Faculty of Resource Science and Technology

ANTIMICROBIAL ACTIVITY OF CRUDE EXTRACT FROM ANTIBIOTIC PRODUCING FUNGI

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This project is submitted in partial fulfilment of the requirements for the degree of Bachelor of Science with Honours
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Declaration

I hereby declare that this thesis entitled “antimicrobial activity of crude extract from antibiotic producing fungi” is the result of my own research work and effort. It has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Signature :

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Date : 2nd July 2012

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List of Abbreviations

DCM	Dichloromethane
DMSO	Dimethyl Sulfoxide
EA	*Enterobacter aerogenes*
EC	*Escherichia coli*
NA	Nutrient Agar
NB	Nutrient Broth
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitor concentration
MTT	Methyl Thiazolyl Tetrazolium
MRSA	Methicillin resistant *Staphyloccus aureus*
OD	Optical Density
PDA	Potato Dextrose Agar
P-S	Penicillin- Streptomycin
SA	*Staphylococcus aureas*
ST	*Salmonella typhi*
TLC	Thin layer chromatography
°C	Celcius
cm	centimeter
mm	milimeter
nm	nanometer
µg	microgram

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ABSTRACT

Fungi have been used for producing medically useful compound. Although there are a large number of fungi species, only a relative few have been found to produce antibiotics. Nowadays, the bacteria become resistance toward antibiotics because of overuse of the antibiotics in the treatment of bacterial infection. The objectives of this study are to determine antimicrobial activity of crude extracts from antibiotic producing fungi and to compare the effectiveness of antibacterial properties among four different crude extracts using three different assays. Evaluation on antimicrobial activity was done on crude extract using four different solvents and five different concentrations. The solvents involved in this research including hexane, dichloromethane (DCM), ethyl acetate, and methanol. The concentration used were 1.0 mg/µl, 0.5 mg/µl, 0.25 mg/µl,0.125 mg/µl and 0.0623 mg/µl. Antimicrobial activity of crude extracts was determined using different bacteria which are three Gram-negative bacteria, *Escherichia coli, Salmonella typhi, Enterobacter aerogenes* and one Gram-positive bacteria, *Staphylococcus aureas*. The disc diffusion assay, broth microdilution assay and direct bioautographic assay was the method used to determine antimicrobial activity using the crude extracts from the antibiotic producing fungi. All of the crude extracts showed different antimicrobial activity toward different bacteria. From the result hexane and ethyl acetate extracts showed active against Gram negative bacteria, *E. aerogenes* and Gram positive bacteria *S. aureas.*

Key words: Fungi, antibiotic, hexane, DCM, ethyl acetate, and methanol

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ABSTRAK

Kulat telah digunakan untuk menghasilkan sebatian berguna dalam perubatan. Walaupun terdapat sebilangan besar spesies kulat, hanya beberapa sahaja telah berjaya menghasilkan antibiotik. Kini, bakteria mempunyai kerintangan terhadap antibiotik disebabkan oleh penggunaan antibiotik yang berlebihan dalam merawat masalah jangkitan bakteria. Objektif utama kajian ini adalah untuk mengkaji aktiviti antimikrob bagi ekstrak mentah daripada kulat yang berpotensi mengasilkan antibiotik dan untuk membandingkan keberkesanan ciri-ciri antibakteria bagi empat jenis ekstrak mentah yang berbeza dengan menggunakan tiga kaedah yang berbeza. Penilaian ke atas aktiviti antimikrob telah dilakukan ke atas ekstrak mentah menggunakan empat pelarut berbeza dan lima kepekatan yang berbeza. Pelarut yang terlibat dalam kajian ini termasuk heksana, diklorometana (DCM), etil asetat, dan metanol. Kepekatan yang digunakan ialah 1.0 mg/µl, 0.5 mg/µl, 0.25 mg/µl,0.125 mg/µl and 0.0623 mg/µl. Aktiviti antimikrob ekstrak mentah telah ditentukan dengan menggunakan bakteria yang berbeza iaitu tiga daripada bakteria Gram negatif, Escherichia coli, Salmonella typhi, Enterobacter aerogenes dan salah satu daripada bakteria Gram positif, Staphylococcus aureas. Disc diffusion assay, broth microdilution assay dan direct bioautographic adalah kaedah yang digunakan untuk menentukan aktiviti antimikrobial menggunakan ekstrak mentah dari kulat yang berpotensi menghasilkan antibiotik. Kajian ini menemui bahawa ekstrak mentah berbeza menghasilkan aktiviti antimikrob yang berbeza. Keputusan kajian menunjukkan heksana dan etil asetat ekstrak menunjukkan aktif aktiviti terhadap bakteria Gram negatif, E. aerogenes dan bakteria positif Gram S. aureas.

