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Antimicrobial producing microbes isolated from soil samples collected from Nanga Merit Forest in Sarawak, Malaysian Borneo

***Samuel Lihan, Chai Sin Lin, Ismail Ahmad, Fazia Mohamad Sinang, Ng Kok Hua and Awang Ahmad Sallehin**

Department of Molecular Biology, Faculty of Resource Science and Technology, Universiti Malaysia Sarawak, Kota Samarahan, Sarawak, Malaysia

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ABSTRACT

This study was carried out to screen for potential antimicrobial producing microbes from soil samples collected from Nanga Merit forest. After primary and secondary screening, one bacterial isolate (B17) and four fungal isolates (F10, F11, F14 and F26) produced antimicrobial activity against Staphylococcus aureus, Salmonella typhi, Escherichia coli and Enterobacter aerogenes. The minimal inhibition concentration (MIC) of the methanol extract from isolate B17 against the four tested bacteria was determined by disk-diffusion and agar well diffusion methods. The MIC values when tested against S. aureus, S. typhi, and E. coli were 25µg/µl whereas the MIC value was 12.5µg/µl when tested against E. aerogenes. In the agar well diffusion method, the respective MIC values were < 1.5625µg/µl for S. aureus, 12.5 µg/µl for both theS. typhi and E. coli, and 9.375 µg/µl for E. aerogenes. Besides, extracts from F26 cultivated on PDA, CDA, and V8 juice agar showed weak activity against E. aerogenes and was not tested further for its MIC values. The extract from B17 also exhibit antifungal activity when tested against test fungus (Fusarium sp.*). Fungal isolates (F10, F11, F14 and F26) cultivated on PDA exhibited great antifungal activities.*

Keywords: Soil microbes, antibacterial and antifungal activity, MIC values

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INTRODUCTION

Antimicrobial agents play the most important role in the treatment of bacterial infections [1] and wide spread efforts have been carried out by many scientists in order to screen for novel antibiotic producing microbes [2]. Through their efforts, many antibiotics have been discovered successfully to combat pathogenic bacteria that cause diseases. Nevertheless, the emergence of new diseases and reemergence of multiple-antibiotic resistance pathogens that render the effectiveness of existence clinically used antibiotics have spurred the needs for the discovery of new antibiotics [3].

Antibiotics can be classified according to their mode of actions. Antibiotics are classified as broad-spectrum antibiotics when they have the ability to affect a wide range of gram-positive and gram-negative bacteria while antibiotics that only effective towards certain group of bacteria are known as narrow-spectrum antibiotics. Several mechanisms of actions of antibiotics have been discovered by scientists. These actions include the inhibition of cell wall, protein and nucleic acids synthesis [4, 5, 6].

There are so many different potential sources where antibiotics can be discovered, such as medicinal herbs [7, 8, 9] and soil. However, soil, which is a naturally occurring loose mixture of mineral and organic particles [10], still

remains the most important target for most researchers in their efforts to discover novel antibiotics which have pharmaceutical values. This is because many microbes especially bacteria that reside in soil have the ability to produce biologically active secondary metabolites such as useful antibiotics. In their natural habitats, bacteria utilize the antibiotics they produce as protective substances by rendering the invasion of other bacterial species. Protection is not the only function of antibiotics. Hence, according to Linares *et al*. [11], antibiotics also act as signaling molecules that bacteria use as a means of communication between cells.

The number of multi-drug resistance pathogenic microbes has increased over time and there are only limited therapeutic drugs that are applicable to combat these pathogens [3, 12]. Therefore, there is a need for continuous discoveries of new antibiotics in order to make treatments under antibiotics remain effective [3, 13]. This study is an attempt to discover novel antimicrobial agents from soil samples from remote area in Sarawak, Malaysian Borneo.

MATERIALS AND METHODS

Soil Sampling, Preparation and Plating

Soil samples were collected from Nanga Merit forest (Site 2). Approximately 1g of the soil samples was dissolved in 9ml of sterile Phosphate Buffer Saline (PBS) buffer (pH 7.0) to make soil suspension. The supernatant from the soil solution was pipette and spread over Nutrient Agar (NA) (Merck, USA) and Potato Dextrose Agar (PDA) (Oxoid, England) plates. The plates were left at room temperature for 5 days for the bacteria and fungus to grow. After 5 days, the number of bacterial fungal colonies was calculated and recorded. The plates were kept at 4^{o}C for 2 days to delay the growth of soil microorganisms.

