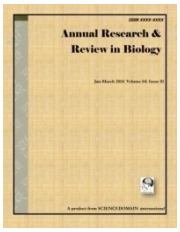

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Anti-Bacterial Activity of Endophytic *Streptomyces* sp. towards *Acinetobacter baumannii*

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Authors' contributions

This work was carried out in collaboration between all authors. Author NB done all the lab works, managed the literature searches and wrote the first draft of the manuscript, with fully supervised by author NMZ in all its aspects. Author AI helped during the collecting of samples. Author AH prepared A. baumannii isolates while author JL contributed in TLC part. Authors NMZ, AH and JL managed in the analyses of the study. All authors read and approved the final manuscript.

Original Research Article

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ABSTRACT

Aims: To screen fifty-six endophytic streptomyces isolates for anti-bacterial activity on two clinical strains of *Acinetobacter baumannii* (carbapenem sensitive and resistant).
Place and Duration of Study: Universiti Kebangsaan Malaysia; between May 2011 and February 2012.
Methods: In this study, 56 isolates of endophytic *Streptomyces* sp. and their extracts were screened for anti-bacterial activity against *A. baumannii*. The compound's profile was obtained for determination of active metabolites

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Results: The bioassay showed that 14 isolates namely "Strain Universiti Kebangsaan" (SUK) had anti-bacterial activity towards *A. baumannii*. Isolates SUK 31, SUK 56 and SUK 15 showed 100% inhibition, while SUK 8 and SUK 10 showed more than 50% inhibition. These 3 isolates proceeded with secondary metabolites extraction using ethyl acetate. Disc diffusion test showed the zone of inhibition towards sensitive and resistant strain of *A. baumannii*; by SUK 15 at 11 and 8 mm, while SUK 31 at 7 and 10 mm, respectively. Minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) of SUK 8 were 1.0mg/mL and 4.0mg/mL, respectively. On the other hand, SUK 15, SUK 31 and SUK 56 had the same MIC and MBC values at 2.0mg/mL. Then again, there was no difference between the MIC and MBC values for both strains of *A. baumannii*. Analysis of profile extract components of one selected extract, SUK 8, showed that there was at least nine polar compounds that had been successfully separated using a Thin Layer Chromatography (TLC) technique.
Conclusion: Endophytic *Streptomyces* sp. exhibits an anti-bacterial activity against resistant and sensitive strains of *A.baumannii*, thus might be a potential drug candidate.

Keywords: Endophytes; Streptomyces sp.; Acinetobacter baumannii.

1. INTRODUCTION

As huge and relatively untapped sources of new medicinal and agricultural products, endophytic microbes, especially actinobacteria, showed enormous potential in the search for natural products [1]. Owing to the excellent tracking record of actinobacteria in the past fifty years, great effort has focused on isolating them from special sources for drug screening. The medicinal plants host numerous endophytic strains, which are expected to produce a wide variety of bioactive metabolites [2].

Actinobacteria are noteworthy as antibiotic producers, especially *Streptomyces* sp., which covers more than 80% of the total antibiotic products. In addition, *Streptomyces* sp. has potential sources for secondary metabolites, and possesses a variety of biological activities. It was estimated that these bacteria synthesized more than 7,000 metabolites [3]. They have become a subject of interest to many studies due to their chemical compound diversities and biological activities [4]. Previous studies proved that *Streptomyces* sp. is used as anti-trypanosomal [5]; anti-malarial; anti-tuberculosis; anti-fungus [4,8]; anti-tumor; and anti-microbes [2,6,7].

Nowadays, microorganisms gain resistance to the existing antibiotics, and become a multi-drugs resistance (MDR). These strains are known as 'ESKAPE' pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* sp.). The 'ESKAPE' indicates their 'escape' from the effects of antibacterial agents or active antibiotics [9]. Recently, *Acinetobacter* sp. has been recognized as a worldwide emerging bacterium of nosocomial outbreaks, particularly in intensive care units (ICUs), which incidence reported to be substantially increased during the past decade. *Acinetobacter baumannii* is found ubiquitously in the environment. It is an aerobic Gram-negative cocobacillus, a non-fermenter of glucose, and a non-motile and non-fastidious bacterium [10].

