Screening for Antimicrobial Activities in Soil Fungal Isolated from Kubah National Park

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(25437)

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List of Abbreviations

CDA  Czapek Dox Agar
°C  Degree Celsius
%  Percent
µg  micro gram
µl  micro liter
FRST  Faculty of Resource Science and Technology
g  Gram
MEA  Malt Extract Agar
MHA  Mueller-Hinton Agar
MHB  Mueller-Hinton Broth
MIC  Minimal Inhibitory Concentration
mg  Milligram
ml  milliliter
MRSA  Methicillin-Resistant *Staphylococcus aureus*

nm  Nanometer
PBS  Phosphate Buffer Saline
PDA  Potato Dextrose Agar

pH  A measurement of acidity or alkalinity of solution [ p stands for “potenz” which means the potential to be while H stands for Hydrogen]
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Screening for Antimicrobial Activities in Soil Fungal Isolated from Kubah National Park

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ABSTRACT

A study was carried out on soil samples collected from Kubah National Park in order to discover novel antibiotics produced by soil microbes. Twenty one samples of soil were analyzed for antimicrobial producing microbes. The test bacteria used in this study were *Escherichia coli*, *Listeria monocytogenes*, *Salmonella enteritidis* and *Klebsiella pneumoniae*. Six fungal isolates (P550 Aⅰa, P550 Aⅰb, P550 Aⅰc, P550 Aⅰd, P550 Aⅱa, P550 Aⅱb) showed strong antibacterial activity against different strains of test bacteria during antibiotic activity screening using agar overlay techniques in the preliminary screening followed by secondary screening. A total of 3 fungal isolates (P550Aⅰa, P550Aⅰb, P550Aⅰc) were selected to undergo antibiotics susceptibility testing and characterization. These 3 fungal isolates showed MIC value and produced inhibition zone compared to the positive control (5× dilutions of penicillin-streptomycin solution). The 3 fungal isolates were subjected to thin layer chromatography and bioautography to characterize their antimicrobial active compound. Three fungal isolates were subjected to Polymerase Chain Reaction (PCR) and gel electrophoresis, however only P550Aⅰb showed band on gel electrophoresis. Upon identification, P550Aⅰb was identified as *Penicillium verruculosum* after being sequenced. All the fungal isolates could be used to produce antimicrobial compound.

Key words: soil, fungal, antibacterial activity, identification

ABSTRAK


Kata kunci: tanah, kulat, aktiviti antibakteria, identifikasi
CHAPTER 1
INTRODUCTION

The term “antibiotic” literally means “against life”. Antibiotics are also known as antibacterial agent. Antibiotics are drugs used to treat infections caused by bacteria that can cause illness to humans and animals. Antibiotic functions to inhibit or destroy the bacterial cells which cause certain disease (Duerden et al., 1993). Antibiotics are secondary metabolites produced by bacteria (Demain, 2000) 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 (1983), only those that have an effect on bacterial cells but not the host cells like human are categorized as useful antibiotics. Nowadays, over 100 different antibiotics are available to cure minor and life-threatening infections.

Antibiotic resistance occurs when effectiveness of drugs, chemical are reduced (Bisht et al., 2009). Scientists are continuously search for novel antibiotic producing microbes because drug resistant strains of pathogen emerge more quickly than the rate of discovery of new drugs and antibiotics (Kumar et al., 2010). Consequently, a numbers of antibiotics that can fight against pathogenic bacteria had been discovered. According to Roberts (1998), there is important to discover new antibiotics as 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 of discovery of novel antibiotics among various sources of antibiotics. According to Dulmage and Rivas (1978), soil microorganisms have
continuously been screened for their useful biological active metabolites such as antibiotics since long ago. The soil samples from Kubah National Park which is undisturbed area were chosen to discover novel antibiotics due to the high probability of isolating novel antibiotics producing soil microorganisms.

There were three main objectives of this study:

i) To determine antimicrobial properties of soil microbes towards test microorganisms.

ii) To isolate and characterize the antimicrobial producing soil microbes.

iii) To determine the components of the isolated antimicrobials.
CHAPTER 2

LITERATURE REVIEW

2.1 Pathogenic microorganisms

Pathogenic microorganisms are microorganisms that are harmless, harmful or beneficial. It can be bacteria, fungi, viruses and protozoan. Those microorganisms that have pathogenic characteristics can cause infections and the capabilities of pathogens to cause infections differ in some degrees (Gilbert, 1977). Pathogenic microorganisms can be found outside the body on the skin surface and mucosal surface or inside the body in the blood and tissues. According to Duerden et al. (1993), pathogens which infect human may come from either exogenous or endogenous sources. Infection transmission can be through direct contact transmission, indirect contact transmission and vector-borne diseases.

