UNIVERSITI PUTRA MALAYSIA

ASSESSMENT ON DIVERSITY AND BIOACTIVE COMPOUNDS PRESENT IN ENDOPHYTIC FUNGI ISOLATED FROM Rhizophora mucronata *IN MATANG MANGROVE FOREST RESERVE, PERAK, MALAYSIA*

TUAN NORAIDA TUAN HAMZAH

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By

TUAN NORAIDA TUAN HAMZAH

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirements for the Degree of Master of Science

April 2018

DEDICATION

In the name of Allah S.W.T., the most Benevolent and ever Merciful
All praise be to Allah S.W.T.

Specially dedicated to:

My Parents

TUAN HAMZAH SAYED MOHD & TENGKU SUPIAH TENGKU BUANG

My Siblings

TUAN SARIF TUAN HAMZAH
TUAN SHARIFATUL MUZLIM TUAN HAMZAH
TUAN SHARIHAZIL TUAN HAMZAH
TUAN SHAHRIHAIRUL AMI TUAN HAMZAH
TUAN SHARIFAH NURHAFISAH TUAN HAMZAH
TUAN SARIFAH SAHARIAH TUAN HAMZAH
SAYED HASSAN BASRI TUAN HAMZAH
SAYED KHALIL KHUSAIRI TUAN HAMZAH

My Fiancée

MUHAMMAD AMIRUL SHAFIQ ZAINAL SHAH

Abstract of thesis presented to the Senate of the Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

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By

TUAN NORAIDA BINTI TUAN HAMZAH

April 2018

Chairman : Prof. Rozi Mohamed, PhD
Faculty : Forestry

Rhizophora mucronata is an important ecosystem entity of the Malaysian mangrove forest. Because the tree grows in an extreme environment, any organism that is isolated from this tree is of huge interest due to its potential in having novel bioactive compounds. This study aimed to assess endophytic fungal diversity isolated from mangrove plant, *R*. *mucronata*, to evaluate the bioactivities exhibited by selected fungal endophytes, and to isolate and identify compounds associated with the selected endophytic fungi extracts. In the present work, a total of 78 fungal isolates were isolated, identified and characterized from the leaf tissues of *R. mucronata*. All strains were identified using primer internal transcribed spacer 1 (ITS1) and internal transcribed spacer 4 (ITS4). The DNA sequences of the strains recorded high similarities to their respective species in the GenBank. Phylogenetic trees were constructed and analyzed using Maximum Likelihood (ML) criteria together with 78 mangrove endophytic fungal sequences and 117 additional sequences of fungal species incorporated from the GenBank. Most of the dominating fungal endophytes were from the genus *Pestalotiopsis*, followed by *Alternaria* and *Cladosporium*. Six isolates representing the genera *Alternaria*, *Fusarium*, *Nigrospora*, *Pestalotiopsis*, *Phoma*, and *Xylaria*, were screened for their antagonism activities. The antagonism tests were evaluated through dual culture and non-volatile compound assay. Dual culture test assay revealed their inhibition percentages against the phytopathogenic fungus *Fusarium solani* between 49-69%, and 1-25% when using non-volatile test assay. The fungal isolates were further screened for their antibacterial activities against four pathogenic bacteria, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Of the six isolates, only *Fusarium lateritium* and *Xylaria* sp. showed antibacterial activities against tested bacteria, with the Minimum Inhibitory Concentration

(MIC) and Minimum Bactericidal Concentration (MBC) ranging from 0.5 to 2 mg/mL. The 1,1-diphenyl-2-picrylhydrazyl(DPPH) radical scavenging assay recorded antioxidant activity in *Xylaria* sp. with 3-fold higher when compared to *F. lateritium*.

