSTRACT

Aims: To isolate and characterise actinomycetes from various sources of soil samples (fruit orchard, dipterocarp forest and oil palm plantation) and to screen these isolates for antibacterial activity against multi-drug resistant bacteria.
Methodology and results: A total of 158 fast-growing actinomycete isolates with different colony morphology were subjected to primary cross-streak and secondary well diffusion screening. Six isolates (OP1E, OP7A, OP2A-C, MG1A, UT9C1 and UT7E) were found to inhibit at least one of the seven multi-drug resistant (MDR) bacteria. MG1A exhibited the strongest and broadest spectrum of antibacterial activity against 6 MDR bacteria tested. These isolates were identified as *Streptomyces* species based on 16S rRNA gene sequence analysis. Further morphological and biochemical analysis revealed that MG1A was highly similar to *S. griseocarneus* (98.36%) whereas OP1E and OP2A-C were similar to *S. parvulus* (99.93% and 99.51% respectively). Preliminary identification using LCMS/MS and database search revealed that the major compound in the extract of OP2A-C could be dactinomycin (1255.4170 g/mol). Other antibacterial compounds in the extracts remain to be identified.
Conclusion, significance and impact of study: Soil actinomycetes with antibacterial activity against MDR bacteria were isolated not only from undisturbed natural soils but cultivated soils. These isolates were characterised, identified and the antibacterial compounds were extracted for further study. The isolates could serve as a potential source for the development of new and sustainable compounds against MDR bacteria.

Keywords: actinomycetes, antibacterial, soils, *Streptomyces*, multi-drug resistant

INTRODUCTION

The emergence of multi-drug resistant (MDR) bacteria due to improper use of antibiotics is becoming a significant global health threat as they spread (Lukasova and Sustackova, 2003). In addition, aquaculture and wastewater treatment plant discharges have been implicated in the distribution and acquisition of the antibiotic resistance genes among bacterial communities in the environment (Marti *et al.*, 2013; Di Cesare *et al.*, 2013). Many species of Gram-positive and Gram-negative bacteria, once considered as innocuous commensals, have now become more widespread and emerged as potential pathogens that are resistant to drugs, generally used to combat nosocomial and community acquired infections. MDR bacteria such as *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella* sp., methicillin resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci (VRE) are among the few main causative agents of local and systemic bacterial infections (Boucher *et al.*, 2009). New strains of MDR bacteria have emerged, example being the *Escherichia coli* O104:H4 bacteria and New Delhi metallo-beta-lactamase-1 (NDM-1) "superbug" bacteria resistant to most available antibiotics (Kumarasamy *et al.*, 2010). The high prevalence of antibiotic resistance in hospital-acquired infections has led to higher healthcare costs due to prolonged hospital stay and prescription of more

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expensive drugs (Leeb, 2004). The increasing prevalence of MDR pathogens coupled with the limitation of strategies such as combinatorial chemistry for development of new therapeutic agents has led to the increasing demand for novel and effective antibiotics from microorganisms such as bacteria or fungi (Projan, 2003).

Actinomycetes, one of the major producers of antibiotics and other bioactive metabolites, have been isolated and screened extensively in drug discovery programs over several decades (Busti *et al*., 2006). These filamentous bacteria have yielded an impressive number of bioactive compounds over the past 50 years and accounted for two-third of naturally occurring antibiotics, contributing significantly to human and plant health (Busti *et al*., 2006; Okami and Hotta, 1988). Many genera of actinomycetes notably *Streptomyces*, *Actinoplanes* and *Nocardia* isolated from terrestrial soils have frequently been found to produce antibiotics with strong growth inhibition on Gram-positive and Gram-negative bacteria as well as fungi (Dhanasekaran *et al*., 2009; Saisivam and Kishan, 2006). Malaysian soil ecosystems are rich sources of actinomycetes capable of producing novel antimicrobials (Goodfellow and Haynes, 1984). In Malaysia, actinomycetes have been isolated from different types of soils such as farms, ornamental garden, herbal garden and forests (Jeffrey *et al*., 2007; Jeffrey, 2008; Jeffrey *et al.*, 2011). In this study, we described the isolation of actinomycetes from soils of oil palm plantation, fruit orchard and dipterocarp forest in Malaysia, for screening and preliminary identification of antimicrobial compounds effective against MDR bacteria of clinical significance. We were interested to know as well if we would get more MDR inhibiting actinomycetes from natural soils compared to cultivated soils.

MATERIALS AND METHOD

Sample collection and actinomycete isolation

A total of 25 different soil samples were collected from a fruit orchard, a dipterocarp forest and an oil palm plantation in Selangor, Malaysia. Soil samples from dipterocarp forest and oil palm plantation were collected randomly whereas the orchard soil samples were collected from 12 different plants rhizosphere (rose apple, lemon, mango, sapodilla, guava, papaya, aubergine, soursop, star-fruit, cassava, ladyfinger and banana). All samples were from 15 cm soil depth and air-dried for 10 days at room temperature, after which 10 g of dried soil from each sample was further air-dried at 30 °C for 7 days. Dried soil samples were serially diluted in sterile saline (up to 10^{-3}) and spread over starch casein agar medium and incubated at 30 °C for 7-21 days (Küster and Williams, 1964). Morphologically distinct actinomycete colonies were picked and sub-cultured for three times on starch casein agar plates.