Carbapenem now plays a prominent therapeutic role, especially in hospitals where MDR strains have become prevalent. Emerging carbapenem-resistant strains limit the effectiveness of the choice of their treatments, which are imipenem and meropenem [11].

Recently, carbapenems were among the last alternatives for treatment. However, the resistance to *A. baumannii* is now a major problem [12]. Thus, the discovery of alternative therapeutic drugs is urgently required.

2. METHODOLOGY

2.1 Endophyte Isolation and Identification

Fifty-six endophytic actinobacteria were successfully isolated from different species of plants from Bukit Bauk, Terengganu; Hutan Simpan Bangi, Selangor; Hutan Simpan Nenasi and Tasik Chini in Pahang; Pasir Mas, Kelantan; and Bukit Panchor, Pulau Pinang and Taman Negara, Kota Kinabalu. The morphological characteristics of the isolates were examined, according to Shirling and Gottlieb, for the International Streptomycete Project [13].

2.2 Test Organisms

The test bacteria, *Acinetobacter baumannii* was isolated from the clinical specimens from Bacteriology Unit, UKM Medical Centre (UKMMC) Malaysia. Gram stained, culture and biochemical tests for species identification were carried out in the hospital laboratory diagnosis UKMMC and were found to be carbapenem-sensitive and carbapenem-resistant as tested by an antibiotic sensitivity test.

2.3 Bioassays of the Actinobacteria

Streptomyces were grown onto nutrient agar (NA) for 14 days. Then, three microliter (µL) bacteria inoculums of tested *A.baumannii* (OD=0.08 at 560nm) were placed about 1.0 cm from the edge of streptomycete colony [14,15] and plates were incubated for 24 hours. A plate of *A.baumannii,* without the presence of endophyte *Streptomyces* sp. was used as negative control. The growths of the bacteria were observed after 24 and 48 hours incubation. Four isolates of endophyte *Streptomyces* sp. that showed the highest percentage of inhibition against tested bacteria were selected for fermentation of secondary metabolites and pursued for sensitivity test.

2.4 Endophyte *Streptomyces* sp. Fermentation and Extraction of Bioactive Metabolites

A few blocks of ISP 2 containing streptomycetes were inoculated into 600mL of nutrient broth, and incubated at room temperature of 28ºC for 17 days on orbital shaker at 140rpm [15]. Culture filtrates were extracted with three half-volumes of ethyl acetate. The organic phase was pulled, followed by evaporation using rotavapor (model Eyela Rotary Vacuum Evaporator N-N series) at 40ºC. The crude extract was dissolved in methanol and was then used for bioactivity test.

2.5 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Determination

The MIC and MBC values of each extract were determined for the bacteria strains using two fold serial microdilution method, carried out in a 96-well microtitre plate as recommended by CLSI 2009. Initially, the ethyl acetate crude extracts were tested with the concentration

range of 0.00391 - 8mg/mL. Bacteria inoculums were prepared into 5 x 10^5 CFU/mL. *E. coli* ATCC 25922 was used as control bacteria while Gentamicin and Polymyxin B were used as positive and negative drug controls. Both drug and bacteria controls were tested at the final concentration (0.0625µg/mL - 128µg/mL), and the extracts were assayed in triplicate. Following incubation under anaerobic conditions, the plate was added with 5µL (1mg/mL) of MTT solution (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). The plate was incubated again for another 2 hours in the dark. The MIC value was taken as the lowest concentration of extract that prevents visible growth while the MBC value was determined by the lowest concentration of the extract that yields no growth following this second subculturing.

