Screening For Antimicrobial Activity Of Fungi In Soil Samples Collected From Kubah National Park

Samuel Lihan, Yue Keong Choon, Ng Kok Hua, Mohd Effendi Wasli

Abstract: Antimicrobial agents including antivirals and antibiotics have saved millions of lives all over the world, but these drugs are losing their effectiveness due to the development of resistance of infectious disease agents towards them. The incidence of antibiotic-resistance towards current drugs has been rapidly increasing but fewer new antibiotics are being developed. This study was carried out on soil samples collected from Kubah National Park, Kuching, Sarawak, Malaysian Borneo, in order to discover novel antibiotics produced by soil microbes. Twenty one samples of soils were analyzed for antimicrobial producing fungi. Potential fungal isolates were tested against *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis* and *Klebsiella pneumoniae.* Six fungal isolates labeled P550AIa, P550AIb, P550AIc, P550AId, P550AIIa, P550AIIb showed strong antibacterial activity against the test bacteria during the antimicrobial activity screening using agar overlay technique in the preliminary screening and secondary screening. Out of the 6 fungal isolates, 3 isolates P550AIa, P550AIb, P550AIc were selected to undergo antibiotics susceptibility testing and further characterization. The crude extract of the 3 fungal isolates were further characterized by MIC, TLC and bioauthography methods. The isolates showed MIC value and produced inhibition zone compared to the positive control (5×dilutions of penicillin-streptomycin solution). The characteristics of the spores produced by the three fungal isolates matched with the description for *Penicillium* spp.. Further confirmation by DNA sequencing of isolate P550AIb revealed its identity as *Penicillium verruculosum.* All the fungal isolates showing antimicrobial activity are potential to be used for producing antimicrobial compound for combating infectious bacterial agents as evidenced in this study.

Index Terms: Antimicrobial activity, Fungi, Identification, Soil.

1 INTRODUCTION

Antibiotic is a drug used to treat infections caused by bacteria that can cause illness to humans and animals. Antibiotic functions to inhibit or destroy the bacterial cells that cause certain disease [1]. In fact, antibiotic is secondary metabolite produced by bacteria [2] to maintain their niche and territory. There are few groups of microorganisms that can be used as sources for clinically useable antibiotics. As stated by Cooke and Gibson [3], only antibiotics that have an effect on bacterial cells but not the host cells are categorized as useful antibiotics. To date, over 100 different antibiotics are available to cure minor and life-threatening infections. Antibiotic resistance occurs when the effectiveness of drugs and chemicals designated to cure diseases are reduced [4].

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Scientists are continuously searching for novel antibiotic producing microbes because drug resistant strains of pathogen emerge more quickly than the rate of discovery of new drugs and antibiotics [5]. Consequently, a numbers of antibiotics that can fight against pathogenic bacteria had been discovered. According to Roberts [6], it is important to discover new antibiotics as the emergence of new diseases and reemergence of multiple-antibiotic resistant pathogens have caused current antibiotics ineffective. There are many sources where antibiotics can be discovered, however, soil is the most important source for the discovery of novel antibiotics. According to Dulmage and Rivas [7], soil microorganisms have continually been screened for their useful biological active metabolites, such as antibiotics since long ago. Therefore, this study was an attempt to discover novel antibiotics from microbes in soil samples from undisturbed area in Sarawak.

2 MATERIALS AND METHODS

2.1 Soil sampling, preparation and plating

Soil samples were collected at four different elevations; 50m, 350m, 450m and 550m above sea level along Ulu Rayu and Summit Trial at Matang Wildlife Centre-Kubah National Park, Sarawak. Samples were collected at 0-10cm using composite augering technique. Approximately 1g of the soil samples was dissolved in 9 ml of sterile Phosphate Buffer Saline (PBS) buffer (pH 7.4) to make soil suspension. The supernatant from the soil solution was pipette and spread over four PDA (BD Difco™, USA) plates. The plates were left at room temperature for 5 days to calculate and record the fungal colonies. Then, the plates were kept at 4°C for 2 days to delay the growth of soil microorganisms.

2.2 Test bacteria

The test bacteria were obtained from Microbiology Laboratory, Department of Molecular Biology, Universiti Malaysia Sarawak. The test bacteria used were gram-positive (Listeria

monocytogenes, Listeria innocua) and gram-negative (Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae).