Screening of actinomycete isolates for production of antibacterial compounds

Actinomycete isolates were screened for antibacterial activity through primary and secondary screening. Primary screening was performed by cross streak method (Madigan *et al.*, 2006) where isolates were streaked at a single straight line across the centre of the nutrient agar plate and incubated at 30 °C for 6 days after which a panel of 8 bacteria (*Bacillus subtilis* ATCC®6051™*, Staphylococcus aureus* ATCC®25923™*, Micrococcus luteus* ATCC®10240™, *Salmonella enterica* ATCC®10708™, *Serratia marcescens* ATCC®8100™, *Pseudomonas aeruginosa* ATCC®9027™*, Proteus mirabilis* ATCC®12453™ and *Escherichia coli* ATCC®11303™) were respectively inoculated as a single streak perpendicular to the actinomycete isolate streak. After incubation at 37 °C for 24 h, inhibition zone (clear zone) formed was measured. Secondary screening on agar well diffusion assay was performed on Mueller Hinton agar plate using the same test pathogens, which were standardised to 0.5 McFarland. Actinomycete isolates with strong antagonistic activities from the primary screening were inoculated into 20 mL of antibiotic production medium (APM: 1% glucose, 2% starch, 0.3% Bacto peptone, 0.3% BBL beef extract, 0.5% Bacto yeast extract, and 0.3% $CaCO_3$, pH 7.0) and cultured at 30 °C for 7 days with shaking at 150 rev/min. The cultures were then centrifuged at 10,000 g for 10 min and 75 µL of the clear spent culture media were dispensed into Mueller Hinton agar wells respectively. Positive and negative controls were 75 µL of ampicillin (1 mg/mL) and sterile APM respectively. After incubation at 35 °C for 16-18 h, the diameter of inhibition zone formed was measured and evaluated based on the Clinical and Laboratory Standards Institute guideline (CLSI, M100-S22, 2012) (Wayne, 2012) for antimicrobial susceptibility. Screening of all isolates was performed in duplicates. Potential actinomycete isolates were then cultured on Difco oatmeal agar (BD, France). Agar plugs of densely sporulating isolates were then preserved in 1 mL glycerol (100%) and stored at -80 °C (Shirling and Gottlieb, 1966).

Extract and antibacterial bioassay

Potential actinomycete isolates were respectively cultured in 100 mL APM rotated at 200 rev/min at 30 °C for 7 days. Subsequently, the cultures were harvested and mixed with ethyl acetate (EA) at 1:1 (v/v) ratio followed by vigorous shaking for 2 h and dried at 55 °C using a rotary evaporator (Buchi, Switzerland). Each of crude extracts was dissolved to a final concentration of 50 mg/mL in 50% methanol and stored at 4 °C.

Crude EA extracts from the isolates were tested for antagonistic activities against a panel of 4 non-MDR microbes (*B. subtilis* ATCC®6051™*, S. aureus* ATCC®25923™, *P. mirabilis* ATCC®12453™ and *E. coli* ATCC®11303™) and 7 MDR bacteria (*Bacillus cereus* ATCC® 9592™, *E. coli* BAA-457 and MDR clinical isolates of *E. coli*, *Klebsiella pneumoniae, Enterobacter* sp.,

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Acinetobacter sp-1 and *Acinetobacter* sp-2 from Doctors Diagnostic Laboratory, India). A total of 75 µL of each crude extract (25 mg/mL) from the respective isolates was tested via the agar well diffusion assay. Five antibiotic discs (Oxoid) of penicillin G (10 units), ampicillin (10 µg), chloramphenicol (30 µg), gentamicin (10 µg), tetracycline (30 µg) were used as either positive or negative controls against the non-MRD bacteria. Antibiotic susceptibility was interpreted as mentioned previously, based on CLSI document (M100-S22).

Morphological and cultural characteristic

Morphological characterisation was performed according to the International *Streptomyces* Project (ISP) (Bartholomew and Mittwer, 1952), whereby potential actinomycete isolates inoculated on the International Streptomyces Project (ISP) media (yeast extract-malt extract agar, ISP-2 and oatmeal agar, ISP-3) was observed on day 2, 4 and 7 of incubation. Gram-staining was performed on smear of 3 days old actinomycete culture (Bartholomew and Mittwer, 1952). Spore chain morphology under undisturbed conditions was performed based on the coverslip culture technique (Bergey and Holt, 1993). The morphology of spores bearing hyphae and spores arrangement was observed under an inverted microscope (Nikon, Japan) at 400× magnification.

Biochemical profiles, nutrient utilisation and molecular identification

Potential actinomycete isolates were grown on ISP-2 agar at 30 °C for 4 h under aerobic condition and assayed for 19 enzymes using the API ZYM kit (BioMérieux, France). These isolates were also inoculated in API 50 CHB medium and assayed for ability to ferment 49 carbohydrates using the API 50 CH kit (BioMérieux, France) according to the manufacturer's instructions.

Identification of the potential actinomycete isolates was further performed by molecular taxonomy via 16S rRNA sequence analysis. Potential isolates were respectively cultured in tryptone-yeast extract broth (ISP-1) for 2 days at 30 °C, with shaking at 150 rev/min. Genomic DNA was extracted using the Macherey-Nagel Nucleospin® Tissue kit and used to amplify he 16S rRNA gene in a 25 µL PCR reaction (0.4 µg of extracted genomic DNA, 0.2 mM dNTPs, 1 U *Taq* DNA polymerase, 1x DNA polymerase buffer and 10 ρmole of universal bacterial 16S rRNA gene forward (F27) and reverse (R1492) primers) (Weisburg *et al*., 1991) with initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 53 °C for 1 min and 72 °C for 2 min, followed by a final extension at 72 °C for 5 min. Expected PCR product (approximately 1.4 kb) was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA) and send to First Base Sdn Bhd (Malaysia) for DNA sequencing in both direction, using the PCR primers as sequencing primers. The 16S rRNA gene sequence obtained for potential actinomycete isolates was searched against related sequences in the NCBI (National Centre for Biotechnology Information) database using Basic Local Alignment Search Tool (BLAST) and aligned using Clustal Omega 2.1. Phylogenetic tree of these isolates was build using the neighbour-joining analysis (Saitou and Nei, 1987) based on their 16S rRNA gene complete sequence and evolutionary distances were computed using the Kimura 2- parameter method, with 1000 bootstrap replications. Only bootstrap values above or equal to 50% are displayed at branch points. Evolutionary analysis was performed using the MEGA6 (Kimura, 1980; Tamura *et al*., 2013).

