ANTIMICROBIAL ACTIVITY SCREENING OF FUNGAL ENDOPHYTES ISOLATED FROM *Scleria sumatrensis* Retz. (FAMILY: CYPERACEAE) AT UNIMAS EAST CAMPUS

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Declaration

I hereby declare that this thesis entitled "Antimicrobial Activity Screening of Fungal Endophytes Isolated from *Scleria sumatrensis* Retz. (Family: Cyperaceae) at UNIMAS East Campus" 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:

Name: Tan Ting Ting

Date: 26th May 2010

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List of Abbreviations

CFU	– Colonies forming unit
CLSI	– Clinical and Laboratory Standard Institute
DNA	– Deoxyribonucleic acid
FDA	– Food and Drug Administration
KY-31	– Kentucky-31
MHA	– Mueller-Hinton agar
MHB	– Mueller-Hinton broth
MRSA	– Methicillin Resistant *Staphylococcus aureus*
NA	– Nutrient agar
NB	– Nutrient broth
OD	– Optical density
PDA	– Potato dextrose agar
psi	– pound per square inch
TCA	– Tricarboxylic acid
VRE	– Vancomycin-resistant enterococci
WHO	– World Health Organization

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Antimicrobial Activity Screening of Fungal Endophytes Isolated from *Scleria sumatrensis* Retz. (Family: Cyperaceae) at UNIMAS East Campus

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ABSTRACT

The increasing prevalence of multiple drug resistant bacteria has posed serious global health problems. This trend has rendered the discovery of new antibiotics to become indispensable. Because fungal endophytes have been recognised as potential sources of antibiotic, a study was carried out on endophytes of wild grass, *Scleria sumatrensis* Retz. In this study, fungal endophytes were isolated from the stems of the grass and then screen for the presence of antibiotics. Two stages of preliminary antibacterial testing were carried out via agar overlay technique using PDA and V8 juice agar, respectively. In preliminary antibacterial screening, the isolate $E5b_1$ was found to be active against three test bacteria which were *Staphylococcus aureus*, *Enterobacter aerogenes* and *Escherichia coli*. The isolates $E1a_1$, $E1a_3$ and $E5a_1$ were active against two test bacteria which were *S. aureus* and *E. aerogenes*, whereas the isolate $E3b_1$ was only active against *E. aerogenes.* The active secondary metabolites of the five endophyte isolates were extracted with methanol and were evaluated for antibacterial activity by using Kirby-Bauer disc diffusion method. The methanol extracts of isolates $E1a_1$, $E1a_3$, $E3b_1$ and $E5a_1$ showed antibacterial activity toward at least one test bacteria which were either *S. aureus*, *E. aerogenes* or both. However, the methanol extract of isolate $E5b_1$ did not show antibacterial activity towards all four test bacteria. Identification of the endophyte isolates to the genus level was predominantly based on the microscopic observations. The genus of isolates $E1a_1$, $E1a_3$, $E5a_1$ and $E5b_1$ were *Phialophora*, *Gilmaniella*, *Cacumisporium* and *Endophragmia*, respectively, whereas isolate $E3b_1$ was recognised as sterile mycelia since it was unable to produce spores under the culture condition. Further study on the secondary metabolites of fungal endophytes should be conducted due to its high potential in medical application.

Keywords: fungal endophytes, *Scleria sumatrensis*, antibacterial activity, agar overlay technique, disc diffusion method