Test Bacteria

The test bacteria used in this study were obtained from the stock culture at Department of Molecular Biology, UNIMAS. The test bacteria include *Staphylococcus aureus*, *Escherichia coli*, *Enterobacter aerogenes*, and *Salmonella typhi*.

Preliminary Selection and Isolation of Soil Microorganisms

The soil microorganisms grown on NA and PDA plates were overlaid by a layer of soft NA (0.75%) that contained test bacteria. Empty plates of NA and PDA overlaid with the test bacteria were used as controls in this study. All the plates were kept at room temperature and the formation of inhibition zone was observed for every 24 and 48 hours. The soil microorganisms that inhibited the growth of test bacteria by producing inhibition zone were selected and isolated in order to obtain pure strain of that particular microorganism. The pure strains of microorganisms were stored as stock culture in slant NA at 4^{o}C for further usage.

Secondary Screening for Bacterial Isolates via Spot Inoculation Method

NA plate was spot inoculated with pure bacterial isolates and incubated at 26^{o}C for 4 days. After that, 2ml of soft NA (0.75%) inoculated with test bacteria were overlaid onto the NA plate containing the pure bacterial isolates. Empty plates of NA overlaid with soft NA containing the test bacteria were used as controls. All the plates were incubated at 26^{o}C and zone of inhibition was observed after 24 hours of incubation period.

Secondary Screening for Fungal Isolates via Agar Overlay Technique

Pure fungal isolates obtained from the preliminary selection were cultivated on two types of media, Czapek Dox Agar (CDA) (Merck, USA) and V8 Juice Agar [14]. After incubated at room temperature for 4 days, these fungal isolates which grown on both media were subjected to secondary screening via agar overlay technique. Soft NA (0.75%) seeded with test bacteria were overlaid onto the CDA and V8 Juice Agar plates containing the pure fungal isolates. Empty CDA and V8 Juice Agar plates overlaid with inoculated soft agar were used as controls. All the plates were incubated at room temperature and zone of inhibition was observed after 24 hours of incubation period.

Extraction of Secondary Metabolites from Pure Isolates

Bacterial and fungal isolates that have shown great antibacterial activities during the secondary screening were selected and cultivated on different culture media (NA, PDA, CDA and V8 Juice Agar). Each of the agar plates that contained the selected bacterial and fungal isolates was dried in a fume hood and then grinded before immersed with methanol 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 usage.

Antibiotics Susceptibility Testing with Methanol Crude Extracts

Two trials were carried out during the antibiotics susceptibility testing with the extracted secondary metabolites from selected bacterial and fungal isolates.

Twenty extracts were subjected to first trial in the antibiotics susceptibility testing. Exactly 1mg of the dried crude extract was weighed and dissolved in 100µl of 100% methanol and 900µl of sterile PBS (pH 7.0). The extract was then diluted to the concentration of 0.5mg/ml, 0.25mg/ml, 0.125mg/ml, and 0.0625mg/ml, respectively. The antibiotics susceptibility testing was carried out based on the disk-diffusion method described by Bopp *et al*. [15].

In second trial, stock solution for all the methanol crude extracts from selected fungal and bacterial isolates was prepared based on the amount of extracts available in order to give the highest concentration. Every extract was weighted and dissolved in 100 µl of 100% methanol and 900µl of sterile PBS (pH 7.0). Antibacterial test was carried out via agar well diffusion method described by Bennett *et al*. [16] with slightly modification.

Determination of Minimum Inhibitory Concentration (MIC) value

Extract showing the ability to inhibit the growth of test bacteria during the antibiotics susceptibility testing was selected for the determination of MIC value. Determination of MIC value was carried based on two methods, which were the disk-diffusion method and agar well diffusion method. From the stock solution, the extract was further diluted to the concentration of 50mg/ml, 25mg/ml, 12.5mg/ml, 6.25mg/ml, 3.125mg/ml, and 1.5625mg/ml (Table 3).

Antifungal Test by Using Crude Extract from Selected Bacterial Isolates

Test fungus (*Fusarium sp.*) was cultured at four corners on PDA. After incubation at 26^{o}C for 5 days, 50µl of 100mg/ml extract was pipette into the well on the center of the plate. The plate was incubated at 26^{o}C for 5 days and the formation of inhibition zone was observed.