The chemical constituents of *F. lateritium* and *Xylaria* sp. were determined via Gas Chromatography (GC) with Mass Spectrometry (MS) and Flame Ionization Detector (FID). These two fungal species were chosen based on their positive activities in several assays conducted in the previous section before, including, antagonism, antibacterial and antioxidant assay. Overall, 69 compounds were identified from ethyl acetate and hexane extracts of *F. lateritium* and *Xylaria* sp..Compounds such as phenylethyl alcohol, phenylacetic acid, cetene, and 2,4-Ditert-butylphenol, were commonly found in all the extracts of both fungal species. Variations of constituents were found in all extracts for both fungal isolates. Hexane extracts of *F. lateritium* and *Xylaria* sp. both recorded a greater variation in chemicals identified. While there were almost similar compounds identified from ethyl acetate extracts of both fungal isolate. Evaluation on the biological activities exhibited by *F. lateritium* and *Xylaria* sp. before, with the presence of these compounds, proved that these two fungal isolates have great potential as antimicrobial agents. Screening the endophytic fungal community associated with *Rhizophora mucronata*, and bioactivities exhibited by the selected isolates has proved that mangroves endophytic fungi could be a potential source in finding the bio-control agent and another important source for vital bioactive compounds.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGUKURAN DALAM KEPELBAGAIAN KULAT ENDOFITIK DAN BIOAKTIF KOMPAUN YANG BERSEKUTU DENGAN *Rhizophora mucronata* DI HUTAN SIMPAN PAYA BAKAU MATANG, PERAK, MALAYSIA

Oleh

TUAN NORAIDA BINTI TUAN HAMZAH

April 2018

Pengerusi : Prof. Rozi Mohamed, PhD
Fakulti : Perhutanan

Rhizophora mucronata adalah entiti ekosistem penting di hutan bakau Malaysia. Oleh kerana pokok bakau ini tumbuh dalam persekitaran yang melampau, mana-mana organisma yang dipencilkan daripada pokok ini mempunyai kepentingan yang besar kerana ia berpotensi dalam menghasilkan kompaun bioaktif yang baru. Kajian ini bertujuan untuk mengakses diversiti kulat endofitik yang di pencilkan dari pokok bakau, *R. mucronata*, untuk mengenal pasti aktiviti biologi yang dihasilkan oleh kulat endofitik yang terpilih, dan juga untuk mengenalpasti kompaun kimia yang terdapat di dalam ekstrak kulat endofitik tersebut. Dalam kajian ini, sejumlah 78 kulat telah dipencilkan daripada tisu daun *R. mucronata* dan diidentifikasi. Semua pencilan telah dikenal pasti dengan menggunakan pencetus penjarak jujukan dalam 1 (ITS1) dan penjarak jujukan dalam 4 (ITS4). Jujukan DNA daripada pencilan yang direkodkan mempunyai persamaan tinggi dengan spesies masing-masing dalam pangkalan data GenBank. Pokok filogenetik telah dibina dan dianalisis menggunakan kriteria *Maximum Likelihood* (ML) bersama-sama dengan 78 jujukan kulat endofitik paya bakau dan 117 jujukan jujukan kulat tambahan dari GenBank. Sebahagian besar kulat endofitik didominasi oleh genus *Pestalotiopsis*, diikuti oleh *Alternaria* dan *Cladosporium*. Enam pencilan mewakili genus *Alternaria*, *Fusarium*, *Nigrospora*, *Pestalotiopsis*, *Phoma* dan *Xylaria*, selanjutnya disaring untuk aktiviti antagonis. Aktiviti antagonis setiap kulat di akses melalui ujian dwikultur dan juga ujian tidak meruap. Ujian dwikultur telah mempamerkan peratusan perencatan terhadap kulat patogenik *Fusarium solani* di antara 49-69%, dan 1-25% apabila menggunakan ujian tidak meruap. Daripada enam pencilan, hanya *Fusarium lateritium* dan *Xylaria* sp. menunjukkan aktiviti antibakteria terhadap bakteria patogenik, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa* dan *Staphylococcus aureus*, dengan

Kepekatan Minimum Perencatan (MIC) dan Kepekatan Minimim Bakteria (MBC) yang terdiri daripada 0.5-2 mg/mL. Ujian (*1,1-diphenyl-2-picrylhydrazyl*) DPPH radikal memerangkap mencatatkan aktiviti antioksidan dalam *Xylaria* sp. sebagai 3 kali ganda lebih tinggi berbanding *F. lateritium*. Dua kulat endofitik, *F. lateritium* dan *Xylaria* sp. telah menunjukkan aktiviti dalam pelbagai ujian yang dijalankan dengan pelbagai reaksi.