Kata kunci: Kulat, antibiotic, heksana, DCM, etil acetat, dan metanol

1.0 INTRODUCTION

Nowadays, as a result of excessive use of antibiotic in treatment of infectious diseases, microorganisms have developed resistance toward antibiotics (Melgarego *et al*., 2008). The antimicrobial resistance particularly among bacteria has become an important issue which has serious implications on the prevention and treatment of infectious diseases (Diarmaid and Dan, 2001). Besides, the emergence and spread of antimicrobial resistance remains a global public health concern. Antibiotic resistance is commonly used to describe the situation when the concentrations of antibiotic needed to kill the bacteria cannot be achieved at the site of the infection (Berdy, 2005). This phenomenon has made the treatment of the bacterial infections become increasingly difficult. Hence, more efforts and researches need to be carried out to discover new antimicrobial drug in order to overcome the widespread of the bacteria resistances.

The antibiotics which have been produced by the fungi are increasing due to the emergence of the bacteria that are resistant toward antibiotics (Bronzwaer, 2002). Antibiotics can be produced by almost all types of living things. They are produced by prokaryotic and eukaryotic organisms belonging to the plant and animal kingdom (Berdy, 2005). Antibiotic also have been successfully used for extensive application in the treatment of infectious diseases of man, animal, and plant (Cassell, 2001). Besides, antibiotics are affective against bacteria because they attack the unique peptidoglycan cell wall or smaller ribosomal unit of the bacteria (Butler and Cooper, 2011).

Penicillin is one of the examples of antibiotic that was discovered accidentally in 1928 by Fleming, who showed its efficiency in laboratory cultures against many disease producing bacteria. This discovery marked the beginning of the development of antibacterial compounds produced by living organisms (Taylor *et al.,* 2003). Since the discovery of penicillin, the first β-lactam antibiotic, in modern medicine fungi turned out to be important antibiotic for curing life treating infectious diseases. Penicillin was introduced as the first antibiotic, since then literally thousands of metabolite which been produced mainly by fungi has been screened for antimicrobial activities (Bronzwaer, 2002).

Crude extract as secondary metabolite was used to test the inhibition of bacterial and fungal growth against human pathogenic bacteria and fungi (Hussain and Ananthan, 2009). Antimicrobial activity can be determined using disc diffusion assay by inoculating the suspension of bacteria through swabbing method on Mueller-Hinton Agar (MHA) plates (Cleidson, 2007). This method described to determine the Minimum Inhibitory Concentration (MIC) of extracts against the standard bacteria strains (Citron and Warren, 2005).

On the other hand, disc diffusion method was used to determine antibacterial and antifungal activities (Al-Fatimi, 2010). Bioautography assay is a method for directly detection of antibacterial compounds on nutrient agar plate. This assay is also practiced as a method to localize antibacterial activity on chromatogram and has found widespread application in the search for new antibiotic. Besides, the broth microdilution method described the modification that was used to determine the MIC of extracts against the standard bacteria strains in liquid culture (Cleidson, 2007).

Objectives of this study are to:

1. determine antimicrobial activity of crude extracts from antibiotic producing fungi.
2. compare the effectiveness of antibacterial properties among four different of crude extracts using three different assays.

2.0 LITERATURE REVIEW

2.1 Fungi

Fungi are one of the organisms that used as a model for the study of various fields in biology including biochemistry, genetic, molecular biology, interaction in environment and also used widely in industry and biotechnology. Fungi also used in research study because they are easily cultured, occupy little space, multiply rapidly and also have short life cycle (Feofilova, 2001). The diversity of fungi in the organization of their life cycles, cellular structure, composition and metabolism is important in mycology study (Wainwright, 1992).