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ABSTRAK

Peningkatan keberleluasan dalam bakteria rintang terhadap antibiotik semakin memburukkan taraf masalah kesihatan di seluruh dunia. Oleh itu, keperluan untuk menghasilkan antibiotik baru dari semasa ke semasa tidak dapat dielakkan. Oleh sebab kulat endofit telah dikenalkan sebagai sumber antibiotik yang berpotensi, maka satu kajian terhadap kulat endofit dalam rumput, Scleria sumatrensis Retz. telah dilaksanakan. Dalam kajian ini, kulat endofit telah dipencilkan daripada ranting rumput. Kemudian, penyaringan bagi menentukan kehadiran antibiotik telah dilakukan ke atas kulat endofit. Dua peringkat penyaringan awalan telah dilaksanakan melalui teknik agar overlay dengan manggunakan PDA dan agar jus V8 maisng-masing. Pada penyaringan awalan, pencilan $E5b_1$ didapati aktif terhadap tiga bakteria ujian, iaitu Staphylococcus aureus, Enterobacter aerogenes dan Escherichia coli. Pencilan-pencilan $E1a_1$, $E1a_3$ dan $E5a_1$ pula didapati aktif terhadap dua bacteria ujian, iaitu S. aureus dan E. aerogenes manakala pencilan $E3b_1$ didapati hanya aktif terhadap E. aerogenes sahaja. Metabolit sekunder yang aktif daripada kelima-lima kulat endofit telah diekstrak dengan menggunakan metanol. Aktiviti antibakteria bagi ekstrak metanol yang diperoleh daripada kelima-lima kulat endofit telah ditentukan melalui kaedah serapan disk Kirby-Bauer. Ekstrak metanol bagi pencilan-pencilan $E1a_1$, $E1a_3$, $E3b_1$ dan $E5a_1$ menunjukkan aktiviti antibakteria terhadap sekurang-kurangnya satu bakteria ujian, iaitu sama ada S. aureus, E. aerogenes atau kedua-duanya. Walaubagaimanapun, ekstrak metanol bagi pencilan $E5b_1$ tidak menunjukkan aktiviti antibakterial terhadap kesemua bakteria ujian. Pengenalpastian kulat endofit kepada peringkat genus adalah terutamanya berdasarkan ciri-ciri mikroskopik kulat endofit. Genus bagi pencilan-pencilan $E1a_1$, $E1a_3$, $E5a_1$ dan $E5b_1$ adalah Phialophora, Gilmaniella, Cacumisporium dan Endophragmia masing-masing. Manakala, pencilan $E3b_1$ adalah dikenalpasti sebagai sterile mycelium kerana ia tidak dapat menghasilkan spora pada keadaan pengkulturan. Memandangkan potensi metabolit sekunder yang dihasilkan oleh kulat endofit dalam bidang perubatan, kajian lanjutan terhadap kulat endofit harus dilaksanakan.

Kata kunci: kulat endofit, Scleria sumatrensis, aktiviti antibakteria, teknik agar overlay, kaedah serapan disk

1.0 Introduction

The emergence of new diseases worldwide is the main consequence of the development of antibiotic resistant bacteria. This phenomenon has caused very serious health problem to the health authorities worldwide. Misuse of antibiotics is one of the major factors that lead to the emergence of antibiotic resistant bacteria (WHO, 2002; Aksoy & Unal, 2008). Some examples of antibiotic resistant bacteria are penicillin-resistant *Streptococcus pneumonia*, vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), multi-resistant *Mycobacterium tuberculosis* and multi-resistant salmonellae. The increasing number of antibiotic resistant bacteria has lead to the effort in discovering new antibiotics from time to time in order to cope with those antibiotic resistant bacteria.

Endophytes have been recognised as a potential source of antibiotic (Schulz *et al*., 2002). Endophytes are fungi which live mutualistically inside the plants without causing diseases to their host plants (Isaac, 1992). The host plants provide shelter and nutrients for the endophytes whereas endophytes will produce certain secondary metabolites, such as toxic alkaloids which will protect the host plants against insects and herbivorous predators. According to Strobel and Daisy (2003), a plant may host one or more endophytes during their life cycle. This fungus can be found within the leaves, roots and stems of both grasses and woody plants.

Much research has been conducted on endophytes for their antimicrobial secondary metabolites in the effort to discover new antibiotics. For instance, *Cryptosporiopsis quercina*, an endophyte resides in *Tripterigeum wilfordii* will produce cryptocandin as secondary metabolite (Strobel *et al.,* 1999). Cryptocandin is a potent antimycotic agent which effectively against human pathogenic fungi such as *Candida albicans*. In addition, phomopsichalasin, secondary metabolite produced by *Phomopsis* sp., shows antibacterial

and antifungal properties towards human pathogenic microbes such as *Bacillus subtilis* and *C. albicans* (Tan & Zou, 2001).

However, the secondary metabolites produced by endophytes are still not fully exploited. During the early decade, they are not yet fully understood (Strobel & Daisy, 2003) and at that time only few secondary metabolites have been isolated from endophytes (Tan & Zou, 2001). Besides, the secondary metabolites produced by endophytes may vary from one species to another. Hence, this study is carried out to identify the species of endophytes present in the wild grass (*Scleria sumatrensis* Retz.) and to determine the antimicrobial activity of secondary metabolites produced by endophytes. This may lead to the discovery of new antibiotics which can deal with those antibiotic resistant bacteria.

The objectives of this study are to:

1. isolate endophytes from a selected wild grass, *Scleria sumatrensis* Retz.
2. identify the species of endophytes that have potentials to produce antibiotics.
3. isolate the antibiotics produced by endophytes.
4. determine the antimicrobial activity of endophytes by Kirby-Bauer disc diffusion method.