2.6 Disc Diffusion Assay

Two and 4 mg extracts were diluted with methanol, pipetted onto two filter paper discs each, and left to get dried in the biosafety cabinet. After that, the disc was placed onto Mueller-Hinton Agar (MHA) with a full lawn of *A. baumannii* culture (OD= 0.08 at 625 nm). The plate was incubated at 37ºC for 24 hours. Then, the diameter of inhibition zone was measured

2.7 Components Profile Separation of Crude Extract SUK 8 using Thin Layer Chromatography

The potentially crude extract, SUK 8, was carried out for compound profile separation using Thin Layer Chromatography (TLC) technique. The crude extract was diluted with ethyl acetate, and then 1µL of the extract was dropped onto the chromatography plate by using capillary tube. It was dried before adding into developing chamber. The developing chamber system was created with different mixtures of solvents and different ratio, and was tested until a good separation of the extract's components was formed. The best solvent system that was found was methanol: chloroform with ratio of 0.5:9.5 and 1:9, viewed under ultraviolet light and dyed with cerium (IV) sulphate. The methanol: chloroform with ratio of 0.5:9.5 and 1:9 was found as the best solvent system for separation of the compounds.

3. RESULTS AND DISCUSSION

All the isolates were identified as *Streptomyces* sp. based on the morphological observation. Table 1 showed the antibacterial bioassay screening test, where 14, out of 56 isolates, were active towards *A. baumannii*. Isolate SUK 31, SUK 37 and SUK 56 showed impressive activity with 100% inhibition against both strains; no colony grew on the NA plate after 24 and 48 h incubations. These isolates were believed to have active compounds, which were able to kill *Acinetobacter* sp. Meanwhile, SUK 15, SUK 8 and SUK 10 classified as active isolates, gave more than 50% inhibition.

Carbapenem such as imipenem, meropenem and doripenem were used as important antibiotics for therapy of various MDR gram-negative infections, especially *Acinetobacter* sp., with no side effect to the patients [16]. Therefore, in this study, a distinction was made between the carbapenem and the non-carbapenem β-lactam antibiotics because carbapenem resistance is a sentinel event for emerging antimicrobial resistance and in itself confers high resistance [10].

The unparalleled results from the bioassay antibacterial and zone inhibition (Table 2) in this study may be caused by various reasons. The bioactive compounds in the crude extract

might be inactivated after going through the fermentation and the extraction process. Also, the culture conditions might be affected due to the production of bioactive compounds [17,18]. Fermentation at high-speed agitation (140rpm) might not be suitable for the growth of *Streptomyces* sp., as it will change the natural state of this endophyte's growth, and might produce different metabolites. Besides, the syntheses of antimicrobial metabolites depend on the medium constituents and other environmental factors such as temperature, pH and incubation time, which show profound influence on antibiotic production [17]. The types of solvents used during extraction also affected the result of this study. Each metabolite needs specific solvent to be dissolved [19]. According to Zin et al. [5], under certain condition, only secondary metabolites, which are soluble in ethyl acetate will be extracted efficiently, although there are other compounds left which may have good activity since they were not soluble with the solvent. Thus, active compounds that react to *A. baumannii* in this study may not be extracted using ethyl acetate. Ethyl acetate is a semi-polar solvent, which absorbs most of the polar and non-polar metabolites. However, not all of the active metabolites can be extracted. Nevertheless, previous studies have shown that ethyl acetate is the best solvent for extracting antimicrobial substances [15,19].

Table 1. Anti-bacteria bioassay: percentage (%) of growth inhibition

Strain		Carbapenem-sensitive (%)		Carbapenem-resistant (%)	
No	**Isolate**	**24 h**	**48 h**	**24 h**	**48 h**
1	SUK 31	100	100	100	100
2	SUK 37	100	100	100	100
3	SUK 56	100	100	100	100
4	SUK 15	100	100	37.8	55.8
5	SUK 8	75	76.9	66.7	71.2
6	SUK 10	64.3	81.5	60	84
7	SUK 7	42.9	44.4	10	42
8	SUK 12	23.6	37.5	20.8	32.7
9	SUK 3	22.2	26	22.2	26.9
10	SUK 21	16.7	12.5	25.9	30.8
11	SUK 25	12.5	15	11.1	46.2
12	SUK 28	12.5	0	11.1	39.7
13	SUK 27	0	20.8	22.2	41
14	SUK 22	0	12.5	11.1	20