LC-MS/MS analysis of antibacterial extracts

EA extracts exhibiting antibacterial activity from all potential isolates were analysed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to determine the amount of compounds present in each extract and the m/z value (mass-to-charge ratio) of each significant compound. The data were obtained in negative mode using the Phenomenex Aqua C18 (50 mm × 2.0 mm × 5 µm) column on an AB Sciex 3200 Q-Trap LCMS/MS of the Perkin Elmer FX-15 UHPLC system. The solvents used were (A) water with 0.1% formic acid and 5 mmol/L ammonium formate; and (B) acetonitrile with 0.1% formic acid and 5 mmol/L ammonium formate. The mobile phase composition was 10% B to 90% B from 0.01 to 8.0 min, hold for 3 min and back to 10% B in 0.1 min and re-equilibrated for 5 min. The samples were filtered with a 0.22 µm nylon membrane and the injection volume is 20 µL of sample (50 mg/mL).

RESULTS

Isolation and screening of actinomycetes

A total of 158 spore-producing, fast growing and morphologically distinct actinomycetes were isolated from soil samples collected from the fruit orchard (50 isolates), dipterocarp forest (42 isolates) and oil palm plantation (66 isolates). Out of these, 37 isolates were found to inhibit the growth of two or more test bacteria in primary screening (9, 13 and 15 from fruit orchard, dipterocarp forest and oil palm plantation respectively). Most of these isolates were effective against Gram-positive compared to Gram-negative bacteria. A secondary screening of these 37 isolates using clear spent culture media of the liquid cultures afforded six potential actinomycetes capable of inhibiting at least 5 of the 8 tested bacteria (inhibition zones of 10 to 41 mm) as depicted in Table 1. All of the six isolates were not inhibiting Gram-negative bacteria effectively. None inhibited the growth of *P. aeruginosa*.

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Table 1: Antibacterial profile of spent culture media of actinomycete isolates via agar well diffusion assay.

Treatment	Diameter of inhibition zone (mm)							
	B. subtilis ATTC 6051	*S. aureus* ATTC 25923	*M. luteus* ATTC 10240	*S. enterica* ATTC 10708	*S. marsencens* ATTC 8100	*P. aeruginosa* ATTC 9027	*P. mirabilis* ATTC 12453	*E. coli* ATTC 11303
MG1A Ψ	10.0	18.0	0.0	9.0	0.0	0.0	13.0	9.0
UT9C1 ‡	14.0	24.0	12.0	16.0	12.0	0.0	15.0	14.0
UT7E ‡	20.0	29.0	36.0	19.0	17.0	0.0	20.0	20.0
OP1E §	29.0	30.0	35.0	0.0	8.0	0.0	20.0	9.0
OP7A §	26.0	26.0	30.0	0.0	8.0	0.0	14.0	0.0
OP2A-C §	29.0	30.0	41.0	0.0	0.0	0.0	18.0	9.0
Ampicillin	40.0	54.0	53.0	48.0	43.0	30.0	40.0	39.0

Ψ, fruit orchard isolate; ‡, dipterocarp forest isolates; §, oil palm isolates. Positive control used was ampicillin. Values are presented as mean.

Antibacterial activity of EA extracts against non-MDR bacteria

Ethyl acetate (EA) extracts from six potential isolates (designated as OP1E, OP7A, OP2A-C, MG1A, UT9C1 and UT7E) demonstrated antagonistic activities (Table 2) against four of the tested bacteria. Most EA extracts compared with clear spent culture media of MG1A, UT9C1, OP7A and OP2A-C (Table 1), showed equal or increased in diameter of inhibition zones against the four non-MDR bacteria, except for EA extracts of UT7E and OP1E which showed decrease in inhibition zones produced. Negative control of 50% LC-grade methanol (solvent for the extracts) showed absence of inhibition zones, indicating inhibitory effect was due solely to the presence of antibacterial compounds in the EA extracts.

Table 2: Antibacterial activities of actinomycete EA extracts on non-MDR bacteria.

Treatment	Diameter of inhibition zone (mm)			
	B. subtilis ATCC6051	*S. aureus* ATCC25923	*P. mirabilis* ATCC12453	*E. coli* ATCC 11303
MG1A	11.0	23.0	21.0	9.0
UT9C1	16.0	24.0	13.0	14.0
UT7E	18.0	25.0	16.0	15.0
OP1E	28.0	27.0	17.0	0.0
OP7A	27.0	30.0	14.0	0.0
OP2A-C	30.0	31.0	19.0	8.0
Ampicillin*	36.0	54.0	35.0	30.0
50% methanol*	0.0	0.0	0.0	0.0

* Controls. Positive: ampicillin (20 µg/well); Negative: 50% methanol (75 µL).

Antibacterial activity of EA extracts against MDR bacteria.