2.2 Multiple-drug resistant pathogenic microorganisms

Antibiotics are mostly prescribed in modern medicine for treatment and prevention of infectious diseases caused by pathogenic microorganism. Increasing use and misuse of existing antibiotics in human and veterinary medicine has developed antibiotics resistance pathogens (Todar, 2008). Antibiotics resistant occur when antibiotics are no longer work against disease-causing bacteria. This is due to exchange of genetic materials between bacterial cells or bacterial chromosome that caused development of antibiotics resistant strains (Cooke and Gibson, 1983; Roberts, 1998; Cirz et al., 2005). Examples of multiple-drug resistant pathogenic microorganisms are methicillin Staphylococcus aureus (MRSA), multi-drug resistant Streptococcus pneumoniae and multiple-drug resistant (MDR) enterococci (Hart, 1998; Hukcke et al., Xu et al.,
Besides bacteria, *Candida albicans* which is a fungal contains characteristics of resistance to different antimicrobial drugs has been studied by the researchers (Gulshan and Rowley, 2007).

Over the past 20 years, antibiotic resistance has increased in virtually every species of bacteria examined and there are only limited therapeutic drugs that can be used for treatment to these pathogens (Roberts, 1998; Demain and Sanches, 2009). Hence, it is important for continuous discoveries of new antibiotics in order to make treatment under antibiotics remain effective (Roberts, 1998; van der Waaij *et al.*, 2000).

### 2.3 Antibiotics

According to Linares *et al.* (2006), antibiotics also act as signaling molecules that bacteria use as a means of communication between cells. Chloramphenicol was an early example. Bacteria utilize antibiotics that being produced as protective substances in natural habitat through preventing invasion of other bacterial species. Function of antibiotics not merely on protection but also have others function as well.

Antibiotics are grouped as broad-spectrum antibiotics and narrow-spectrum antibiotics. A broad-spectrum antibiotic able affects 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 antibodies. Several mechanisms of actions of antibiotics have been discovered by scientists, these actions include the inhibition of cell wall, nucleic acids and protein synthesis (Lambert, 1977; Brooks *et al.*, 2001; Tortora *et al.*, 2007). The production of antibodies can be divided into three main groups of microorganisms which are Gram-positive rod shape bacteria such as *Bacillus*, actinomycetes and fungi such as *Cephalosporium* and *Penicillium* (Tortora
et al., 2007). *Penicillium* fungi are versatile and opportunistic. The examples of penicillin for antibiotics that had been identified and presently in use under genus *Penicillium* are *Penicillium notatum* and *Penicillium chrysogenum*.

### 2.4 Sources of natural occurring antibiotics

According to von Bubnoff (2006), scientists are continually searching for novel antibodies producing microorganisms from extraordinary places such as higher plants, animals, marine microbes, deep sea mud and seaweeds. Anticancer agent paclitaxel (Taxol) from the yew tree and the antimalarial agent artemisinin from *Artemisia annua* are clinically useful drugs that had been isolated from plants (Prasad et al., 2010). Besides, animal also can be used to search for novel antibiotics. Bacteria and other microorganism can live in close association with higher organism in the marine environment. Epibatidine, which is a series of antibiotic peptides were obtained from the skin extracts of the Ecuadorian poison frog (Gomes et al., 2007). It is a potent analgesic compound. In addition, study showed that lichens have high potential to produce beneficial antibiotics (Burkholder et al., 1944).

There are many sources where antibiotics can be discovered. However, soil still is the main target for researchers because soil contains the most diverse population of bacteria in the environment and the number of unique microbial niches is available since there is large diversity of soil types. Most soil microbe population have not been studied. Another reason is there are many microbes especially bacteria that reside in soil have capability to produce biologically active secondary metabolites such as useful antibiotics.
2.5 Antibiotic producing soil microbes

Most antibiotics producing microbes and their secondary metabolites can be found in soil. *Penicillium* derived from word ‘brush’ which refers to appearance of spores in *Penicillium*. Genus *Penicillium* which is sexual phase falls into order Eurotiales while genus of blue or green mold fungi that exists as asexual are referred as deuteromycetes. *Penicillium* is dominant in cooler conditions; particularly the cool temperate zone and it can be found in soil, decaying vegetations and seed (Pitt, 1994).