2.3 Preliminary selection and isolation of soil microorganisms

The soil microorganisms grown on PDA plates were overlaid by a layer of soft NA (0.75%) containing the test bacteria. Four empty plates of PDA overlaid with the test bacteria were used as control. All the plates were incubated at room temperature and observation for the formation of the inhibition zone was carried out for every 24 and 48 hours. The soil fungi that inhibited the growth of test bacteria by producing inhibition zone were isolated and stored at -20^{o}C in glycerol stock.

2.4 Secondary screening for fungal isolates via agar overlay technique

Pure fungal isolates obtained from the preliminary selection were cultivated on PDA. After incubated at room temperature for 4 days, these fungal isolates were subjected to secondary screening via agar overlay technique. Soft NA (0.75%) which seeded with test bacteria were overlaid onto the growing fungal isolates. Four empty PDA plates overlaid with soft agar were used as controls. All the plates were incubated at room temperature for 24 hours and the zone of inhibition was observed.

2.5 Extraction of secondary metabolites from pure isolates

Fungal isolates showing great antibacterial activities during the secondary screening were selected and cultivated on PDA. The agar plates containing the selected fungal isolates were dried in a fume hood and grinded before they were immersed with hexane solvent. After 4 days of immersion, the solvent was filtered and then concentrated by using rotary evaporator at 40^{o}C. The collected crude methanol extracts were kept at 4^{o}C for further testing.

2.6 Antibiotics susceptibility testing with hexane crude extracts

Antibiotics susceptibility testing was carried out based on disk diffusion method described by Bopp *et al*. [8]. Approximately 1mg of the dried crude extract was weighted and dissolved in 5µl of 100% methanol and 95µl of Mueller Hinton Broth (MHB) (Oxoid, UK) at pH7.0. The extract was diluted to concentration of 0.5 mg/ml, 0.25mg/ml, 0.125 mg/ml and 0.0625mg/ml, respectively.

2.7 Determination of minimum inhibitory concentration (MIC) value

Determination of MIC value was carried out using disk diffusion method. The test bacteria that seeded were arranged on Mueller Hinton Agar (MHA) (Oxoid, UK) plate with seven 6mm antibiotic-free filter discs (Whatman No.3). Aliquot of 10µl of different dilution of antibiotics was dropped onto the discs with concentration of 1.0mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0.0625mg/ml, respectively. Positive and negative control of antibiotics-free filter discs on the MHA plate was pipetted with 10µl of 5x dilutions penicillin-streptomycin solution and MHB contained 100% methanol. The plate was incubated at 37ºC for 24 hours. The diameter of the inhibition zone formed around the disc was measured.

2.8 Thin Layer Chromatography (TLC)

1.0mg/ml of fungal crude extract was prepared by adding 0.1mg of crude extract and 100µl of hexane. Sample solvent was prepared in chamber by adding hexane and dichloromethane in ratio of 1:3.5 in total of 25ml. Next, 6µl of 1.0mg/ml crude extract was dropped twice on each spot on silica plate. The TLC was run until solvent reach on top. The TLC plate was dried and then visualized under ultraviolet developing camera.

2.9 Bioautography

Soft NA (0.75%) seeded with test bacteria was overlaid onto TLC plate. Positive and negative control of silica gel plates were pipette with 100µl of 5x dilutions penicillin-streptomycin solution and 100µl of distilled water. All the plates were kept at 37ºC overnight in an incubator and the formation of inhibition zone on silica gel plates was observed. The inhibition zone was added with 300µl of MTT solution and left for 4 hours. The TLC plates with the soft agar on them were dried and the results were observed.

2.10 Characterization of pure fungal isolates

Characterization of pure fungal isolates was carried out based on macroscopic and microscopic examinations. Pure fungal isolates showing great antibacterial activities during secondary screening were cultivated on PDA, CDA (Merck, USA) and MEA (Oxoid, UK). The different growth morphologies, including top colour, mycelium mat, mycelium end reverse colour, medium colour and perimeter were observed and recorded. Intact structures of the fungi, including structure of hyphae, conidia, spores and conidiospores were observed under light compound microscope.

2.11 PCR identification

The ribosomal DNA (rDNA) of the pure fungal isolate was amplified in polymerase chain reaction (PCR) using universal forward primer ITS1 and reverse primer ITS4 as described by White *et al*. [9]. The PCR product was subjected to agarose gel electrophoresis at 90V for 40 minutes. The DNA fragment in the agar was excised and purified by using QIAquick Gel Extraction Kit (Qiagen, USA). The purified PCR product was sent to 1st Base Sdn Bhd, Malaysia for DNA sequencing. The sequence of amplified fragment was blasted with the available 18S rRNA gene sequences deposited in the NCBI GenBank by using BLAST.