Konstituen kimia dua spesies kulat endofitik telah ditentukan melalui Gas Chromatography (GC) dengan Mass Spektrometri (MS) dan Flame Pengionan Detector (FID). Kedua-dua spesies kulat ini dipilih kerana aktiviti positif mereka dalam beberapa ujian yang telah dijalankan sebelum ini, termasuk ujian antagonis, antibakteria dan antioksidan. Secara keseluruhan, 69 kompaun telah dikenal pasti daripada ekstrak etil asetat dan heksana *F. lateritium* dan *Xylaria* sp.. Kompaun seperti *phenylethyl alcohol*, *phenylacetic acid*, *cystiene*, *cetene* and *2,4-Ditert-butylphenol*, telah ditemui dalam ekstrak kedua-dua spesies kulat. Kedua-dua ekstrak heksana *F. lateritium* dan *Xylaria* sp., telah merekodkan lebih banyak kepelbagaian dalam kompaun kimia yang dikenal pasti. Walaubagaimanapun, untuk ekstrak etil asetat, sebatian yang telah dikenal pasti daripada kedua-dua isolat kulat adalah hampir sama. Penilaian mengenai aktiviti biologi yang dipamerkan oleh *F. lateritium* dan *Xylaria* sp. dalam seksyen sebelum ini, dan juga dengan pengenalpastian kompaun-kompaun penting seperti ini, membuktikan bahawa dua ekstrak kulat tersebut mempunyai potensi yang besar sebagai antimikrob. Saringan komuniti endofitik kulat yang terdapat di dalam tisu daun *R. mucronata* dan bioaktiviti yang dipamerkan oleh pencilan yang dipilih membuktikan bahawa kulat endofitik yang dipencilkan dari pokok bakau boleh menjadi satu sumber dalam pencarian agen kawalan biologi. Selain itu, ia juga penting bagi tujuan pengekstrakan kompaun-kompaun yang mempunyai aktiviti anti-mikrobial.

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This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

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This is to confirm that:

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Name of Chairman of Supervisory Committee: Prof. Dr. Rozi Mohamed

Signature: ____________________
Name of Member of Supervisory Committee: Dr. Razak Terhem

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LIST OF ABBREVIATIONS

A	Alpha
B	Beta
ha	Hectare
km	Kilometer
^{O}C	Degree Celcius
µg	Microgram
µL	Microliter
µM	Micromolar
mM	Millimolar
ng	Nanogram
PCR	Polymerase Chain Reaction
µm	Micrometre
MMFR	Matang Mangrove Forest Reserve
CCD	Charged Couple Device
dbh	Diameter at breast height
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
E	East
EV	Electric vehicle
FID	Flame ionization detector
G	Gram
GC	Gas Chromatography
H	Hour
ITS	Internal Transcribed Spacer

M	Metre
Mg	Milgram
Min	Minute
ML	Maximum likelihood
GTR	General time reversible
G+I	Gamma distributed with invariant sites
mL	Millilitre
MS	Mass spectrometry
NCBI	NationalCentre for Biotechnology Information
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
DPPH	1,1-diphenyl-2-picrylhydrazyl
NaOCl	Sodium hypochlorite
BSV	Bootstrap value
PEA	Phenylethyl alcohol
DTBP	2,4-Ditert-butylphenol
FORDIA	Forest Research, Development and Innovation Agency
Rpm	Revolutions per minute
RI	Retention index
UPM	Universiti Putra Malaysia
IBS	Institute of Bioscience