*Penicillium* is filamentous fungi which have round and unicellular conidiospores. Glucans are commonly present in cell wall of *Penicillium* species. The small hyphae in *Penicillium* can lead in difficulty for detection of protoplasmic movement and smaller peripheral growth zones. *Penicillium* are osmotolerant and heterotrophic.

*Penicillium* is important in antibiotics productions for example, penicillins that produced by *Penicillium chrysogenum* and other species may produce useful antibiotics such as griseofulvin, mycophenolic acid (Hamzah et al., 2009). A study conducted by Bhagobaty and Joshi (2009) 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.
CHAPTER 3

MATERIALS AND METHODS

3.1 Sources of materials

3.1.1 Soil samples

Soil samples were collected by Department of Plant Science and Environmental Ecology from Kubah National Park and the soil samples were put into plastic bag under aseptic condition. The soil samples were labeled specifically and kept in Laboratory.

3.1.2 Test microorganisms

Test bacteria were revived from Microbiology Laboratory, UNIMAS and used for preliminary testing. Two types of test bacteria were used, which were gram positive (Listeria monocytogenes) and gram negative bacteria (Escherichia coli, Salmonella enteritidis and Klebsiella pneumoniae).

3.2 Preparation of soil samples and plating

One gram of soil was weighted and put into 15ml Falcon tube to which 10ml of sterile Phosphate Buffer Saline (PBS) buffer at the pH 7.4 was added. Using vortex mixer, the mixture of soil and PBS was homogenized and then left for 1 hour to allow the large soil particles to completely settle. Subsequently, by using sterile cotton swabs, 100μl of the supernatant from soil suspension was pipetted and spread over four Potato Dextrose Agar (PDA) (BD Difco™) plates. Each plate was labeled specifically and left at room temperature (28ºC) for five days for calculating and recording the
fungal colonies. Before preliminary antibiotic screening being carried out, the plates were kept at 4°C for two days in order to delay the growth of soil microorganism.

3.3 Cultivation of test bacteria

Cultivation of test bacteria and preliminary selection were carried out as described by Fankhauser (2005) and J-Nkanga & Hagedorn (1978). Test bacteria were cultured in LB at 37°C for 18-24 hours before use. By using spectrophotometer at wavelength of 520nm, the turbidity of the bacteria was determined and optical density (OD) value was adjusted to 0.6-0.8. 0.75% (w/v) soft Nutrient Agar (NA) was prepared and kept at 50°C until use.

3.4 Preliminary selection via antibiotic activity screening

After determination of the OD value, 100μl of test bacteria was pipetted and mixed well with 2ml of 0.75% (w/v) soft NA. Then, the mixture of the soft agar was overlaid on PDA plates grown with soil microorganisms from plating before the soft NA solidified. Four empty plates of PDA that overlaid with the test bacteria were used as control. The plates were kept at room temperature (28°C) and observation for the formation of the inhibition zone was carried out for every 24 hours and 48 hours.

3.5 Selection and isolation of soil microorganisms

Soil microorganism was selected and isolated from preliminary selection. In preliminary selection, soil microorganisms that inhibited the growth of test bacterial by producing inhibition zone were selected and isolated in order to obtain pure strain of that particular microorganism.
3.6 Secondary screening for fungal isolates via agar overlay technique

After soil microbes inoculated, pure fungal isolates were selected. Five fungal isolates per plate was undergo preliminary selection and cultivated on PDA. After incubation at room temperature (26ºC) for 4 days, these fungal isolates which grow on PDA was subjected to secondary screening via agar overlay technique, whereby 2ml of 0.75% soft NA which seeded with test bacteria was overlaid onto the growing fungal isolates. Four empty PDA plates which overlaid with inoculated soft agar were used as controls in this experiment. These plates were incubated at room temperature (26ºC) for 24 hours. Next, zone of inhibition was observed.

3.7 Antibacterial activity of soil microbe extracts

3.7.1 Cultivation of selected fungal isolates

During secondary screening, fungal isolates that showed good antibacterial activities was selected and cultivated on different culture media with 20 plates per isolate. These culture media used was PDA. Selected fungal isolates was cultivated on PDA at room temperature (26ºC) until they reached full plate. All plates with covers removed were placed inverted in secluded area for drying.

3.7.2 Extraction of secondary metabolites

Agars were immersed with hexane solvent in conical flasks before it was grinded separately with mortar and pestle after drying. After 4 days of immersion, the solvent was filtered and agar residual from the previous filtration was re-immersed in new hexane solvent for another one day before further filtration was carried out.
By using rotary evaporator at 40°C, hexane solvent which might contain potential secondary metabolite was concentrated and then placed in desiccators for another 3 days for the removal of excess moisture. The crude hexane extracts was collected, weighted and kept in 1.5ml eppendorf tube at 4C for further usage. Aluminum foils were used to cover eppendorf tubes to prevent the crude hexane extracts has direct contact with light source.