3 RESULTS

3.1 Selection of potential antibiotic producing soil microbes

During the first round of preliminary selection, a total of 29 plates showed formation of zone of inhibition. After the fourth round of sub-culturing in preliminary selection for set I, II, III and IV, a total of 24 fungal isolates were successfully isolated. The isolates of fungal and the bacteria to which they show antimicrobial activity are shown in Table 1.

Table 1. Potential antibiotics producing fungal isolates showing activity in preliminary screening

Set I = *Klebsiella pneunoniae*	Set II = *Salmonella enteritidis*	Set III: *Listeria monocytogenes*	Set IV: *Escherichia coli*
P50AE Ic	P50AEIIa	P50AEIIIa	-
P350AIa	P50AEIIb	P50AEIIIb	
P350AIb	P350AIIa	P50AEIIIc	
P350AIc	P550AIIa	P350AIIIa	
P450B1I a	P550AIIb	P350AIIIb	
P450B1I a (2nd)		P350AIIIc	
P550AI a			
P550AI b			
P550AI c			
P550AI d			
P550AI e			
P550AI f			
P550AI g			
Total: 13	Total: 5	Total: 6	Total: 0

3.2 Secondary screening

Secondary screening was carried out to further confirm the antimicrobial activities possessed by selected fungal isolates from preliminary screening. In secondary screening, 6 isolates were showing the ability to inhibit the growth of test bacteria, which were P550AIa, P550AIb, P550AIc, P550AId, P550AIIa and P550AIIb (Table 2). Fig. 1 shows the zone of inhibition for positive fungal isolates cultivated on PDA during the confirmation in secondary screening.

3.3 Antibiotics susceptibility testing with hexane crude extracts

Three fungal isolates (P550AIa, P550AIb and P550AIc) were chosen to undergo disk diffusion test by using hexane crude extracts at various concentrations in order to determine the MIC value. In this disk diffusion test, 5 test bacteria (*Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis*, *Klebsiella pneumoniae*, and *Listeria innocua*) were used. From the first round of the disk diffusion test, 2 fungal isolates (P550AIb and P550AIc) showed antimicrobial activity against different test bacteria. P550 AIb was most active against *Listeria innocua* and *Salmonella enteritidis*, whereas P550AIc produced the largest zones of inhibition against *Salmonella enteritidis.* On the other hand, P550AIa did not show any zone of inhibition against the different species of test bacteria. In second round of the disk diffusion test, all the three fungal isolates showed antimicrobial activity by inhibiting one or more microorganisms (Fig. 2). Hexane extracts of P550 AIb showed the most active antimicrobial activity against different test bacteria. It produced the largest zones of inhibition in all range of concentration against *Listeria innocua*. P550 AIa was most active against *Salmonella enteritidis* and *Klebsiella pneumoniae*. On the other hand, P550AIc showed less and smaller zone of inhibition compared to the other two fungal isolates. MIC value was measured by the lowest antibiotic concentration that is able to cause the formation of inhibition zone around the disc (Table 3).

Table 2. Measurement of zone of inhibition for positive fungal isolates cultivated on PDA in secondary screening

Codes	Test bacteria[a]			
	KP	SE	LM	EC
P50AE Ia	-	-	-	-
P50AEI b	-	-	-	-
P50AE Ic	-	-	-	-
P150A I	-	-	-	-
P350AIa	-	-	-	-
P350AIb	-	-	-	-
P350AIc	-	-	-	-
P450B1Ia	-	-	-	-
P450B1I a (2nd)	-	-	-	-
P550AIa	-	-	34	-
P550AIb	44[b]	-	-	-
P550AIc	34	-	-	-
P550AId	43	-	-	-
P550AIe	-	-	-	-
P550AIf	-	-	-	-
P550AIg	-	-	-	-
P50AE IIa	-	-	-	-
P50AE IIb	-	-	-	-
P250A II	-	-	-	-
P350AIIa	-	-	-	-
P550AIIa	39	-	-	-
P550AIIb	62	-	-	-
P50AE IIIa	-	-	-	-
P50AE IIIb	-	-	-	-
P50AE IIIc	-	-	-	-
P350A IIIa	-	-	-	-
P350A IIIb-	-	-	-	-
P350A IIIc	-	-	-	-
P550A IIIa	-	-	-	-

[a]Test bacteria species: KP: *Klebsiella pneumoniae*, SE: *Salmonella enteritidis*, LM: *Listeria monocytogenes*, EC: *Escherichia coli*. [b]Zone of inhibition (size was measured in mm from the edge of fungal colony to the border of the corresponding inhibition zone), - : no activity (0 mm)