Prior to screening using the extracts, seven MDR bacteria were tested against five antibiotics, selected based on their antibiogram profile (Table 3). Both *Acinetobacter* MDR bacteria were resistant to all five antibiotics tested, while the rest were sensitive to one to three of the five antibiotics tested. All MDR bacteria were resistant to penicillin G. Extract of MG1A exhibited the strongest and broadest spectrum of antibacterial activity against all MDR bacteria except one of the *Acinetobacter* (Table 3). EA extract of UT9C1 is the second most effective (after MG1A) inhibiting MDR bacteria except *Acinetobacter* sp-2. None of the extracts (like the antibiotics tested) were able to inhibit MDR *Acinetobacter* sp-2. Extracts from actinomycetes isolated from oil palm plantation (OP1E,OP7A and OP2A-C) produced substantial inhibition zones (ranged from 29 to 31 mm) against *B. cereus* ATCC® 9592™ with extract of OP2A-C being the only one inhibiting *B. cereus* ATCC® 9592™, *E. coli* BAA-457 and *E. coli* clinical isolates. All three extracts of oil palm actinomycetes inhibited the growth of *B. cereus* ATCC® 9592™ equally well like chloramphenicol, gentamicin and tetracycline.

Actinomycete molecular identification

All six potential isolates belong to the genus *Streptomyces* based on 16S rRNA sequence analysis (Figure 1). The 16S rRNA gene of MG1A (1403 bp) showed 98.36% identity to *S. griseocarneus* DSM40004 while isolate UT9C1 (1392 bp) and UT7E (1390 bp) respectively showed 99.14% and 99.64% identity to *S. polychromogenes* NBRC13072. Isolate OP1E (1399 bp) and OP2A-C (1402 bp) showed 99.93% and 99.51% identity to *S. parvulus* NBRC13193 respectively, while OP7A (1401 bp) showed 99.93% identity to *S. gramineus* JR-43. The multiple sequence alignment of 16S rRNA gene sequences using Clustal Omega 2.1 showed the highest similarity between OP1E and OP2A-C (99.28%), followed by UT9C1 and UT7E (98.18%). MG1A isolate was the least identical with the other 5 isolates with 93.97% similarity to OP1E and 97.19% to UT7E respectively.

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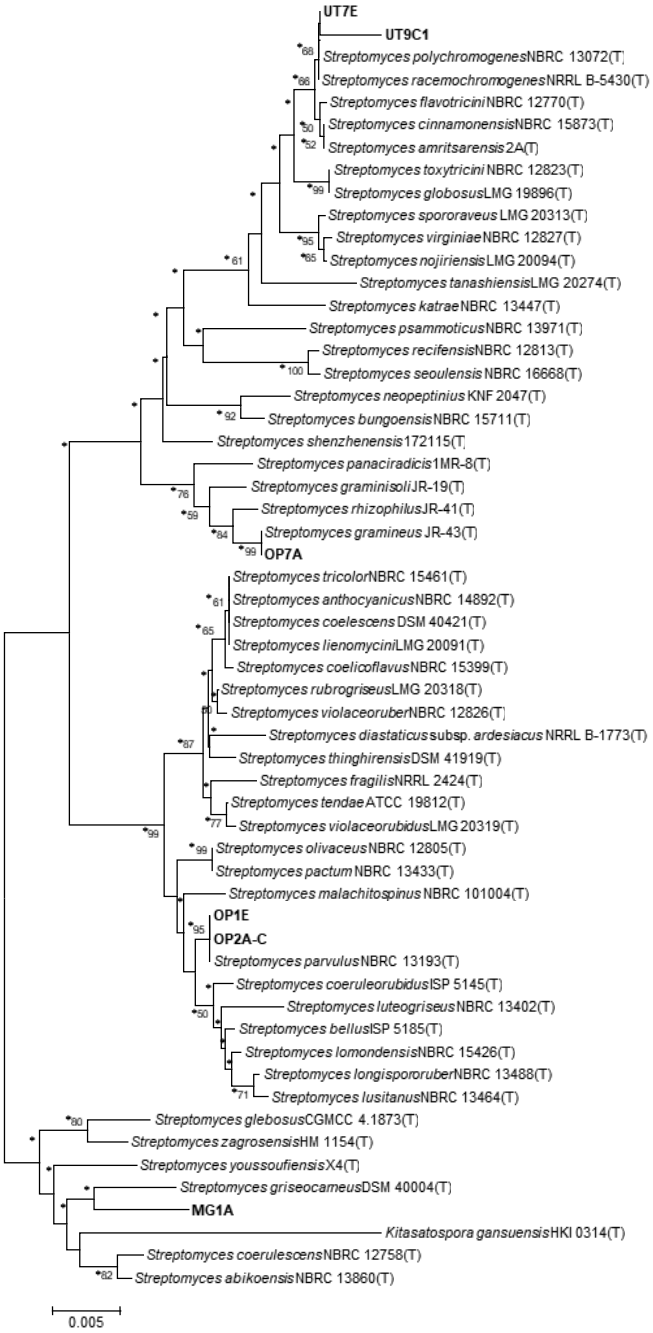

Figure 1: Phylogenetic dendogram of the six potential actinomycete isolates. Neighbour-joining analysis was performed based on almost complete sequence of 16S rRNA gene (OP1E, 1399 bp; OP2A-C, 1402 bp; OP7A, 1402 bp; MG1A, 1402 bp; UT7E, 1389 bp; UT9C1, 1381 bp). *Kitasatospora gansuensis* HKI 0314(T) was used as out-group. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated. Asterisks denote branches that were also recovered with both unweighted pair group method with arithmetic mean (UPGMA) and maximum-parsimony approaches. Number at nodes are bootstrap values based on 1000 re-samplings, only bootstrap values above or equal to 50% are displayed at branch points. Bar, 1% sequence divergence. Evolutionary analyses were conducted in MEGA6.