Antifungal Testing by Using Selected Pure Fungal Isolates

Selected pure fungal isolates were cultivated at four corners on PDA at 26^{o}C for 2 days. After that, test fungus (*Fusarium sp.*) was cultured on the center of the PDA surrounded by the other four pure fungal isolates. The plates were incubated at 26^{o}C for 1 week. The growth behavior for both the fungal isolates and test fungus were observed.

Characterization of Pure Antibiotics Producing Bacterial Isolates

Gram-staining was carried out for pure bacterial isolates selected for characterization. Next, five biochemical tests, which including the oxidase test, Simmons citrate test, Methyl Red (MR), Voges-Proskauer (VP), and Sulfide Indole Motility (SIM) test were performed for the identification of pure bacterial isolate. The growth characteristics on NA for the pure bacterial isolates which including the forms, colours, margins elevations of the bacterial colonies, and also the cell shapes and arrangements under light compound microscope were observed and recorded. The identification of the bacterial isolates was based on Bergey's Manual of Determinative Bacteriology [17].

Characterization of Pure Antibiotics Producing 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 different media (PDA, V8 Juice Agar and CDA). The different growth morphologies which include the top colour, reverse colour, perimeter, mycelium mat, mycelium end, and medium colour changes were observed and recorded. The identification of these fungal isolates was based on the Bergey's Manual of Determinative Bacteriology [17]; Bergey's Manual of Systematic Bacteriology [18]; Illustrated Genera of Imperfect Fungi [16]; and Introduction to Food-Borne Fungi [19].

RESULTS AND DISCUSSION

Soil samples from remote area (Nanga Merit Forest, site 2) were chosen as samples for analysis in this study based on the likelihood of the presence of native microorganisms that have not previously been studied by other researchers. These native soil microorganisms have higher probability to produce novel antimicrobial substances. In this study, a total of 30 bacterial and 38 fungal isolates from different depths were successfully isolated via preliminary screening and selection. Among these isolates, only 23 bacterial isolates and 26 fungal isolates were selected to undergo secondary screening. The reduction in the isolates number was because some of the isolates were appeared to be the same species based on their physical appearances and growth morphologies.

From this study, it was noticed that the number of microbes that could be obtained were decreasing with the depth of the soil. This indicated that the depth of the soil and its organics constituents did influence the population and distribution of soil microbes [20, 21]. According to Sewell [22], bacteria can be found most abundantly in the first three inches from the soil surface with the number decreasing and only few amount of bacteria presence when the depth reached six feet. Besides, the results obtained from this study has shown to be similar with the research conducted by Tangjang *et al*. [23] where they found out that there was greater amounts of bacterial and fungal populations in the top soil (0-10 cm) if compared to that of other depths. This might be due to the higher organics

contents found in the top soil [23] where humus is abundantly presence, especially for the forest floor that is often covered by wilted leaves that tend to decompose.

Table 1: Antibacterial activities shown by different pure bacterial isolates

Codes	Test Bacteria[a]			
	ST	SA	EC	EA
B1	-	-	-	-
B2	-	+	-	-
B3	-	+	-	-
B4	-	-	-	-
B5	-	+	-	-
B6	-	-	-	-
B7	-	-	-	-
B8	-	-	-	-
B9	-	-	-	-
B10	-	-	-	-
B11	-	-	-	-
B12	-	+	-	-
B13	-	+	-	-
B14	-	+	-	-
B15	-	-	-	-
B16	-	-	-	-
B17	++	+++	+++	+++
B18	-	-	-	-
B19	-	-	-	-
B20	-	-	-	-
B21	-	-	-	-
B22	-	-	-	-
B23	-	-	-	-

[a]Test bacterial species: ST: Salmonella typhi; SA: Staphylococcus aureus; EC: Escherichia coli; EA: Enterobacter aerogenes. [b]Zone of inhibition: +++ Very strong activity; ++ Strong activity; +: Weak activity; -: No activity.