Table 3. The minimal inhibition concentration (MIC) using disk diffusion test of the fungal isolates

Fungal isolate	Test bacteria	1.0mg/ml		0.5mg/ml		0.25mg/ml		0.125mg/ml		0.0625mg/ml		MIC value (mg/ml)
		R1 R2	A	R1 R2	A	R1 R2	A	R1 R2	A	R1 R2	A	
P550AIa	*Listeria innocua*	-	-	-	-	-	-	-	-	-	-	-
	Salmonella enteritidis	0.65 0.70	**0.68**	0.80 0.60	**0.70**	0.90 1.05	**0.98**	1.00 0.95	**0.98**	-	-	**0.125**
	Klebsiella pneumoniae	0.65 0.70	**0.68**	1.00 0.90	**0.95**	0.90 0.90	**0.90**	0.75 0.80	**0.78**	-	-	**0.125**
	Escherichia coli	-	**-**	-	**-**	-	**-**	-	-	-	-	**-**
	Listeria monocytogenes	-	**-**	-	**-**	-	**-**	-	-	-	-	**-**
P550AIb	*Listeria innocua*	1.10 1.05	**1.08**	1.10 1.00	**1.05**	1.10 0.90	**1.00**	0.80 0.90	**0.85**	0.80 0.70	**0.75**	**0.0625**
	Salmonella enteritidis	0.800. 80	**0.80**	0.80 0.80	**0.80**	-	**-**	-	**-**	-	-	**0.5**
	Klebsiella pneumoniae	0.80 0.85	**0.83**	-	**-**	-	**-**	-	**-**	-	-	**1.0**
	Escherichia coli	-	**-**	-	**-**	-	**-**	-	**-**	-	-	**-**
	Listeria monocytogenes	1.05 1.10	**1.08**	1.20 1.10	**1.15**	1.00 0.90	**0.95**	0.90 0.85	**0.88**	-	-	**0.125**
P550AIc	*Listeria innocua*	-	-	-	-	-	-	-	-	-	-	**-**
	Salmonella enteritidis	1.00 1.00	**1.00**	-	-	-	-	-	-	-	-	**1.0**
	Klebsiella pneumoniae	-	**-**	-	-	-	-	-	-	-	-	-
	Escherichia coli	-	**-**	-	-	-	-	-	-	-	-	-
	Listeria monocytogenes	0.80 0.80	**0.80**	-	-	-	-	-	-	-	-	**1.0**

Note: R1: First reading 1, R2: Second reading, A: Average

3.4 Thin Layer Chromatography

In thin layer chromatography, ret ention factor (*Rf*) value is a ratio of the distance travelled by the solute/ distance travelled by the solvent. Retention factor gives a quantitative measure of specific components and their properties in a mixture. It can help to identify components of a mixture. Results of thin layer chromatography for the 3 fungal isolates are shown in Fig. 3. The *Rf* values for P550AIa, P550AIb and P550AIc were 0.45, 0.52 and 0.40 respectively.

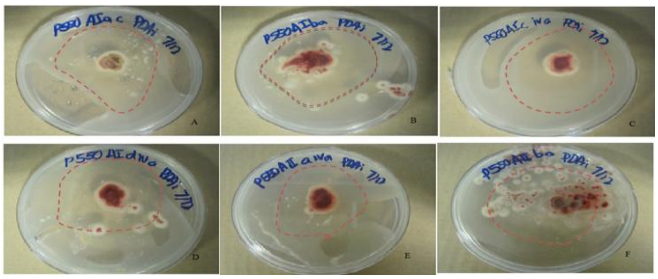

Fig.1. Zone of inhibition by positive fungal isolates on PDA plates during confirmation of secondary screening. A: P550AIa against *Listeria monocytogenes,* B: P550AIb against *Klebsiella pneumoniae,* C: P550AIc against *Klebsiella pneumoniae,* D: P550AId against *Klebsiella pneumoniae,* E: P550AIIa against *Klebsiella pneumoniae,* F: P550AIIb against *Klebsiella pneumoniae.* Dotted line circle indicate the clear zone of inhibition.

3.5 Bioautography

Bioautography is a qualitative technique for screening of antimicrobial activity of natural products and it gives an idea of presence or absence of substances with antimicrobial activity. *Listeria innocua* was added as an additional test bacterium in bioautography. The result of the bioautography is shown in Table 4 and the diagrams of results were shown in Fig. 4. P550AIa showed inhibition against *Salmonella enteritidis* and *Listeria monocytogenes*. P550AIb and P550AIc showed inhibition against *Listeria monocytogenes* and *Listeria innocua*. All the three fungal isolates did not show zone of inhibition against *Klebsiella pneumonia*e and *Escherichia coli*.