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Morphological characteristic

All except MG1A isolate produced diffusible colour pigments that would change the colour of ISP-3 agar (Figure 2). UT9C1 and UT7E, displayed similar morphology on ISP-2 but differently on ISP-3 agar; UT9C1 excreted dark brown pigment on ISP-3 but not UT7E. Instead, bright yellow pigmentation was observed. OP1E, OP7A and OP2A-C showed similar morphology on both ISP-2 and ISP-3 agar, secreting yellow to orange colour pigments. All of these six isolates possess typical morphology of *Streptomyces*, consisting of branching mycelia with straight, flexuous or spiral spore bearing hyphae (Figure 3). No sporangium or sclerotium was observed. UT9C1, UT7E and MG1A isolates formed straight or flexuous spore chains whereas isolates OP1E and OP2A-C produced spiral spore chains. MG1A possessed distinct straight spore chain with minimal flexuous.

Biochemical profiles

Biochemical analysis using the API ZYM test (Table 4) demonstrated the presence of esterase and leucine arylamidase activity and the absence of β-glucuronidase and α-fucosidase activity in all 6 isolates tested. All isolates were able to utilise D-glucose, esculin and glycerol (Table 4) as carbon source. The morphological and biochemical characteristics of MG1A, OP1E and OP2A-C isolates were further compared to the published characteristics of their closest relatives (Table 5). The colours of both aerial and substrate mycelia of MG1A were identical to *S. griseocarneus,* both did not produce diffusible pigments but utilised very similar carbon sources. Their enzyme activity profiles were identical. OP1E and OP2A-C are similar to *S. parvulus* in morphology, carbon utilisation and moderately similar enzyme activity profile (Table 5). The other three isolates (UT9C1, UT7E and OP7A) however were not similar to their respective closest relatives.

LC-MS/MS

LC-MS/MS analysis indicated the presence of unknown compounds in EA extracts of MG1A, UT9C1, UT7E, OP7A and OP1E with molecular weights that did not match with any of the known *Streptomyces* antibiotics in the PubChem Compound Database (NCBI). The LC profile of the OP2A-C EA extract showed two UV detectable peaks with retention time (RT) of 0.92 min and 5.41 min respectively. The mass of the major peak [M-H] was at (m/z) 1253.8 (Figure 4) which matched dactinomycin $C_{62}H_{86}N_{12}O_{16}$ (Figure 5) produced from *S. parvulus.* The negative mode spectrum of dactinomycin produced by Havlicek and Spizek (2014) corroborated the mass spectrum of the bioactive compound analysed from OP2A-C extract.

Table 3: Antibacterial activity of actinomycete EA extracts against MDR clinical isolates.

Treatment	Diameter of inhibition zones (mm)						
	B. cereus * ATCC 9592	*E. coli* * BAA-457	*E. coli* †	*K. pneumoniae* †	*Enterobacter* sp †	*Acinetobacter* sp-1 †	*Acinetobacter* sp-2 †
MG1A	**11.0**	**12.5**	**19.0**	**13.0**	**15.5**	**15.0**	0.0
UT9C1	**12.0**	**11.0**	**11.0**	**11.0**	**10.5**	**11.0**	0.0
UT7E	**10.0**	**11.0**	9.0	0.0	9.0	0.0	0.0
OP1E	**29.0**	0.0	0.0	0.0	0.0	0.0	0.0
OP7A	**29.0**	0.0	0.0	0.0	0.0	0.0	0.0
OP2A-C	**31.0**	**11.5**	**15.0**	0.0	0.0	0.0	0.0
Penicillin G (10 units)	7.0 (R)	0.0 (R)	8.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)
Ampicillin (10 µg)	8.0 (R)	0.0 (R)	**20.5 (S)**	0.0 (R)	0.0 (R)	0.0 (R)	0.0 (R)
Chloramphenicol (30 µg)	**22.5 (S)**	0.0 (R)	**22.5 (S)**	0.0 (R)	**25.0 (S)**	0.0 (R)	0.0 (R)
Gentamicin (10 µg)	**26.0 (S)**	**23.0 (S)**	**21.0 (S)**	0.0 (R)	9.5 (R)	9.5 (R)	0.0 (R)
Tetracycline (30 µg)	**21.5 (S)**	0.0 (R)	0.0 (R)	**16.5 (S)**	7.0 (R)	8.5 (R)	9.5 (R)

* ATCC cultures; †, Clinical isolates; (R), resistant; (S), sensitive; interpretation based on CLSI document (M100-S22).

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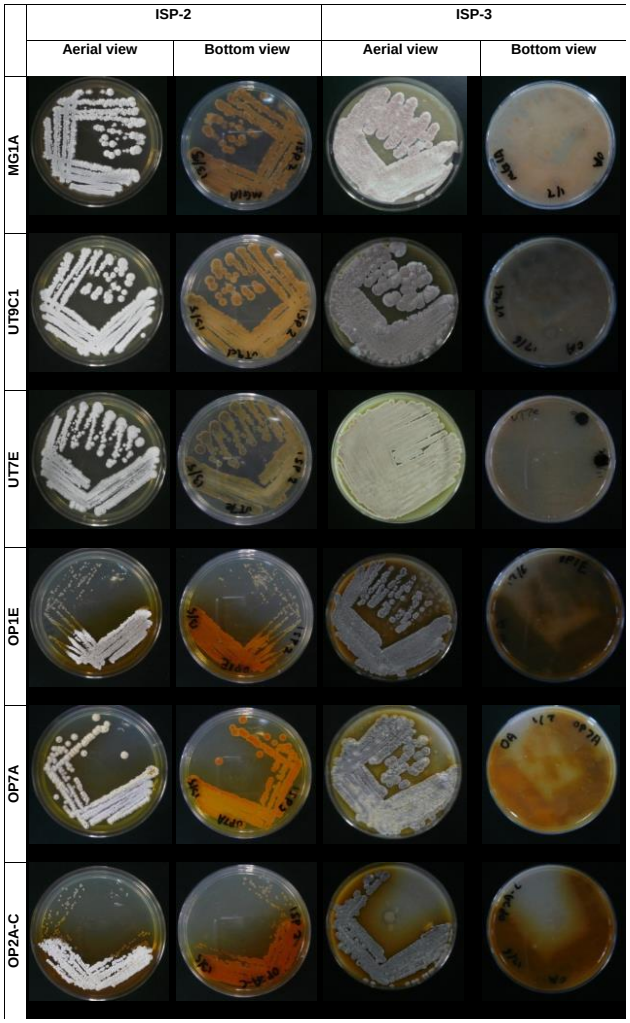

Figure 2: Appearance of OP1E, OP7A, OP2A-C, MG1A, UT9C1 and UT7E actinomycete isolates on ISP-2 and ISP-3 agar.