2.0 Literature Review

2.1 Antibiotics

Antibiotics are secondary metabolites with antimicrobial properties produced by a particular microorganism in order to compete for nutrients or living space by direct killing (bactericidal) or inhibiting the growth (bacteriostatic) of other microorganisms (Hogg, 2005). Antibiotics are effective against both fungal and bacterial infections but antibiotics do not work against viral infections. Besides antibiotics produced by microorganisms, there are also semi-synthetic antibiotics which are derived from the naturally produced antibiotics. For example, ampicillin is a semi-synthetic antibiotic derived from Penicillin G by which the core structure of the penicillin molecule (6-aminopenicillanic acid) is retained with the addition of chemically synthesised side chains onto the core structure.

Antibiotics are classified into four major groups based on their antimicrobial mechanism (Hogg, 2005). The mechanisms exerted by antibiotics are the inhibition of cell wall synthesis (group I), disruption of cell membranes (group II), interference with protein synthesis (group III) and interference with nucleic acid synthesis (group IV). Among these mechanisms, antibiotics belonging to group I and group III show higher therapeutic properties and better selective toxicity compared to the other groups. Therefore, group I and group III are more effective in treating microbial infections.

Antibiotics can be natural products synthesised by microorganisms or synthetic compounds synthesised by chemists. Soil microorganisms are the main antibiotic producing microorganisms. Nevertheless, other microorganisms such as endophytes have been thoroughly studied in order to discover new antibiotics from them.

2.2 Antibiotic Resistance in Bacteria

Bacteria which show no significant effect to a particular antimicrobial agent, such as penicillin are considered to be resistant to that particular antibiotic. The emergence of antibiotic resistant bacteria has increased the demand for new antibiotics (WHO, 2002). There are four major mechanisms which render bacteria to become resistant to antibiotic (Hawkey, 1998; Chartrand *et al*., 1996). These mechanisms include antibiotic modification or antibiotic inactivation, decreased membrane permeability or increased antibiotic efflux, alteration in the target site and production of an alternative target. Among these mechanisms, antibiotic modification is the most common and well-known mechanism which render antibiotic resistance in bacteria (Hawkey, 1998).

Antibiotic resistance in bacteria can be induced by either intrinsic or acquired pathways (Wickens & Wade, 2005). Intrinsic or innate antibiotic resistance pathway is a naturally occurring event in bacteria. The bacteria may become resistant to a particular antibiotic even though they are not previously exposed to that antibiotic. For instance, *Acinetobacter baylyi* is resistant to a number of antibiotics such as β-lactam antibiotics by which it is induced by intrinsic pathway (Gomez & Neyfakh, 2006).

On the other hand, acquired antibiotic resistance pathway usually exists in bacteria which have been previously exposed to the antibiotic. This is mainly caused by gene mutation or the introduction of resistance genes into the bacteria (Hawkey, 1998; Wickens & Wade, 2005). Mutation is spontaneously occurred in bacteria in which the bacterial genes are mutated and become resistance to a particular antibiotic. Introduction of new gene into the bacteria is usually achieved via conjugation, transformation. transposition or transduction (Wickens & Wade, 2005).

2.3 *Scleria sumatrensis* Retz. (Family: Cyperaceae)

Scleria sumatrensis Retz. is commonly known as nutrush (ITIS, 2010). It is a type of robust grass which can be found throughout the year and it is also spread across a wide range of habitat. The habitat of *S. sumatrensis* is ranged from dry grassy forests to wet forests (PIER, 2006). This is mainly due to its ability to withstand the keep changing and extreme environmental conditions.

Many studies were carried out to discover bioactive compounds from the endophytes reside within grasses belonging to Cyperaceae family. For instance, *Mapania* sp. which belongs to Cyperaceae family was studied in order to discover the bioactive endophytic streptomycetes reside within the plant (Zin *et al*., 2007). The endophytic streptomycetes was found to be active against some pathogenic fungi such as *Aspergillus fumigates, Mycosphaerella fijiensis, Pythium ultimum, Sclerotinia sclerotiorum, Rhizoctonia solani* and *Phytophthora erythroseptica*.

2.4 Endophytes

The term "endophytes" was first used by De Bary in 1866 with the purpose of distinguishing between those fungi which live within healthy plant tissues from epiphytes, fungi which live on the outer surface of the host plant. However, endophytes are not only referred to fungi, it can also refer to bacteria, algae and insects that colonised within the host plant (Schulz & Boyle, 2005). According to Carroll (1988), endophytes can be transmitted vertically through the host seeds or horizontally through the spores generated by the host plants. Endophytes can be found in both temperate and tropical rainforests (Strobel & Daisy, 2003). However, Bills *et al*. (2002) stated that tropical endophytes produce more active secondary metabolites compared to temperate endophytes.