Table 2. Disk diffusion assay: diameter of inhibition zone

Inhibition zone size (Carbapenem-sensitive)		
Extract disc	**2 mg**	**4 mg**
SUK 8	-	-
SUK 15	11 mm	12 mm
SUK 31	8 mm	8 mm
SUK 56	-	-
Inhibition zone size (Carbapenem-resistant)		
Extract disk	**2 mg**	**4 mg**
SUK 8	-	-
SUK 15	7 mm	8 mm
SUK 31	10 mm	10 mm
SUK 56	-	-

Meanwhile, the MIC and MBC values of different ethyl acetate crude extracts of the isolates are stated in Table 3 and Table 4. The result showed that SUK 8 had MIC value of 1.0 mg/mL for both strains of *A. baumannii* with MBC value at concentration of 4.0mg/mL, compared to other isolates, while SUK 15, SUK 31 and SUK 56 showed the same values of MIC and MBC (2.0 mg/mL). From the result, the activities of the extracts against both strains of *A. baumannii* were similar.

Table 3. MIC of SUK 8, SUK 15, SUK 31 and SUK 56

Minimum Inhibition Concentration (MIC) mg/mL		
Isolate	**Carbapenem-sensitive**	**Carbapenem-resistant**
SUK 8	1.0 mg/mL	1.0 mg/mL
SUK 15	2.0 mg/mL	2.0 mg/mL
SUK 31	2.0 mg/mL	2.0 mg/mL
SUK 56	2.0 mg/mL	2.0 mg/mL

Table 4. MBC of SUK 8, SUK 15, SUK 31 and SUK 56

Minimum Bactericidal Concentration (MBC) mg/mL		
Isolate	**Carbapenem-sensitive**	**Carbapenem-resistant**
SUK 8	4.0 mg/mL	4.0 mg/mL
SUK 15	2.0 mg/mL	2.0 mg/mL
SUK 31	2.0 mg/mL	2.0 mg/mL
SUK 56	2.0 mg/mL	2.0 mg/mL

There has been scarcity of information on the antibacterial effects of *Streptomyces* sp. against multidrug resistant *A. baumannii* strains. A Study by Lee et al. [20] showed the extract from *Streptomyces* sp. KH29 has good antimicrobial activities against 41 isolates of multi-drugs resistance of *A. baumannii*. In this study, the ethyl acetate extracts of SUK 8, SUK 15, SUK 31 and SUK 56 were tested against both strains of *A. baumannii* (carbapenem-sensitive and carbapenem-resistant). Antibacterial activity of SUK 8 showed the lowest MIC value (1.0mg/mL). This study is similar to the study by Arasu et al. [17], where the same MIC value was obtained when *Streptomyces* sp. extract was used against other Gram-negative bacteria.

SUK 8 crude extract was further evaluated for its compound profiling. The result showed that SUK 8 extract had eight polar ultraviolet (UV) active fractions separated under UV light observation, and another one active fraction was seen after dyeing with cerium (IV) sulphate, making a total of nine successfully separated components. Fig. 1 shows the TLC plate after observation under UV light, using solvents system methanol and chloroform with ratio of 0.5:9.5 and 1:9. Meanwhile, Fig. 2 shows the TLC plate after dyeing with cerium (IV) sulphate.