Fungi are achlorophyllous, heterotrophic, eukaryotic and spore-bearing organism surrounded by a well defined cell wall made up of chitin, with or without fungal cellulose, along with many other complex organic molecules. Fungi usually obtain food by absorption, except a few lower groups where they take in food by ingestion (Kornfeld, 2002). Fungi play such a dominant role in human society that it could be readily argued that it is the most important biotechnologically useful organisms. One of the most economically important uses of fungi is in the industrial production of biochemicals such as organic acid that involve citric, fumaric, lactic and gibberellins (Abraham, 2001).

The term ‘endophyte’ is used to define fungi or bacteria that occur inside asymptomatic plant tissues. Fungal endophytes are ubiquitous and are dominated by Ascomycota (Ganley, 2005). People in the India subcontinent have a long history using medical plants to cure various diseases. Medical plants of Western Ghats of India are reported to have a diverse community of endophyte fungi (Raviraja, 2005). Few studies on the endophytic fungi of these plants have been

conducted. The present study was undertaken to investigate the diversity of endophytic fungi and their seasonal colonization pattern in medical shrub species commonly used in Malnad region, Western Ghats of Karnataka, Sourthern India. Besides that, a total of 6125 fungal endophytes were isolated from 9000 leaf segments of 15 medicinal shrubs growing in Malnad region during winter, monsoon and summer seasons. These fungal isolates belonged to Ascomycota, Coelomycetes, Hyphomycetes, Mucoromycotina and sterile forms (Fernando, 2008). In addition, fungal endophytes have been recognized as a respiratory on novel compounds of immense value in agariculture, industry and medicine (Tan and Zou, 2001)

2.2 Test Bacteria

Staphylococcus aureas is a commonly bacteria that is easy to find on the skin and in the nose of healthy persons. This bacterium has the ability to grow comparatively well under conditions of high osmotic pressure and low moisture. Among the entire *Staphylococcus* group, *S. aureus* is the one that cause most infection (Stoppler, 2009). The bacteria gained their pathogenicity via production of many toxins that increases the ability to invade the body or damage the tissue (Tortoro, 2004).

Escherichia coli is one of bacteria that are commonly found in the gut of warm blooded organisms. There are several types of *E. coli* exist as part of the normal flora in human gut and also have several beneficial functions, such as for production of vitamin K2. They also can prevent harmful bacteria, known as pathogenic bacteria, because of establishing themselves in the intestine (Sondi, 2004).

Enterobacter aerogenes is a Gram-negative bacteria and rod shaped microorganism from Enterobacteriaceae family. This microorganism is important nosocomial pathogen that responsible for various infections, including lower respiratory tract infection, skin and soft-tissue infection and wound infection. *Enterobacters* species possess inducible beta-lactamases, which are undetectable in vitro but are responsible for resistance action during treatment. Physicians treating patients with *Enterobacter* infections are adviced to avoid certain antibiotics, because resistant mutants can quickly appeared (Sandra and Tenney, 2000).

Salmonella typhi is bacteria which can cause disease in human. This bacterium is the causive agent of typhoid fever. Although typhoid fever is not widespread in the United States, it very common in under developed countries and causes a serious often fatal disease. The main source of *S. typhi* is from drinking infected water. Food may also be contaminated with *S. typhi* if it was washed or irrigated with contaminated water (Kidgell, 2002).

2.3 Antibiotics

Originally, the term antibiotic referred only to organic compounds produced by bacteria and fungi, which are toxic to other organisms. Antibiotics represent the single contribution of drug therapy for the health care of increasing population of the world, and provide effective control of many microbial pathogens that have been the cause of death of human and animals (Makut and Owolewa, 2011). Antibiotics produced by fungi, are widely used in current chemotherapy especially the penicillin, cephalosporin and fusidic acid, which have antibacterial and antifungal activity (Romanowski, 2007).