Table 2: Antibacterial activities shown by fungal isolates cultivated on V8 Juice Agar and CDA, respectively

Codes	V8 Juice Agar				CDA			
	Test Bacteria[a]				Test Bacteria[a]			
	ST	SA	EC	EA	ST	SA	EC	EA
F1	-	-	-	+	+	-	+	+
F2	-	-	-	+	-	-	+	+
F3	-	-	-	+	-	+	-	+
F4	-	-	-	+	+	+	+	+
F5	-	-	-	+	-	-	-	+
F6	-	-	-	-	-	-	-	-
F7	-	-	-	-	-	-	-	-
F8	-	-	-	-	-	-	-	-
F9	-	-	-	-	-	-	-	-
F10	+++	+++	+++	++	+	+	+	+
F11	-	+++	+++	+++	+	+	++	+
F12	+	-	-	-	+	-	++	-
F13	-	-	-	-	-	-	-	-
F14	-	+++	+++	+++	-	+	+	+
F15	-	-	-	-	-	-	-	-
F16	-	-	-	-	-	-	-	-
F17	-	++	-	++	-	++	+	++
F18	-	-	-	+	-	-	+	+
F19	-	-	-	+	+	+	+	++
F20	-	-	+	+	-	-	+	+
F21	-	+	-	+	-	+	-	+
F22	-	++	-	++	-	+	-	+
F23	-	-	-	-	-	-	-	-
F24	-	+	-	+	-	-	-	+
F25	-	-	-	-	-	-	+	-
F26	+	++	+++	+++	++	+	+++	+++

[a]Test bacterial species: ST, Salmonella typhi; SA, Staphylococcus aureus; EC, Escherichia coli; EA, Enterobacter aerogenes. [b]Zone of inhibition: +++ Very strong activity; ++ Strong activity; +: Weak activity; -: No activity.

Table 1 shows the bacteria codes and their activities against test bacteria used in this study. Among the 23 pure bacterial isolates that were subjected to secondary screening, only one bacterium isolate (B17) showed very strong activities against the 4 species of test bacteria used in this study (Table 1) and thus B17 was selected for further

antibiotics screening test. On the other hand, there were 4 fungal isolates (F10, F11, F14 and F26) cultivated on V8 Juice Agar and 1 fungal isolate (F26) cultivated on CDA exhibited very strong activities against at least 3 species of test bacteria used in this study (Table 2). Hence, these 5 fungal isolates were subjected to further antimicrobial test. Besides, some synergistic effects could also be seen where clear zone existed between some fungal isolates cultured on CDA when tested against some of test bacteria which they ordinarily cannot inhibit that particular test bacterium by their own. The isolates showing synergistic effects were the combination of F3, F17, F20 and F21 was active against *S. aureus*, *E. coli* and *E. aerogenes*; combination of F2, F5 and F18 was active against *E. coli* and *E. aerogenes*; and combination of F5 and F18 was active against *S. aureus*.

In antibiotics susceptibility testing with methanol crude extracts, only the extract from B17 and its first dilution exhibited the ability to inhibit the growth of the 4 species of test bacteria used in this study. This result was in parallel with the result obtained during secondary screening, where the isolate exhibited strong anti-bacterial activities. Extract from this isolate was active against *S. aureus* (gram-positive bacterium). Besides, this isolates exhibit similar result when tested against the *E. coli* and *S. typhi* whereby the size of inhibition zone was the same. If compared to the activity on different species of test bacteria, isolate B17 possessed greater ability to inhibit the growth of gram-positive bacteria rather than gram-negative bacteria.

In this study, extracts from other fungal isolates were shown to give negative results when tested against different species of test bacteria except for isolate F26 which was extracted from three different agar (PDA, CDA, and V8 Juice agar). These extracts could only inhibit one strain of test bacteria, *E. aerogenes*. Dilutions for the extracts of F26 obtained from PDA and CDA showed that the later possessed the ability to retard the growth of *E. aerogenes* while the former could not. The result also revealed that extracts from V8 Juice agar possessed greater activity when tested against *E. aerogenes* if compared to that of CDA. This might suggest that different culture medium can affect the ability of fungal isolate to produce active biological substances which have antimicrobial activity.