The unique pattern of secondary metabolites in this extract should be further evaluated. Thin Layer Chromatography (TLC) is a chemical screening approach, which is simple and inexpensive for visualizing the secondary metabolite pattern of a sample. The separation pattern of the metabolites from a microorganism on TLC plates, as well as their chemical reactivity towards some staining reagents under defined reaction conditions, enables the visualization of an almost complete picture of the secondary metabolites (also known as their metabolic fingerprints) [21] which are useful in the search for products of interest. Each

isolate gives a particular secondary metabolite pattern, which demonstrates the quantity of secondary metabolites in the extract [22]. This study showed that one or more separated compounds, obtained from *Streptomyces* sp. of SUK 8, may have antimicrobial potential against *A. baumannii.* Butyl, propyl, esther, cyclohexane and heptanes-2,3-dione were identified using the GC-MS library data and was reported to be the most preferable compounds to have antimicrobial effects [23]. On the other hand, Yu et al (2010) [24] reported that endophytic *Streptomyces* sp., which resides in different plant hosts, contained different bioactive compounds such as alkaloids, peptides, steroids, terpenoids, phenols, quinines and flavonoids. Therefore, extract SUK 8 may consist of one or more bioactive compounds from these classes.

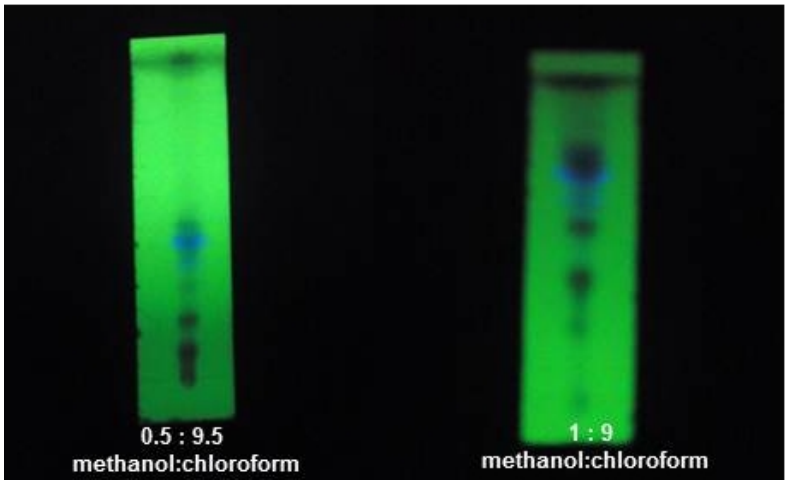

Fig. 1. Profile compounds of ethyl acetate crude extract of SUK 8 under UV light

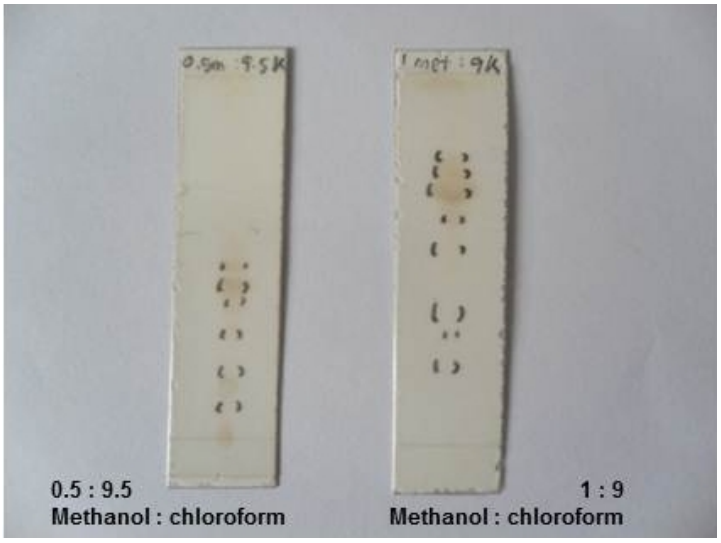

Fig. 2. Profile components of ethyl acetate crude extract of SUK 8 after dyeing with cerium (IV) sulphate

4. CONCLUSION

Isolates SUK 8, SUK 15, SUK 31, and SUK 56 are promising antimicrobial agents against *Acinetobacter* sp. However, isolation and structure elucidation of the bioactive compounds in these isolates need to be further considered for the next research study.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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