According to Carroll (1988), most endophytes infect a small fraction of a host population. But, for some endophytes, the infection can spread throughout the host population. The interaction between host plant and endophytes is usually recognized as mutualism in which both host plant and endophytes are benefited from each other. In addition to mutualism, the interaction between host plant and endophytes can also be facultative saprophytes and even parasitic based on the ecological role played by the endophytes within the host plant (Schulz & Boyle, 2005).

2.4.1 Fungal endophytes

According to Schulz and Boyle (2005), fungi reside within the tissues of the host plant without causing visible disease symptoms are termed "fungal endophytes". Fungal endophytes can be divided into three ecological groups that are mycorrhizal fungi, balansiaceous or grass endophytes and non-balasiaceous endophytes. Fungal endophytes are more common and more widely studied by researchers as compared to bacterial endophytes (Strobel & Daisy, 2003).

Generally, the interaction between fungal endophytes and their host plants is recognised as mutualism. The host plants provide nutrients as well as protection from abiotic stresses and epiphytic competition to the fungal endophytes. In contrast, fungal endophytes produce secondary metabolites to protect the host plants against both abiotic and biotic stresses such as phytopathogens and herbivorous predators. Nevertheless, the interaction between fungal endophytes and their host plants may also shift to slightly pathogenic or antagonistic (Arnold, 2007).

2.5 Secondary Metabolites from Endophytes

Secondary metabolites produced by endophytes can be widely applied in many areas, such as medical, agricultural and industrial exploitation (Strobel & Daisy, 2003). A variety of secondary metabolites are produced by endophytes and each metabolite has its own unique function. Secondary metabolites produced by endophytes can be classified into antibiotics or antimicrobial agents, antiviral compounds, insecticides, antioxidants, anticancer agents, antidiabetic agents and immunosuppressive compounds.

Among this classification, secondary metabolites with antibiotic properties are best studied by researchers in order to discover new antibiotics which can be used to deal with human pathogenic microorganisms. However, secondary metabolites successfully isolated from endophytes are relatively less than secondary metabolites isolated from fungal phytopathogens and fungal soil isolates (Tan & Zou, 2001).

Antibiotics produced from endophytes are effective against phytopathogen as well as human and animal pathogenic microorganisms (Strobel & Daisy, 2003). According to Miller *et al*. (1998), *Pseudomonas viridiflava* is an endophyte associated with many grass species and lettuce. *P. viridiflava* will produce ecomycins, an antifungal agent which actively against human pathogenic fungi, such as *Cryptococcus albicans* which causes candidiasis.

Moreover, *Streptomyces* NRRL 30562, an endophyte in *Kennedia nigriscans* produced a variety of antibiotics which possess different biological activities toward target organism (Castillo *et al*., 2002). The antibiotics produced are called munumbicins. Munumbicins are actively against Gram positive bacteria and multi-drug resistant bacteria such as *Mycobacterium tuberculosis*. A derivative of munumbicin called munumbicin D is effective against malarial parasite, *Plasmodium falciparum*.

2.6 Grass-Endophytes Symbiosis

Grasses are usually lacking of certain secondary metabolites which are fatal to herbivores and pests (Bacon & White, 2003). Hence, grasses are easily infected and killed by phytopathogens. Nevertheless, endophytes associated with grasses will produce a variety of secondary metabolites which may protect the host from grazing animals and confer the ability to withstand environmental stresses, such as drought to the host. Besides, endophytes-infected grasses will also show improved growth and better reproduction capability (Marks *et al*., 1991). Much research has been conducted to discover the species of endophytes reside within the grasses.

Lolium arundinaceum, formerly known as *Festuca arundinacea* (Darbyshire, 2007), has been well studied by researchers due to its high survival rate in the ecological system worldwide (Rudgers & Clay, 2007; Sullivan *et al*., 2007). The grass species is frequently infected by the wild type KY-31 endophyte *Neotyphodium coenophialum.* The host grass provides living environment and photosynthate such as sugar to the fungus. *N. coenophialum* utilises the sugar and convert it to a variety of primary metabolites through both glycolysis and TCA cycle (Scott, 2001). Subsequently, the primary metabolite will be used as a substrate for the synthesis of secondary metabolite such as ergovaline. Ergovaline is a member of ergot alkaloid (nitrogen-based alkaloid) and it exhibit neurotoxicity effect to insects and mammal herbivores (Tan & Zou, 2001).