Isolates	Spore chain morphology	400× magnification
OP1E	Open loops, primitive spirals	
OP7A	Spirals	
OP2A-C	Spirals	
MG1A	Straight to flexuous	
UT9C1	Flexuous	
UT7E	Flexuous	

Figure 3: Spore chain morphology of OP1E, OP7A, OP2A-C, MG1A, UT9C1 and UT7E actinomycete isolates observed using inverted microscope (400× magnification).

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Table 4: Enzyme activity and carbohydrate utilisation profiles of actinomycete isolates.

Enzyme activity	MG1A	UT9C1	UT7E	OP1E	OP7A	OP2A-C
Alkaline phosphatase	+	w	+	+	+	+
Esterase (C 4)	+	+	+	+	+	+
Esterase Lipase (C 8)	w	w	w	w	w	w
Lipase (C 14)	w	-	-	-	-	-
Leucine arylamidase	+	+	+	+	+	+
Valine arylamidase	+	-	w	-	+	-
Cystine arylamidase	+	w	w	w	+	w
Trypsin	+	w	+	w	w	w
α-chymotrypsin	+	-	+	w	w	-
Acid phosphatase	w	w	+	+	+	+
Naphthol-AS-BI-phosphohydrolase						
α-galactosidase	w	+	+	+	+	+
β-galactosidase	-	-	-	-	+	-
β-glucuronidase	-	-	-	+	+	-
α-glucosidase	-	-	-	-	-	-
β-glucosidase	-	-	-	+	+	w
N-acetyl-β-glucosaminidase	w	+	+	+	-	-
α-mannosidase						
α-fucosidase	+	-	+	+	+	+
	+	-	w	+	+	w
	-	-	-	-	-	-
Carbohydrate utilised:						
Glycerol	+	+	+	+	w	+
L-arabinose	w	-	-	+	-	+
D-ribose	-	-	-	+	+	+
D-xylose	-	-	-	w	-	w
D-galactose	-	-	-	+	-	+
D-glucose	+	+	+	+	+	+
D-fructose	-	-	-	-	-	w
D-mannose	-	-	-	+	-	+
L-rhamnose	-	-	-	+	-	+
D-mannitol	-	-	-	+	-	+
Methyl-αD-mannopyranoside	-	-	-	-	-	+
Methyl-αD-glucopyranoside						
Amygdalin	-	-	-	-	-	w
Arbutin	-	-	-	-	-	w
Esculin	-	w	-	-	-	-
Salicin	+	+	+	+	+	+
D-cellobiose	-	w	-	-	-	w
D-lactose (bovine origin)	-	-	-	+	w	+
D-saccharose (sucrose)	-	-	-	+	-	w
D-trehalose	-	-	-	-	-	-
D-raffinose	-	-	-	w	-	+
Amidon (starch)	-	-	-	-	-	-
Glycogen	+	+	-	+	-	+
Gentiobiose	+	+	-	+	w	+
D-arabitol	-	-	-	w	-	+
	-	-	-	+	-	+

+, positive; -, negative; w, weak reaction.

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Table 5: Phenotypic and biochemical comparison of isolate MG1A, OP1E, OP2A-C and their closest phylogenetic neighbours *S. griseocarneus* and *S. parvulus.*

Characteristics		MG1A	*S. griseocarneus**	OP1E	OP2A-C	*S. parvulus**
Spore chain		RF	V	RA	S	S
Ariel mass colour	ISP-2	Grey	Grey	Grey	White	Grey
	ISP-3	White	White	Grey	Grey	Grey
Reverse side colour	ISP-2	Brown	Brown	Orange	Orange	Yellow/ Brown
	ISP-3	Brown	Brown	Brown	Brown	Yellow/ Brown
Diffusible pigment	ISP-2	None	None	Yellow	Yellow	Yellow
	ISP-3	None	None	Orange	Orange	None
Carbon utilisation	Glucose	+	+	+	+	+
	Arabinose	-	-	+	+	W
	Sucrose	-	-	-	-	-
	Xylose	-	-	-	-	+
	Inositol	-	+	-	-	-
	Mannitol	-	-	+	+	+
	Fructose	-	-	-	w	+
	Rhamnose	-	-	+	+	+
	Raffinose	-	-	-	-	-
	Cellobiose	-	-	+	+	-
API ZYM substrate tested	Alkaline phosphatase	+	+	+	+	
	Esterase (C4)	+	+	+	+	
	Esterase Lipase (C8)	w	+	w	w	
	Lipase (C14)	w	+	-	-	
	Leucine arylamidase	+	+	+	+	
	Valine arylamidase	+	+	-	-	
	Cystine arylamidase	+	+	w	w	
	Trypsin	+	+	w	w	
	α-chymotrypsin	+	+	w	-	nd
	Acid phosphatase	w	+	+	+	
	Naphthol-AS-BI-phosphohydrolase	w	+	+	+	
	α-galactosidase	-	-	-	-	
	β-galactosidase	-	-	+	-	
	β-glucuronidase	-	-	-	-	
	α-glucosidase	-	+	+	w	
	β-glucosidase	w	-	+	-	
	N-acetyl-β – glucosaminidase	+	+	+	+	
	α-mannosidase	+	+	+	w	
	α-fucosidase	-	-	-	-	

*Data adapted from Wink (2009). R, rectus or straight; F, flexibilis; S, spiral; RA, retinaculum apertum; V, verticillate; +, positive reaction; -, negative reaction; w, weak reaction; nd, not determined.