CHAPTER 1

INTRODUCTION

1.1 General

Fungal species identification is estimated to have reached up to 1.5 million fungal species (Hawksworth, 2004), and of these, only 7% were being identified (Schmit and Muller, 2007). Of these values, marine fungal species constituted roughly about 1,500, since it was last recorded by Hyde et al. (1998) which excluded lichens and also many more fungi that are not yet fully described (Thatoi *et al*., 2013). These values, however, are increasing annually, since most researchers are starting to realize how vital it is to discover the undiscovered. Different areas, or regions might provide the researchers with varies fungal distribution. This is due to fungal behaviors which sometimes can be selective towards specific niches and ecosystem. Despite the limited information available on these fungal species, hints that captured by most researchers are, it is vital for them to investigate more on the fungal diversity, hoping to discover novel fungal species from the various niche (de Souza Sebastianes *et al*., 2013). The variety of fungal species might render different benefits. Thus, this makes it an important task for the researchers in investigating the fungal diversity and abundance, but also the bioactive compounds they potentially produced. Previously, high numbers of described metabolites have been linked to the investigation of the terrestrial sources of where the fungi have been isolated (Bugni and Ireland, 2004). Therefore, natural products chemists and pharmacologists have diverted their attention from studying typical areas to areas that were less investigated. Examples of such scarcely explored niches that might offer a significant amount of unprecedented bioactive compounds are ecological areas such as oceans and rainforest such as mangroves. Mangroves are unique intertidal forest wetlands formed at the interface between land and sea which typically occurred in tropical and sub-tropical latitudes with diverse organisms. The global extent of mangroves are 152, 360 km2, with 51,050 km2 of them are from Southeast Asia (Spalding, 2010). In Southeast Asia, Indonesia harbored the most mangroves with 31,890 km2 coverage. While Malaysia ranked second with 580,000 ha of mangroves (34,000 ha are in Sabah, 140,000 ha in Sarawak, and 100,000 ha in Peninsular Malaysia). Mangrove forests protect coastlines from wave action and prevent coastal erosion, besides reducing damages inland caused by storms. They are well adapted to their extreme environmental conditions of high salinity, fluctuations in sea level, high temperatures and anaerobic soils, through pneumatophores roots, salt excreting leaves and viviparous water dispersed propagules. Not only mangrove plants but organisms including fungi, inhabiting the environment will also have to adapt in their own ways to survive the harsh conditions of the mangroves. Out of a large number of estimated fungal species, Hyde listed 120 fungal species, originated from 29 mangroves around the globe (Hyde, 1990). These included 87 Ascomycetes, 31 Deuteromycetes, and 2 Basidiomycetes. Up to the year 1995, 169 species of fungi have been described to be isolated from Malaysian mangroves (Alias *et al*., 1995). Fungi adapted themselves with the environment by altering their metabolic pathways and producing unique secondary metabolites that may help them to survive the conditions (Ronsberg *et al*. 2013). These facts make endophytic fungus isolated from such environment as a promising target in the search for novel secondary metabolites (Debbab *et al*. 2012).

Secondary metabolites are produced resulting from the modification and combinations of reactions from primary metabolic pathways (Roopa *et al*., 2015). These bioactive compounds commonly found in various plant species despite different genera or family, and various metabolites can be expressed from a single species under different environmental conditions (Shukla *et al*., 2014). The commonly distributed metabolite groups are alkaloids, polyketides, terpenes, and steroids (Roopa *et al*., 2015). There were a tremendous number of significant bioactive compounds that have been extracted from the plants, for example, taxol or paclitaxel which was extracted from *Taxus brevifolia* (Zhao *et al*., 2010). Taxol is a chemical substance that has been widely used as anticancer drugs and also effective against non-cancerous conditions like the polycystic kidney disease (Zhou *et al*., 2010; Malik *et al*., 2011). Taxol extraction from the tree of *T. brevifolia* has been limited due to several factors such as the slow-growing of Taxus tree, low yield of taxol production from Taxus tree, besides may cause death to the tree, due to the removal of the Taxus barks (Rooppa *et al*., 2015). This limitation has caused researchers to find the alternative method for isolating compounds like taxol and other bioactive compounds (Roopa *et al*., 2015) from organisms such as bacteria and fungi.