Table 3: Antibacterial activity of various concentration of methanol extract of B17

Concentration of extract		100[d]	50	25	12.5	6.25	3.125	1.5625	+[b]	-[c]	MIC value (mg/ml)
[a]Test bacteria		Zone of inhibition (mm)									
(i) Disk-diffusion method (10µl/disk)	SA	16	15	12	6	6	6	6	15	6	25
	ST	10	9	8	6	6	6	6	20	6	25
	EC	10	9	8	6	6	6	6	17	6	25
	EA	15	13	11	8	6	6	6	26	6	12.5
(ii) Agar well diffusion method (30µl/well)	SA	25	22	20	17	15	14	12	20	6	<1.5625
	ST	15	13	11	8	5	5	5	25	6	12.5
	EC	15	13	11	8	5	5	5	23	6	12.5
	EA	14	13	12	12	11	8	5	26	6	9.375

[a]Test bacteria species: ST: Salmonella typhi; SA: Staphylococcus aureus; EC: Escherichia coli; EA: Enterobacter aerogenes. [b]Positive control: (5×dilution of penicillin-streptomycin). [c]Negative control (10% methanol solvent). [d]: mg/ml.

Crude extract from isolate B17 was chosen for the determination of MIC value (Table 3) by using both the disk-diffusion and agar well diffusion methods (Figure 1). It was observed that the crude extract of B17 was most effective against *S. aureus* (gram-positive bacteria) if compared to that of others gram-negative bacteria. Additionally, the effectiveness for the crude extract obtained from B17 to inhibit the test bacteria *S. typhi* and *E. coli* were the same for both of the disk-diffusion and agar well diffusion methods used (Table 3). The effectiveness of the crude extracts of B17 against different species of test bacteria was in the order of: *S. aureus* > *E. aerogenes* > *S. typhi*, *E. coli*. In fact, MIC value can be influenced by several factors such as the aeration, period of incubation, type of culture medium, nature of test microorganisms, and the size of inoculums. Therefore, standardization is important in carrying out the test. Besides, extract from isolate should be pure enough in order to fully characterize the activity of an antibiotic [24].

In addition, it was observed that besides anti-bacterial properties, B17 also exhibited the capabilities to inhibit the growth of test fungus (*Fusarium* sp.) used in this study. This indicated that B17 might have great potential to become an effective anti-fungal agent besides as an anti-bacterial agent that could be applied in agricultural industries, husbandry and medical fields. However, more species of test fungi were needed in this testing in order to prove the anti-fungal properties exhibited by B17. On the other hand, 4 fungal isolates (F10, F11, F14 and F26) that were selected for the antifungal test showed that all these 4 fungal isolates exhibited the capabilities to inhibit the growth of the test fungal (*Fusarium* sp.).