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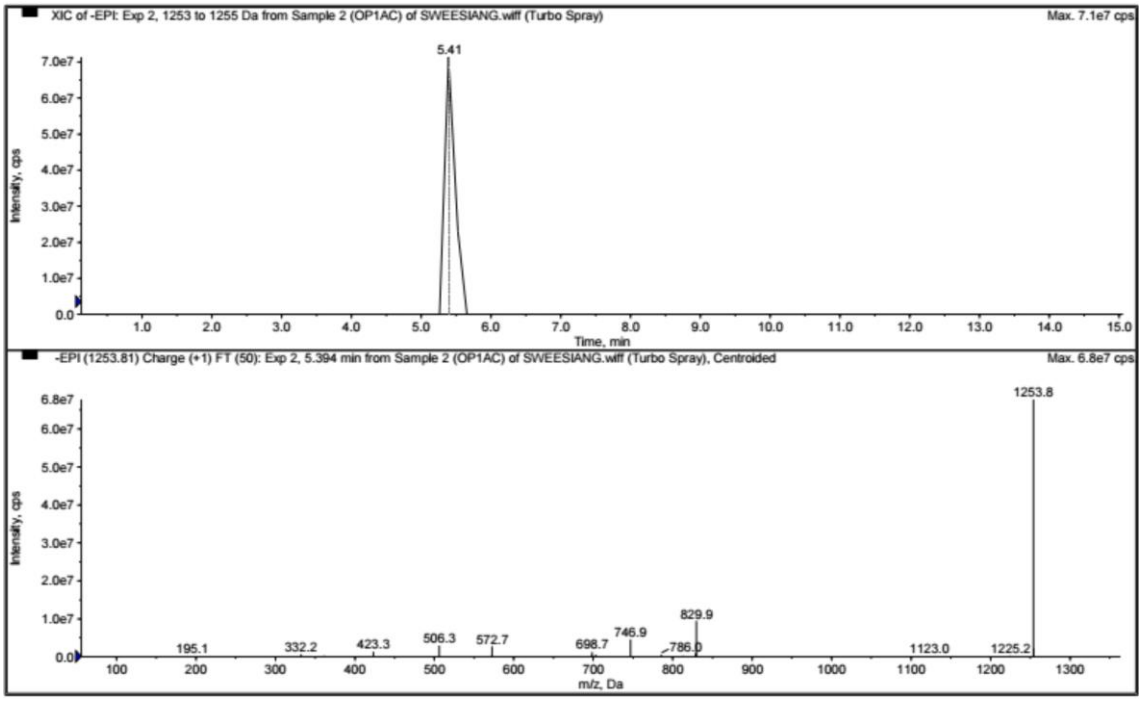

Figure 4: Mass spectral analysis of antibacterial extract OP2A-C from Table 3. The mass of the major peak detected at 5.4 min was at [M-H] (m/z) 1253.8.

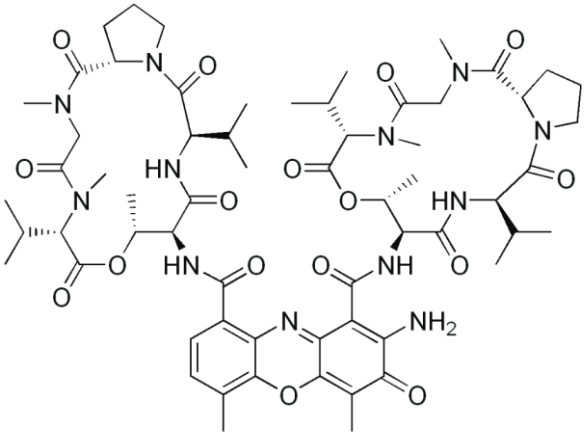

Figure 5: Dactinomycin, $C_{62}H_{86}N_{12}O_{16}$, (MW of 1255.4170 g/mol) produced from *S. parvulus* (Rahman *et al*., 2010).

DISCUSSION

Dactinomycin emerged by chance as one of the most useful chemotherapeutic agents in paediatric oncology after its discovery. Its initial intended use as antibacterial substance was shelved due to its toxicity (D'angio, 2014). The most important biochemical effects of Dactinomycin are binding to single- and double-stranded DNA and subsequent inhibition of RNA and protein synthesis (Dobson *et al*., 2008). The antibacterial activity from previous studies (Shetty *et al*., 2014) supported our findings that Dactinomycin-like contained in the extract of OP2A-C is potent against most Gram-positive bacteria and MDR clinical isolates such as *B. cereus* ATCC9592. Except for *E. coli,* the Dactinomycin-like inhibits Gram-negative bacteria weakly. The outer membrane of the cell wall structure of Gram-negative bacteria which excludes antibacterial properties from penetrating the cell could partially be accounted for the low inhibition activities observed in most Gram-negative bacteria (Oskay, 2009; Dehnad *et al*., 2010). Still, the identity of this Dactynomycin-like compound from OP2A-C uncovered from our preliminary chemical investigation will need to be confirmed via NMR.