3.6 Characterization of pure fungal isolates

The 3 pure fungal isolates (P550AIa, P550AIb, P550AIc) selected from secondary screening were cultivated on different growth media (PDA, CDA, MEA) for characterization. The purpose to culture fungal isolates on different media was to

compare the growth characteristics induced by different

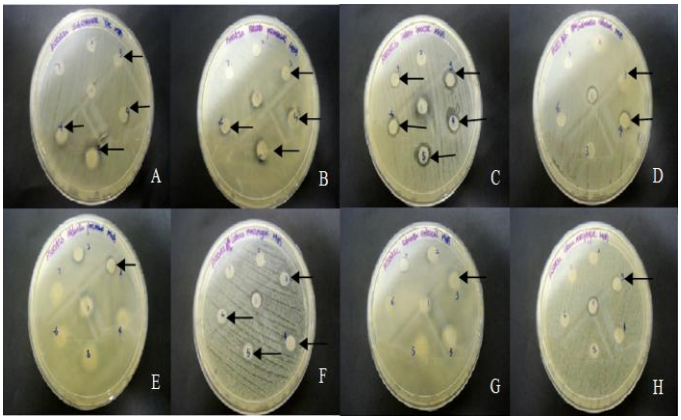

Fig 2. The second round of MIC value determination via disk diffusion method with positive control (5× dilutions of penicillin-streptomycin solution) at middle and start with negative control, concentration of 1.0mg/ml, 0.5mg/ml, 0.25mg/ml, 0.125mg/ml and 0.0625mg/ml in a clockwise position. A: P550AIa *S. enteritidis*, B: P550AIa *Klebsiella pneumoniae*, C: P550AIb *Listeria innocua*, D: P550AIb *S. enteritidis*, E: P550AIb *Klebsiella pneumoniae*. F: P550AIb *Listeria monocytogenes*, G: P550AIc *Salmonella enteritidis*, H: P550AIc *Listeria monocytogenes.* The arrow heads indicated zone of inhibition.

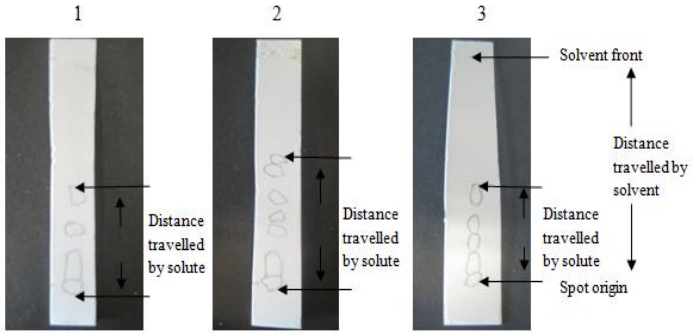

Fig.3. Thin layer chromatography when view under ultraviolet light chamber after immersed with samples solvent. 1: P550AIa, 2: P550AIb, 3: P550AIc.

Table 4. Growth inhibition diameters (mm) of crude extracts obtained by bioautography method against different test bacteria.

	EC	SE	KP	LM	LI
P550AIa	-	4	-	5	-
P550AIb	-	-	-	3	3
P550AIc	-	-	-	5	4

EC: *Escherichia coli,* SE: *Salmonella enteritidis,* KP: *Klebsiella pneumoniae,* LM: *Listeria monocytogenes,* LI: *Listeria innocua.*

culture medium. From the observation, it was revealed that different growth medium influenced the growth and physical appearances of the fungal isolates. Most of the fungal isolates cultured on CDA have a slower growth if compared to that of the same fungal isolates cultivated on PDA and MEA, respectively. It was observed that P550AIa, P550AIb and P550AIc that grown on CDA had slowest growth rates compared to others culture medium. Besides, the colour of the fungal colonies varied significantly when cultured in different growth media.