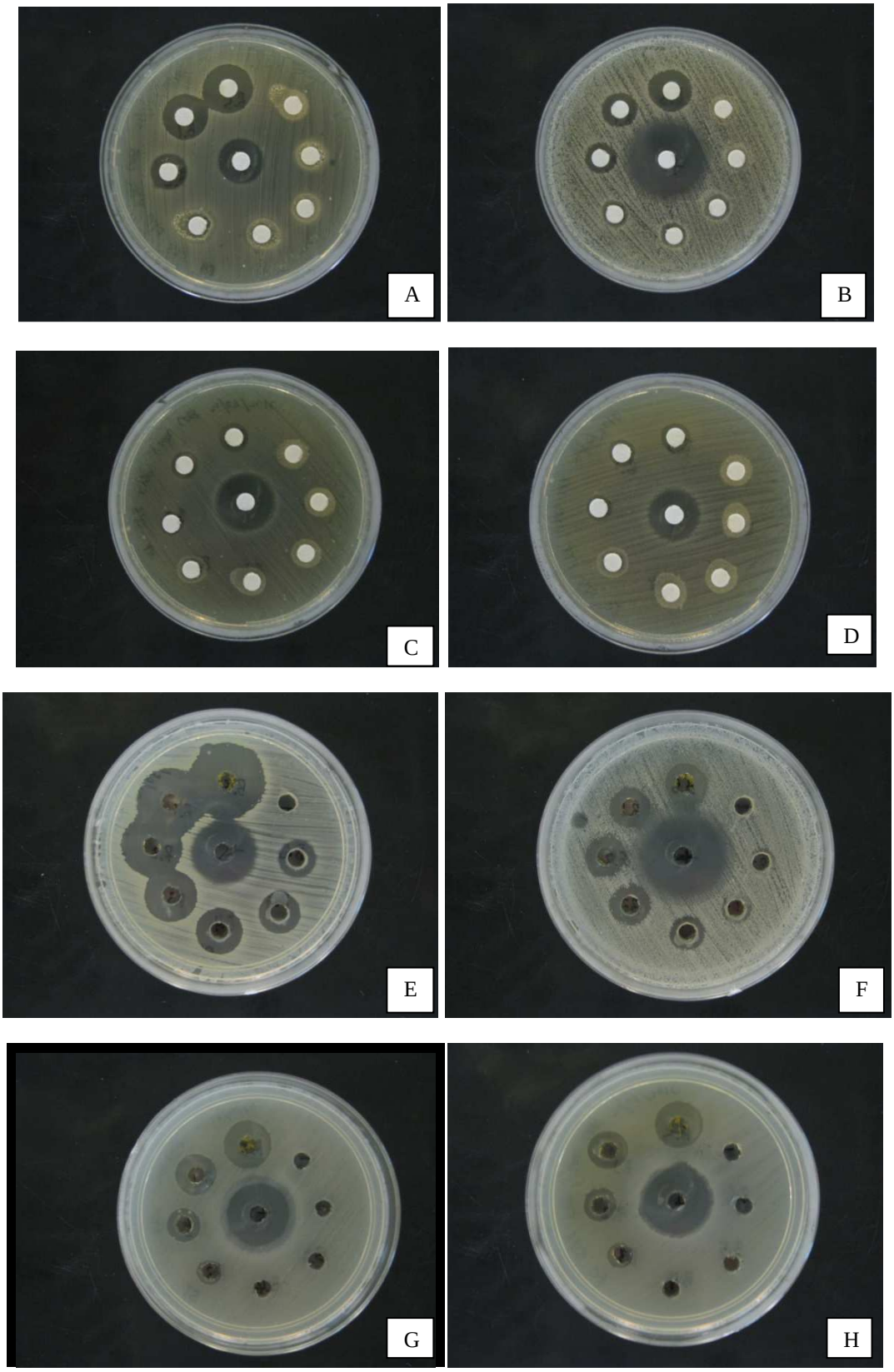

Figure 1: Determination of MIC value via disk-diffusion method (plate A, B, C, D) or agar well diffusion method (plate E, F, G, H) with positive control (5×dilution of penicillin-streptomycin) at the middle and start with the concentration of 100mg/ml, 50 mg/ml, 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml, 1.5625 mg/ml, and negative control (10% methanol) in a counterclockwise position. Plate A and E (*S. aureus)*; B and F (*E. aerogenes)*; C and G (*S. typhi*) and D and H (*E. coli)*.

Table 4. Morphology and biochemical characterisation of bacterial isolate, B17

Gram staining		-
Physical characteristics	Forms	Circular
	Colour of colonies on NA	Yellow
	Margins	Entire
	Elevation	Low Convex
	Shape	Coccus
	Arrangement	Cluster in chain
Biochemical tests	Indole	-
	Methyl Red (MR)	-
	Voges-Proskauer (VP)	+
	Citrate utilisation	+
	Oxidase test	-
	Hydrogen sulfide (H_2S)	-
	Motility	-

[a]+: Positive result; [b]-: Negative result

For the characterization of pure antibiotic producing isolates, B17 was gram-negative cocci in which its genus remained unidentified after referring to 9th edition of Bergey's Manual of Determinative Bacteriology. This was because none of the genus described in the *Manual* matched exactly with the characteristics of B17 stated. However, there was a possibility that this bacterial isolate might be classified under the Genus *Phenylobacterium*. This is because all the results for biochemical tests (Table 4) and most of the characteristics described were fitted to the descriptions for this genus as described in the *Manual* except for the positive result of VP test in this study that was different from the negative result as described in the *Manual*. To eliminate this possibility, repetitions need to be performed in order to confirm that there were no false positive results obtained for the biochemical tests. Besides, extra biochemical tests need to be performed for the ease of identification for this bacterial isolate.

In this study, 11 fungal isolates were selected for characterization. Out of the 11 fungal isolates characterized, 7 isolates (F2, F3, F5, F17, F18, F20 and F21) were proposed to be classified under the genus *Penicillium* sp., whereas another four isolates (F10, F11, F14 and F26) were proposed to be classified under the genus *Streptomyces* sp. based on their macroscopic and microscopic examinations.

In conclusion, it was found out that there is high potential to discover useful antibiotics producing microbes in Nanga Merit Forest with some possibly novel strains that have not yet been studied by other researchers. Therefore, more research works can be conducted on this remote area in order to explore novel antibiotics and their producing microbes which are residing in it.

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