Screening of antibiotic has been widely performed for about 50 years, and new antibiotics are still being found. However, the possibility of discovering new antibiotics through random screening of microorganisms such as actinomycetes and other bacteria producing antibiotics is reduced nowadays. Because of that, new approaches are required for finding new antibiotics efficiently. The term ‘antibiotic’ literally means ‘against life’. An antibiotic was originally defined as a substance, produced by one microorganism (Berdy, 2005).

Many of the microbial products including antibiotics are considered to be secondary metabolites. It is because they seem to have no direct role in those aspects of metabolism which support necessary functions in the cell namely energy production, growth and reproduction. There is a great structural variety among the secondary metabolites. Some antimicrobial active and some are not. Antibiotic has a powerful action on a wide range of infectious bacteria include in both, gram positive and gram negative (Weinstein, 2003). The streptomycin is example of antibiotic that was isolated in 1944 by Waksman, a Microbiologist, from a species of soil bacteria, called *Streptomycesgriseus*, particularly *Tubercle bacilli*, and has proved to be very valuable against tuberculosis (Stewart and William, 2001). In 1947, another antibiotic, chloromycetin was discovered by Burkholder (Cars, 2001).

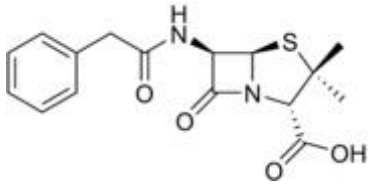
Antibiotic had been used by human in three ways. Two of the ways are in human medicine, which is the use of antibiotics to treat sick animals and the prophylactic use of antibiotics to prevent diseases. The third one is on agriculture, which has no parallel to human medicine and used as growth promoters. That is, the long-term administration of low levels

antibiotics to some animals increases the weight gain per unit of food and allows animals to reach full weight more quickly (Darmaid and Dan,2001).

Various types of antibiotics work as bactericidal or bacteriostatic. Bactericidal antibiotic kills the bacteria generally by either interfering with the formation of the bacterium's cell wall or its cell contents in the cytoplasm. Penicillin, daptomycin, fluoroquinolones and metronidazole are some example of bactericidal antibiotics (Figure 2.1). Whereas, bacteriostatic antibiotic inhibit the bacteria from multiplying by interfering with bacterial protein production, DNA replication, or other aspects of bacterial cellular metabolism which can subsequently stop the bacteria multiplication (Martyn, 2008).

In addition, although the concept of antibiotics has evolved over time to include plant and animal products as well as synthetic and semisynthetic compounds that been used in theraphy, the word 'antibiotic' has become entrenched as a descriptor for any molecule produced in the laboratory with the ability of cidal (killing) or static (inhibitory) to inhibit specific group of microorganisms (Grace, 2006). The range of bacteria or other microorganisms that is affected by a certain antibiotic is expressed as its spectrum of action.

The spectrum have classified into two which are broad spectrum or narrow spectrum. Broad spectrum is referring to the antibiotics effective against prokaryotes that kill or inhibit a wide range of Gram-positive and Gram-negative bacteria. However, narrow spectrum is when antibiotics effective mainly against Gram-positive or Gram-negative bacteria. If antibiotic against a single organism or disease, they are referred to as limited spectrum (Todar, 2009).



Penicillin G

Figure 2.1: Molecular structure of an antibiotic Penicillin G.

2.4 Antibiotic Resistant

Antibiotic resistance is the natural consequence of the selective pressure imposed by antibiotic drugs upon bacteria populations. Antibiotic resistance occurs when pathogenic microorganisms are capable to inactive antibiotics or survive under the selective pressure of antibiotic. The rapid emergence and spread of antibiotic resistance genes is due to the consumption of large amount of antibiotics (Cleidson, 2007). Antimicrobial agents represent the greatest advance in modern curative medicine.

Resistance to antimicrobial agents has existed since before they were introduced into human probably because most of the classes of compounds used clinically are produced also by microorganisms in the environment (Helen, 2001). The introduction and increasing clinical use of each antimicrobial agent and also followed by an increasing isolation rate of resistant bacteria. Resistance was shown in a short period of time by mutational target alteration against antibiotic such as quinolones, rifampicin, fucidic acid and mupirocin after their introduction into clinical use (Diarmaid and Dan, 2001).