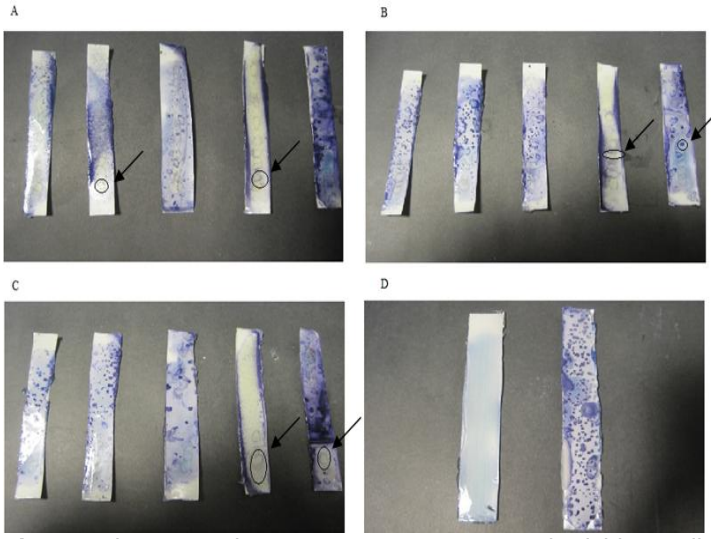

Fig.4: Bioautography. A: P550AIa - *Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, Listeria monocytogenes, Listeria innocua* (from left to right). B: P550AIb - *Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, Listeria monocytogene, Listeria innocua* (from left to right). C: P550AIc- *Escherichia coli, Salmonella enteritidis, Klebsiella pneumoniae, Listeria monocytogenes, Listeria innocua* (from left to right). D: Positive control (left), negative control (right). The circle and head arrow indicate the zone of inhibition.

3.7 Molecular identification

From the PCR amplifications of the 3 pure fungal isolates (P550AIa, P550AIb, P550AIc), only isolate P550AIb showed the formation of DNA band when run with 1.0% TBE agarose gel electrophoresis. The sequence of the amplified fragment of isolate P550AIb was compared with query sequences in NCBI GenBank by using NCBI BLAST search. From the blast analysis, P550AIb showed the highest homology (99% identity similarity) with the *Penicillium verruculosum*.

4 Discussion

Ulu Rayu and Summit Trial at Matang Wildlife Centre - Kubah National Park were chosen as the sampling site in this study based on the assumption that the surface soil samples from these undisturbed areas contain native microorganisms, which might have higher probability to produce novel antimicrobial substances that have not previously been studied by other researchers. According to Sarawak Soil Classification, soils at Matang Wildlife Centre–Kubah National Park derived from mixture of coarse sandstone intersparsed with sedimentary rocks which consist of mainly shale. Such soil corresponds to Ultisols under the USDA Soil Taxonomy [10]. Salar and Aneja [11] reported that the frequencies of fungal species isolated are corresponded with the altitude. This might be due to the higher moisture content and high organic carbon in soil samples in high altitude. Selection and isolation of soil