3.8 Antibiotics susceptibility testing with hexane crude extracts

3.8.1 Preparation of test bacteria

As described by Fankhauser (2010), test bacteria was inoculated into 3ml of Mueller-Hinton Broth (MHB) (Oxoid) and incubated at 37°C for 18 to 24 hours. Using spectrophotometer with uninoculated MHB as blank and reference, the turbidity of the test bacteria was measured at wavelength of 550nm. The reading of OD of each test bacteria was adjusted to 0.168.

3.8.2 Disk-diffusion method

Antibiotics susceptibility testing was carried out based on disk-diffusion method described by Bopp et al. (1999). Extracts were tested using disk-diffusion method. One milligram of the dried crude extract was weighted and dissolved in 5μl of 100% methanol and 95μl of MHB at pH7.0. After that, extract was diluted to concentration of 0.5 mg/ml, 0.125 mg/ml and 0.625mg/ml respectively.
3.8.3 Determination of minimum inhibitory concentration (MIC) value

During antibiotics susceptibility test, extract that showed the ability to inhibit the growth of four different species of test bacteria was selected for MIC determination value.

For disk-diffusion method, test bacteria which seeded were arranged on MHA plates with seven 6 mm antibiotic-free filter disks (Whatman No.3). Aliquot of 10μl of different dilution of antibiotics was dropped onto the disks with concentration of 0.5μg/disk, 0.25μg/disk, 0.125μg/disk and 0.625μg/disk, respectively. One of the antibiotics-free filter disks with the concentration of 1.0μg/disk was dropped with aliquot of 10μl of stock solution. Positive and negative control of antibiotics-free filter disk on the MHA plate was pipette with 10μl of 5x dilutions penicillin-streptomycin solution and MHB contained 100% methanol. The plates were sealed, placed inverted and incubated at 37ºC for 24 hours. After that, the diameter (mm) of the inhibition zone formed around the disk was measured. The lowest antibiotic concentration that able to cause the formation of inhibition zone around the disk was MIC value.

3.9 Thin Layer Chromatography (TLC)

1.0mg/ml of each fungal crude extract were prepared by adding 0.1mg of crude extract and 100μl of hexane. Samples solvent was prepared in chamber by adding hexane and dichloromethane in ratio of 1:3.5 in total of 25ml. Silica gel plates were prepared by measuring 7.5cm with drawing a parallel line at 1.0 cm above the lower border and 0.5 cm below the upper border of silica gel plates. Then, 6μl of 1.0mg/ml crude extract was dropped twice on each spot on silica plates. The TLC was run until solvent reach on top 0.5cm before the TLC plate was took out of the container and let
it dry in a ventilated place. Dry TLC plates were visualized under ultra violet developing camera.

3.10 Bioautography

Test bacteria were cultured in NB at 37°C for 18-24 hours before use. By using spectrophotometer at wavelength of 550nm, the turbidity of test bacteria were determined and optical density (OD) value were adjusted to 0.168. After determination of OD value, 200μl of test bacteria were pipetted and mixed well with 4ml of 0.75% of soft NA which then were overlaid on plate of TLC and swab if needed. Positive and negative control of silica gel plates was pipette with 100μl of 5x dilutions penicillin-streptomycin solution and 100μl of distilled water. The plates were kept at 37°C overnight at incubator and observed the formation of inhibition zone on silica gel plates. Add 300μl of MTT solution on inhibition zone and left for 4 hours. Cut the TLC plates and agar on it into a new petri dish. Let it dry and observed the result.

3.11 Characterization of pure fungal isolates producing isolates

Observations were conducted on 7-days old fungi cultures that incubated at room temperature by using naked eyes and stereo microscope. Based on macroscopic and microscopic examinations, characterization of pure fungal isolates was carried out. Pure fungal isolates that showed great antibacterial activities during secondary screening were cultivated on PDA, CDA and MEA until fungi reached their maximum growth. Observed and recorded the different growth morphologies which include top color, mycelium mat, mycelium end reverse color, medium color and perimeter.
Intact structure of the fungi which including structure of hypae, conidia, spores and conidiospores was observed by preparing slide culture of each fungal isolates. Before an approximately 5mm × 5mm agar cube contained part of the fungal isolate placed on slide, the slide was immersed in 70% (v/v) ethanol, flamed and left to cool. A cover slip was placed on plasticin. The cover slip was pressed until it touched the surface of agar cube. Before observation under light compound microscopic was carried out, prepared slide culture was placed in sterile Petri dishes and incubated at room temperature (26ºC) for 3 days.