Streptomyces parvulus species produces Dactinomycin almost exclusively (>95%) (Meienhofer and Atherton, 1973). Given OP1E matches closely to OP2A-C (99.28%) and *Streptomyces parvulus* NBRC13193 (99.93%), OP1E could be producing another variety of Dactinomycin, only slightly weaker than that of OP2A-C. More than 41 varieties of Dactinomycin have been reported, mainly with variations in the peptide portion of the structures (Singh *et al*., 2010). Yet, compounds analysed from OP1E and four other isolates did not match any *Streptomyces*-produced antibiotics in the database. The compounds produced could therefore be novel and interesting, especially MG1A and UT9C1; both though demonstrated moderate antibacterial activities, they inhibited a broad range of MDR bacteria.

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The colours of aerial mycelium and substrate mycelium of actinomycetes have been reported to be medium-dependant (Seo, 2006) and serve as one of the important phenotypic characteristics for taxonomical studies, despite limited information on the chemical nature of the pigments (Aria and Mikami, 1972; Dastager *et al*., 2006). Isolates with abundant powdery spores are usually classified into streptomycete-like strains with distinct 'earthy' geosmins odour (Getha and Vikineswary, 2002). Some of the main colours observed of the aerial spore mass are grey, white, brown, green, blue and red. The grey streptomycete-like isolates usually will be the highest in occurrence compared to the others (Shariffah-Muzaimah *et al.*, 2015). The non-streptomycete-like strains are usually small, compact or slimy colonies with colours ranging from orange, red-brown or brown to black (Getha and Vikineswary, 2002). All the six potential actinomycete isolates in this study sporulated massively on ISP-2 agar signifying their identities as members of the genus *Streptomyces*. The precise mechanism of the medium-dependent phenotype in actinomycetes is unknown but it is believed that nutrients availability might influence the sporulation of the organism which will further assist in classifying them (Pridham *et al.*, 1958; Kim *et al*., 2005).

The biochemical analysis performed on all six isolates revealed results which were in accordance to that reported for actinomycetes. Jiang *et al*. (2013) reported more than 80% of 233 actinomycete strains isolated from animal feces displayed esterase and leucine arylamidase enzyme activities. Santala *et al*. (2004) reported more than 90% actinomycete strains studied were positive for leucine arylamidase while less than 10% were able to produce β-glucuronidase and α-fucosidase. The comparison of biochemical and enzyme activity on the isolates with their close relatives (based on phylogenetic analysis) revealed high similarity and ability to utilise the same nutrients. This suggests they are convicted *Streptomyces*. Interestingly, all three isolates from oil palm plantation in this study were able to use cellulose as carbon source. Cellulolytic ability was not reported in *S. parvulus* except from a marine origin (Shaik *et al*., 2017), which could dispose cellulosic wastes added to its environment. The palm wastes in the oil palm plantation could have triggered in the isolates, the ability to utilise cellulose.

Most naturally occurring antibiotics and new bioactive metabolites are discovered from Streptomycetes (Liu *et al.*, 2013). Selective isolation incorporating the use of different pretreatment techniques; antibiotics, organic solvents, heat, salt, pH, complex carbon and nitrogen sources are among the few strategies adopted in addition to targeting uncommon and rare endophytic and symbiotic actinomycetes from medicinal plants and insects to harness unique candidates (Lazzarini *et al.*, 2000; Berdy 2012). Enrichment techniques utilising a range of growth media to trigger the production of bioactive metabolites are also constantly reported. Our strategy targeting natural forest and cultivated soils for unique actinomycetes with antibacterial activities against MDR clinical isolates demonstrated cultivated soils could harbour unique actinomycetes (MG1A from rhizosphere of mango tree and OP2A-C from oil palm plantation). Land converted for agriculture, pastures and alike tend to have lower biodiversity of bacteria (Rodrigues *et al*., 2013), which would have lowered the chances of finding unique actinomycetes. Instead, more actinomycetes were isolated from soils collected from oil palm plantations than soils collected from the forest. Shange *et al*. (2012) along with other studies (Burke *et al*., 2003; Waldrop *et al*., 2000; Lauber *et al*., 2008; Lee-Cruz *et al*., 2013) found actinomycetes showed higher abundance in agricultural and pasture soils compared to forest soils. The activities performed for cultivation such as tillage, high fertilizer application and residues left from crops which resulted in significantly higher soil organic carbon, total nitrogen and nutrient cycling as observed by Shange *et al*. (2012) and Lee-Cruz *et al.* (2013) could have attributed to the higher abundance of actinomycetes isolated from cultivated soils. In addition, the actinomycetes are resistant to land use and change and would not be depressed in terms of overall diversity except certain taxonomic groups (Lee *et al*., 2013). In order to compete with other microbes, the actinomycetes like MG1A and OP2A-C could have upped their strategies utilising available nutrients and activated the genes to produce assorted antibiotics in cultivated soils, unexpectedly producing broad and effective antibacterial properties against MDR clinical isolates. The indications from this study are that MDR inhibiting fast-growing actinomycetes could be isolated not necessarily only from natural soils but cultivated soils as well, with the latter probably a better target.

CONCLUSION

The less explored Malaysian soil ecosystems represent a rich resource for actinomycete species and antibiotics. In this study, 6 potential actinomycete isolates (OP1E, OP7A, OP2A-C, MG1A, UT9C1 and UT7E) with significant antibacterial activity were isolated and characterised from soil samples of three different bio-habitats in Malaysia. In this study, three actinomycete isolates and extracts; MG1A, UT9C1 and OP2A-C exhibited potential growth inhibitory activity against more than three MDR clinical isolates. Based on morphology, biochemistry and 16sRNA sequence data, MG1A is related to *S. griseocarneus,* OP1E and OP2A-C to *S. parvulus*, UT9C1 and UT7E to *S. polychromogenes* and OP7A to *S. gramineus.* Future studies are required to purify, identify and validate the identity of the antimicrobial compounds from these isolates as well as the synergistic effect of the compounds against MDR clinical isolates.

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CONFLICT OF INTEREST

All authors declared no conflict of interest.

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