microorganisms were carried out by agar overlay technique. There was a slightly modification in the agar overlay technique employed for preliminary selection in this study. The incubation temperature of the plates was changed from 37^{o}C to 26^{o}C in order to ensure the survival of fungal isolates. This modification was successful and it reduced much of workload. Without modification, each fungal isolates needed to be isolated and tested individually with test bacteria before undergo further analysis and this would require a lot of time. Soil microorganisms showing inhibition zone were isolated. Sub-culturing was carried on PDA plates to obtain pure culture. The subsequent growth on the fresh media, PDA constitutes a subculture or passage. The preserved microorganism was not in a stage of established growth until it was thawed or hydrated and grown for the first time. There were 29 fungal isolates isolated during the first round of sub-culturing. During the fourth round sub-culturing for preliminary screening, a total of 24 fungal isolates were successfully isolated (Table 1). The consistence results obtained through preliminary screening from the first round until the fourth round confirmed that agar overlay technique was a good technique for preliminary selection. In fact, agar overlay technique in preliminary selection is simple to be carried out and able to produce clearer zones when compared to Kirby-Bauer method [12]. Agar overlay technique also does not demand a cautious adjustment of the turbidity during standardization of inoculums and this makes it more favourable to be employed in clinical laboratory [13]. Secondary screening was carried out to further verify the antimicrobial activities occupied by the selected microbial isolates from primary screening. In addition, secondary screening aims to select only the most potential microbes for further characterization and identification. In this study, 6 fungal isolates (P550AIa, P550AIb, P550AIc, P550AId, P550 AIIa and P550AIIb) showed strong antibacterial activities against different strains of test bacteria (Table 2). This implied that agar overlay technique could increase the efficiencies for the selection of potent microorganisms. In fact, for the fungal isolates that did not exhibit any activities against at least one of the test bacteria used, they were actually not producing the desired antibiotics but they were located within the clear inhibition zone produced by other soil microbes during primary screening. Therefore, they were selected and subjected to secondary screening. The 3 fungal isolates (P550AIa, P550AIb and P550AIc) were chosen to undergo disk diffusion test by using hexane crude extracts at various concentrations to determine MIC value. MIC value was determined through disk diffusion method instead of broth-based method because disk diffusion was a quick and simple method [14]. The disk diffusion method was faster as the interpretation can be made after 24 hours of incubation. In disk diffusion test, aliquot of test bacteria was spread onto solidified MHA instead of mixing the inoculums with melted MHA by inoculating agar before pouring [15]. During the first round of the disk diffusion test, isolate P550AIb was active against *Listeria innocua* (gram-positive bacteria) by producing inhibition zone which was larger than the positive control (5× dilutions of penicillin-streptomycin solution) while no inhibition zone was exhibited against *Escherichia coli* (gram-negative bacteria). During the second round of the disk diffusion test, P550AIb showed strong activity against *Listeria innocua* by exhibiting the clear inhibition zone in all range of concentration while no inhibition zone was exhibited against *Escherichia coli* (Table 3). The result of the hexane extract in isolate P550AIb during second round disk diffusion test was compatible with the result obtained during the first round disk diffusion test. This was further supported by the research conducted by Kambezi and Afolayan [16] and El-Mahmood [17] where hexane extracts are more active against gram-positive bacteria than gram-negative bacteria because gram-negative bacteria is more resistant to action of antibacterial agents. Thus, isolate P550AIb contained an active antimicrobial compound which can exhibit inhibition zone towards *Listeria innocua*. The hexane extracts of some isolates, such as P550AIa in first round disk diffusion test did not show inhibition zone when tested against different species of test bacteria. In addition, some results produced by the extracts of fungal isolates were not in agreement with the results for the first time and second time disk diffusion test. According to Shaaranin [18], some active biological substances did not dissolve easily in solvent. Thus, there were differences between results obtained for the first time and second time disk diffusion test. MIC value was measured by the lowest antibiotic concentration that able to cause the formation of inhibition zone around the disc which was comparable to the positive control (5×dilutions of penicillin-streptomycin solution) when tested against different test bacteria. The potential of P550AIa, P550AIb and P550AIc to be further studied was showed by small MIC value (Table 3). This result has further supported that remote area have soil microbes which are able to produce useful antimicrobial compound. Thin layer chromatography allows the fast determination of composition of complex mixtures and isolation of substances in micro amounts [19]. The separation of bioactive compound that present at hexane crude extract was well-separated by TLC plates. This can be shown through the R*f* value calculated by the distance travelled by the compound divided by distance travelled by the solvent [20]. Based on the results obtained from thin layer chromatography, P550AIc showed small R*f* value of 0.40, which indicated the pigments were absorbed strongly and were moving slowly along the silica gel plate. In contrast, P550AIb showed a high R*f* value of 0.52, which indicated the pigments were adsorbed not very strong and were moving further up through the silica gel plate [21]. All the 3 fungal isolates (P550AIa, P550AIb and P550AIc) had antimicrobial activity against at least one of the test bacteria. This activity was denoted by white spots against a red-purple background on the TLC plates after spraying with methylthiazolyltetrazolium chloride (MTT). *Listeria innocua* was added as an additional test bacterium in bioautography because it showed resistant to many type of antibiotics. As shown in Table 4, hexane crude extracts of P550AIa, P550AIb, P550AIc on TLC plates showed an inhibition zone against *Listeria monocytogenes*, whereas *Listeria innocua* was inhibited by most of the crude extracts on TLC plates. In fact, different incubation times and temperatures are important in bioautography in order to yield a better result [22]. However, there are some absences of inhibition zone on silica gel plates. According to Suleiman *et al*. [23], the absence of inhibition of microbial growth on silica gel plate was due to the evaporation of active compound, photo-oxidation or tiny amount of active compound. In addition, absence of zone of inhibition in bioautography might due to the solvent that remained on TLC plate became too acidic or too alkaline after remained for long drying time and hence inhibited possible bacterial growth [24]. Besides, synergism could also explain the absence of antimicrobial activity on separated compounds