3.12 Molecular Identification via Polymerase Chain Reaction and DNA Sequencing

3.12.1 DNA Extraction

Firstly, studied fungi were allowed to grow into full plate culture. All the mycelium were scrapped and placed in pestle and mortar. Then, the mycelium was grinded in liquid nitrogen and the slurry was transferred into 2 milliliter (mL) micro centrifuge tubes. Next, 0.5mL of pre-warmed extraction buffer was added into the tube. The tube was inverted several times to mix the solution. The tube was incubated at 70ºC for 30 minutes (min). After that, 0.5mL of chroform: isoamyl alcohol was added and the tube was inverted several times before subjected to centrifugation at 10000 gravity unit (g) for 5 min at room temperature. Then, 1.0mL of precipitation buffer was added. The tube was inverted for 2 min and centrifuged at 13000g for 15 min at room temperature. All the supernatant were discarded. After that, 0.35mL of 1.2 Molar (M) sodium chloride and one volume of chloroform: isoamyl alcohol were added into the tube and mixed vigorously using vortexer. The tube was centrifuged again at 10000g for 5 min at room temperature and the upper phase formed was transferred into new
2mL microcentrifuge tube. Then, 0.42mL of isopropanol was added and mixed by inversion. After incubation at -20°C for 15 min, the tube was centrifuged at 13000g for 20 min at 4°C. All the supernatant were removed. After that, 1mL of 70% ethanol was added and the tube was centrifuged again at 13000g for 3 min at 4°C. The DNA pellet was air-dried at room temperature. Finally, the DNA was resuspended in DNase-free water and stored at -20°C.

### 3.12.2 PCR Amplification

The ribosomal DNA was amplified in Polymerase chain reaction (PCR) using universal primer ITS1 (forward) and ITS4 (reverse) primers. The sequences of the primers were showed in Table 1 (White et al., 1990).

<table>
<thead>
<tr>
<th>Primer design</th>
<th>Sequence (5’ to 3’)</th>
<th>Primer size (bp)</th>
<th>Expected Amplicon size</th>
</tr>
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<tr>
<td>ITS1</td>
<td>TCCGTAGGGTGAACCTGC</td>
<td>10</td>
<td>602bp</td>
</tr>
<tr>
<td>ITS4</td>
<td>TCCTCCGCTTATTGATATGC</td>
<td>10</td>
<td>602bp</td>
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</table>

The constituents of PCR reaction mixture are shown in Table 2. All PCR reagents were mixed gently and briefly centrifuged to collect all drops from wall of tube. The thermal cycling programme for PCR amplification using ITS1 and ITS4 primers were performed according to the adjusted parameter shown in Table 3.
Table 2: 1× reaction mixture for PCR amplification with ITS1 and ITS4

<table>
<thead>
<tr>
<th>PCR Reagents</th>
<th>Quantity (μl)</th>
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<tr>
<td>PCR Master Mix, 2× (Promega, USA)</td>
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<tr>
<td>ITS1</td>
<td>0.5</td>
</tr>
<tr>
<td>ITS4</td>
<td>0.5</td>
</tr>
<tr>
<td>DNA template</td>
<td>3.0</td>
</tr>
<tr>
<td>Nuclease free water</td>
<td>8.5</td>
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<tr>
<td>Total final volume</td>
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Table 3: Step cycle, temperature and duration for PCR amplification

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<th>Step Cycle</th>
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<tr>
<td>Initial denaturation</td>
<td>94°C for 2 min</td>
</tr>
<tr>
<td>Denaturation</td>
<td>94°C for 1 min</td>
</tr>
<tr>
<td>Annealing</td>
<td>53°C for 0.75 min</td>
</tr>
<tr>
<td>Extension</td>
<td>72°C for 1 min</td>
</tr>
<tr>
<td>Final extension</td>
<td>72°C for 5 min</td>
</tr>
</tbody>
</table>

3.12.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was conducted with 1% (w/v) agarose powder (Promega, USA) in 50ml of Tris-acetate-EDTA buffer (TAE) and stained with ethidium bromide. The gel was electrophoresed at 90V for 40 minutes.

3.12.4 PRC Product Purification

The amplified DNA fragments were purified by using QIAquick Gel Extraction Kit (Qiagen, USA). A total of 50μl PCR product was subjected to purification according to the manufacturer protocol.