Resistance can spread through species selection, mutation, gene epidemics, and strain epidemics. Resistance can arise through mutations, which can be defined as random and spontaneous genetic changes. Antibiotic do not cause mutations occur, but their use clearly generates an intense pressure for the selection of resistance mutants that arise naturally at low frequency. Bacteria can acquire genetic resistance determinants from other bacteria, either as plasmid or chromosomal insert. Besides, plasmid and some chromosomally inserted transposons are often freely transmissible and their epidemic spread allows resistance to extend to diverse organisms (Helen, 2001).

The rapid emergence of mutational resistance can swiftly reduce the effectiveness of an antibiotic. Methicillin resistant *Staphyloccus aureus* (MRSA) were first reported in the early 1960's and are now regarded as a major hospital acquired pathogen worldwide. The term methicillin resistant is historically used to describe resistance to any of this class of antimicrobials. Fluoroquinolones were originally active against MRSA, but staphylococci have an efflux pump and resistance arises cause by mutation of gene call *norA*. Because of that, most MRSA are now resistant to antibiotics (Helen, 2010).

2.5 Antibiotic-Producing Microorganisms

The ability of certain fungi and bacteria to produce chemical substances which inhibit or destroy pathogenic organisms (Waksman, 1952) has supported the fact that these unrelated groups are involved in most of the natural antibiotics production (Todar, 2009). These substances are hypothesized to confer a selective advantage to the producer when competition is significant

microbial fitness. A fungus such as *Penicillium chrysogenum* is an important industrial organism due to its ability to produce several types of β-lactam antibiotic (Hogg, 2005).

Different antibacterial properties could be obtained through the substitution of R-group subtituent of the penicillium nucleus (Frisvad and Andersen, 2008).The ability to produce antibiotics has been found mainly in fungi of the group Aspergillales and also in a few bacteria. The streptomycetes are remarkable for the chemical diversity of antibiotic that they produce. Altogether about 2,000 antibiotics have been characterized so far, but only 50 are using therapeutically. Antibiotic produced by fungi, are widely used in current chemotheraphy especially the penicillin, cephalosporin and fucidic acid, which shows antibacterial and antifungal activity (Grace, 2006).

A number of antibiotic drugs have been discovered from soil-inhabiting microorganisms which include 20% from fungi, 70% from actinomycetes and 10% from eubacteria (Makut and Owolewa, 2011). Antibiotics are produced by many microorganisms in various ecological conditions. Producers of antibiotic can be found in rivers, lakes, decaying plants and animal remains. However majority of microorganisms that produce antibiotic are soil inhabitants (Chandra, 2010).

2.6 Crude Extract

Crude extract was used to test the inhibition of bacterial and fungal growth against human pathogenic bacteria and fungi (Hussain and Ananthan, 2009). The fungal extract mostly contains compounds from the secondary metabolism, but also some primary metabolites (Smedsgaard and Nielsen, 2005). A profile of secondary metabolites is compiled by the mycologist and chemist is based on fungal extracts. It consists of compounds produced on one or more media and includes antibiotics (Frisvad and Andersen, 2008). Fungi, plants, lichen fungi, and actinomycetes are the four groups of organisms are particularly good producers of secondary metabolites, whereas yeasts, protozoa, and animals are less efficient producers. Therefore, secondary metabolites have mostly been used in plant and fungal taxonomy (Frisvad and Larsen, 2007).

2.7 Antimicrobial Assay

Antimicrobial assay is a method to discover antibiotic. The current available screening methods for the detection of antimicrobial activity of natural product are classified into three groups, disc diffusion assay, broth microdilution assay and direct bioautography assay (Cleidson, 2007). Antimicrobial activity of crude extract by using disc diffusion technique was carried out with careful consideration of factors, such as inoculation size, incubation temperature ,as well as type and depth of agar to ensure that the test are standardized, reproducible and reflected the effectiveness of the antimicrobial agents (Rennie *et al*., 2007).