on bioautography after determining the MIC value of the crude extract. The presence of synergism was indicated by the lower activity compared to the expected activity, after the compounds based on bioautography were isolated and characterized [23]. In fact, the bioautography method is known as a qualitative technique for the screening of antimicrobial activity of natural products and it gives an idea of presence or absence of substances with antimicrobial activity [24]. Bioautography is an efficient assay to detect antimicrobial compound present in extracts because it permits localization of activity in complex matrix and promote target-directed isolation of active constituents [25]. This method is economic and allows a greater bioassay-directed fractionation of bioactive compounds. Bioautography in combination with TLC separation is unique and greatly utilised in research on antibiotic substances [22]. Based on microscopic examinations in this study, the conidiospores branching for fungal isolates P550AIa, P550AIb and P550AIc were predominantly biverticillate. According to Barnett and Hunter [26], the conidiophores of *Penicillium* end in phialides. *Penicillium* has a long dry chain of conidia [27]. The characteristics of the spores produced by these 3 fungal isolates were matched with those described by Güçlü *et al.* [28] for *Penicillium* spp. where fungal colonies which used PDA as culture medium showed conidia green, subglobose and smooth. In addition, the colour of these fungal isolates cultivated on CDA were mostly compatible with the colour of *Penicillium* colonies, which includes white, shade of green, yellow-brown and yellow aerial mycelium. According to Samson and van Reenen-Hoekstra [27], cultivation of fungal isolates on CDA is appropriate for identification. Culture medium, growth condition and culture maturity are factors that can affect the colour of spore mass and there can be none of the colour match with the established groups [29]. This condition can cause a problem in the process of determination of fungal isolates. Different culture medium could result in different growth morphology for the same isolates due to the different components and nutrients in the culture medium which can induce different growth of the fungal isolate. Optimal culture medium is essential as it supplies the essential nutrients to the fungi for their growth condition, allowing the fungi to exhibit their phenotypic characteristics. Besides, type of medium used has direct connection with the growth rate of fungi [30]. The characteristics of the spores produced by the fungal isolates (P550 AIa, P550 AIb and P550 AIc) were matched with description for *Penicillium* spp. Therefore, these 3 fungal isolates were tentatively identified under genus *Penicillium* but with different species. DNA extraction from filamentous fungi is difficult due to its high polysaccharide contents. Genomic DNA of P550AIb was successfully extracted from full plate culture using CTAB method as described by Scott and Arnold [31] with modifications. Initially, grinding the cells frozen by liquid nitrogen enabled the disruption of the cell wall in order to release the cellular constituents into the extraction buffer. Chloroform/isoamyl extraction was performed in water-bath for 30 minutes to remove the increased amount of protein [32]. Addition of CTAB/NaCl solution ensured the protein in the cell was not separated from the rest of the solution with the DNA. NaCl solution kept all molecules in good condition and prevented unwanted aggregation. In fact, CTAB is often considered as the standard method in fungal DNA extraction and therefore is more preferable [32]. CTAB method is indeed cheaper than the cost of using commercial kits, which will increase the cost of laboratory tests. The amplification of 18S rRNA with ITS1 and ITS4 primers has been successfully performed and 18S rRNA gene was chosen as a target for PCR amplification because the sequence data is widely used in molecular analysis to reconstruct the evolutionary history of organisms. Through the 18S rRNA gene sequence analysis, isolate P550AIb was identified as *Penicillium verruculosum* with 99% identity similarity. A study conducted by Peyronel [33] showed that *Penicillium verruculosum* have colonies velutinous or floccose to funiculose, mycelium white to bright yellow and the conidial mass green. In addition, *Penicillium verruculosum* can grow well at 37°C. Besides, a study conducted by Bhagobaty and Joshi [34] showed that under in-vitro conditions, culture broth of fungal endophyte, *Penicillium verruculosum* RS7PF associated with roots of *Potentilla fulgens L.* showed the ability to promote seed germination in *Vigna radiata* (Green gram) and *Cicer arietinum* (Chick pea). *Penicillium verruculosum* is able to produce Indole Acetic Acid (I.A.A) with its own machinery which ultimately promotes seed germination. This is further supported by [35] that analysis of unique fungus yield from isolation of *Potentilla fulgens L.* by using fungal specific 18S rRNA primers showed that this unique fungal shared a 98% homology with *Penicillium verruculosum* (Genbank accession number AF510496). This indicates that this unique fungus maybe is a new endophytic strain of *Penicillium verruculosum.* It was examined to have a role in establishment of seedlings and micropropagation of plant.

5 Conclusion

From this study, the three antibiotic-producing fungal isolates (P550AIa, P550AIb and P550AIc) were tentatively identified as *Penicillium* spp. and isolate P550AIb was further identified as *Penicillium verruculosum* after being sequenced. In conclusion, there is high potential to discover useful antibiotics producing soil fungi from the study site with some possibly novel strains that have not yet been studied by other researchers.

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