Endophytic fungi isolated from mangroves have illustrated its ability in producing tremendous novel bioactive compounds (Elavarasi *et al*., 2012). They were proved to be a potential reservoir of natural compounds with a vital pharmacological activity that could be an advantage in the development of novel medicinal agents (Zhang *et al*., 2006). There are more than 200 endophytic fungal species that have been successfully isolated and identified from mangrove plants and have been demonstrated to be a well-established source for structurally diverse and biologically active secondary metabolites (Pang and Mitchell, 2005; Li *et al*., 2009). These endophytic fungi carry a vital role as potential biological control agents, sources of novel compounds for disease treatment and crop protection. Many have reported that novel secondary metabolites that were extracted from mangrove fungi are widely used in the pharmaceutical industry as primary compounds for several drugs includes antiviral, anticancer, antibiotic and immunosuppressive drugs (Blunt *et al*., 2013). Generally, the production of secondary metabolites that are potentially useful for pharmaceutical and agricultural applications is wide-ranging among mangroves endophytic fungi (Maria *et al*., 2005). There were a variety of chemical entities with unique structures and potent bioactivities have been isolated from mangrove endophytic fungi and suggested to have a high potential to be exploited in various fields such as agriculture, medicine and industrial field (Nithya and Muthumary, 2011). One interesting example is a compound that was extracted from unknown endophytic fungi, isolated from mangrove plant, *Kandelia candel* and was characterized as cyclic depsipeptide 1962A. The compound exhibited significant cytotoxic activities when tested against human breast cancer during MTT assay (Huang *et al*., 2007). While, in an investigative study performed by Lin et al. (2008), two polyketides that were extracted from endophytic fungi, *Penicillium* sp., which was previously isolated from a mangrove plant, *Aegiceras corniculatum*, also exhibited strong cytotoxic activity.

1.2 Problem Statement

In Matang Mangrove Forest Reserve (MMFR) Perak, in the year 2000-2009 working plan, the research needs including, evaluating the economic viability of using stumps for charcoal purposes, the impact of agricultural bunds on mangrove vegetation, and floristic diversity and conservation. While in the present working plan of 2010-2019, the management has recommended several new and additional research needs that shall be conducted in MMFR, including screening for pharmacological activities of mangrove plant species. Despite the million benefits own by the mangrove endophytic fungi, no studies on microorganisms particularly fungi associated with mangrove plant have been performed in MMFR. Matang Mangrove Forest Reserve is known as the best-managed mangrove forest. However, the organisms present in the environment is not well studied. Previous studies (de Souza Sebastianes *et al*., 2013; Li *et al*., 2016), have described that there was a variety of fungal diversity isolated from several mangrove species involving regions such as Brazil and China. While in Malaysia, there were only several studies investigating the organisms associated with mangrove species and its benefits. More research is needed in order to discover the potential of these mangrove endophytic fungi. Not only the genetic diversity of the isolated mangrove endophytic fungi but also the potential bioactivities that could be exhibited by the fungi. To date, only several studies on the mangrove endophytic fungi have been conducted in Malaysia particularly on the diversity and the production of bioactive compounds.

1.3 Justification

Matang Mangrove Forest Reserve is globally known as the best-managed mangrove forest. However, the organism documentation since the previous plan included only macro-organisms such as vegetations, faunas such as crabs and birds. While an ecosystem is made up of not only macro-organisms but microorganisms such as bacteria and fungi. Therefore, assessing the genetic diversity of mangroves endophytic fungi associated with the mangrove plants could be used in establishing the diversity of organisms in MMFR. Along with the state forestry management work plan, to screen the pharmacological properties of the mangrove plant, this study will provide in-depth investigation focusing not only the mangrove plant but also organisms associated with the mangrove plant. Evaluating the biological activities potentially exhibited by the fungal endophytes could be a preliminary step in investigating the potential benefits owned by the mangroves endophytic fungi. Identification of chemical constituents of these mangrove endophytic fungi may prove that mangroves harbor not only variety of fungal species, but also fungal with diverse bioactive compounds.

1.4 Objectives

The general objective of this study was to assess the genetic diversity of mangrove endophytic fungi and its potential bioactivities isolated from mangrove plant, *Rhizophora mucronata*, from Matang Mangrove Forest Reserve (MMFR), Perak.

The specific objectives of this study include:

1. To assess the genetic diversity of mangrove's endophytic fungi isolated from the mangrove plant, *Rhizophora mucronata* by DNA sequencing of the ITS1 and ITS4 regions.
2. To evaluate potential bioactivities exhibited by selected endophytic fungal species via *in vitro* studies.
3. To determine the chemical constituents of the selected endophytic fungal species via GC-MS and GC-FID.

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