Moreover, *Neotyphodium lolii,* an endophyte that reside within *Lolium perenne* (perennial ryegrass) shows a similar symbiotic relationship as *L. arundinaceum* and *N. coenophialum* (Schardl & Moon, 2003). *N. lolii* possesses a pathway of biosynthesis of secondary metabolites that is similar to the pathway shown in *N. coenophialum*. However,

instead of producing ergovaline as in *N. coenophialum*, *N. lolii* tends to produce indole-diterpene alkaloids such as loliterm B which is a mammalian toxin (Panaccione, 2001).

2.7 Antibacterial Screening Test

Antibacterial screening test is applied to determine the susceptibility of the bacteria toward a particular antimicrobial agent. Many methods were developed for antimicrobial activity screening, for instances, agar overlay technique, Kirby-Bauer disc diffusion method, agar well diffusion method and tube dilution assay (Hogg, 2005). Among these methods, Kirby-Bauer disc diffusion method is the most commonly used method in determining the antimicrobial activity exhibited by the particular antimicrobial agent.

2.7.1 Agar overlay technique

Agar overlay technique can also be called as agar diffusion method (Janssen *et al*., 1986). It is a technique developed to determine the degree of antimicrobial activity for a particular antimicrobial agent towards microorganisms. According to Hostettmann (1998), this technique is widely applicable to initial or preliminary antimicrobial screening of antimicrobial agents for a wide range of microorganisms. Moreover, agar overlay technique is much easier to perform and even it will produce clearer and sharper zone of inhibition compared to disc diffusion method (Barry & Badal, 1982).

2.7.2 Kirby-Bauer disc diffusion method

Kirby-Bauer disc diffusion method is the most commonly used method in antibacterial screening test. According to Boyle *et al.* (1973), Kirby-Bauer disc diffusion method is the

standard method recommended by FDA for use in antimicrobial activity screening analysis. This is mainly due to the simplicity and rapidity of Kirby-Bauer disc diffusion method in determining the antimicrobial activity of a particular antimicrobial agent. According to Huys (2002), the degree of susceptibility of microbes to the antibiotic is directly proportional to the diameter of the zone of inhibition formed around the disc. The larger the zone of inhibition formed around the disc, the higher the degree of susceptibility of the microbes to the particular antibiotic.

3.0 Materials and Methods

3.1 Preparation of Culture Media

3.1.1 Preparation of PDA

PDA medium in Petri dish was prepared by dissolving 39g of PDA powder in 1000ml of distilled water. Then, the PDA solution was heated until boiling by hot plate with stirrer to completely dissolve the powder. The PDA solution was sterilised by autoclaving at 121^{o}C with the pressure of 15psi. The sterilised PDA solution was poured into Petri dishes with approximate 13ml each and left at room temperature until the PDA solidified.

3.1.2 Preparation of V8 juice agar

A can of V8 juice with a volume of 163ml was filtered through cheesecloth. The filtrate was collected in the beaker whereas the fiber left behind the cheesecloth was discarded. A total of 100ml of V8 juice was measured by using measuring cylinder in preparing 500ml V8 juice agar.

A total of 7.5g of Bacto agar and 1.0g of $CaCO_3$ were measured and dissolved in 400ml of distilled water. Then, the 100ml of V8 juice was added into the Bacto-$CaCO_3$ mixture. The solution was heated until boiling by using hot plate with stirrer to completely dissolve the powder and evenly distribute the V8 juice throughout the solution. This was followed by autoclaving the solution at 121^{o}C and 15psi. The sterilised V8 juice agar suspension was poured into Petri dishes with approximate 13ml each and left at room temperature until the V8 juice agar solidified.

3.2 Samples Collection

Ten samples of wild grass, *Scleria sumatrensis* Retz., were collected from five areas at the East Campus of UNIMAS (Appendix A). The samples were placed in separate unused polythene bags and brought to the laboratory.

3.3 Surface Sterilisation

The collected samples were washed thoroughly with sterile distilled water and then air dried at room temperature prior to surface sterilisation. The stem of the *S. sumatrensis* was cut into 3cm pieces while the other parts of the wild grasses were discarded. The stems were first soaked in 5% Clorox for 5 minutes. This was followed by soaking the stems in 70% ethanol for further 5 minutes. Finally, the stem samples were soaked thrice in sterile distilled water for 1 minute each.

3.4 Endophytes Cultivation

The stems were dry-blotted on the sterile filter paper to remove the excess water present on the stems. Subsequently, the stems from the same wild grass were placed on the PDA plate. The PDA plates were incubated at room temperature for 5 days or 120 hours until sufficient growth of endophytes was established.

3.5 Subculture of Endophytes

The endophytes growing from the cut ends of the cultured stems were subcultured onto new PDA plates (first subculture). Five days after incubating at room temperature, the