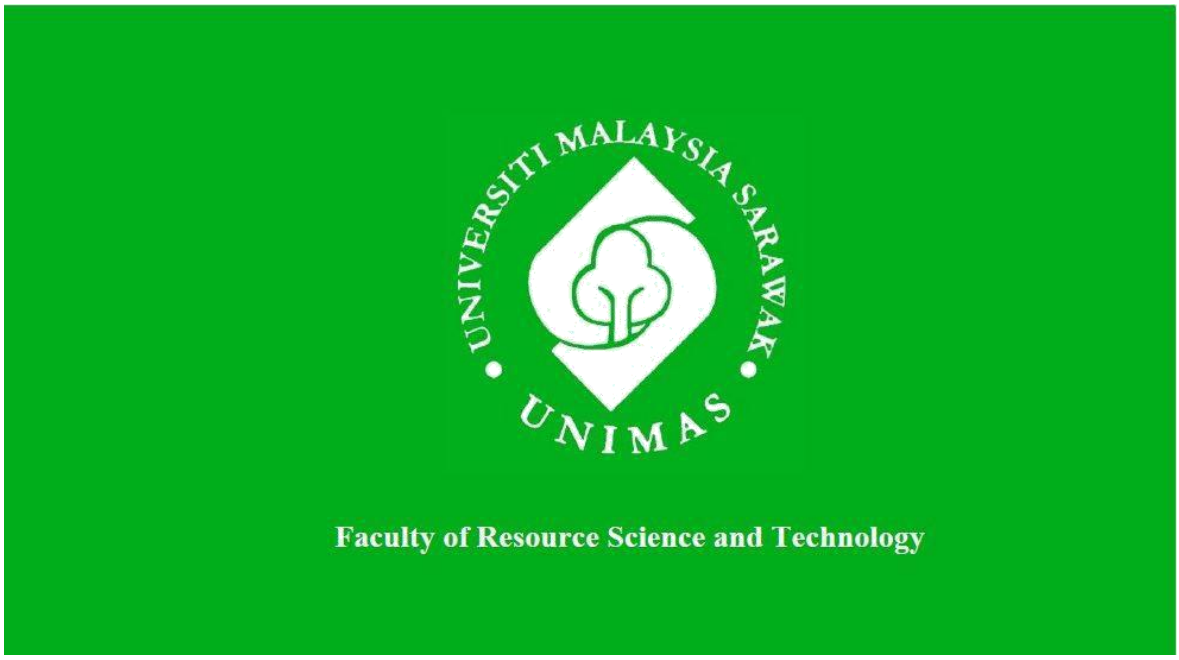

Bioactivities of Methanol Extracts from *Rhizophora apiculata* Blume and *Rhizophora mucronata* Lam. Barks

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Bachelor of Science with Honours
(Plant Resource Science and Management)
2015

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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree qualification of this or any other university or institution of higher learning.

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Bioactivities of Methanol Extracts from *Rhizophora apiculata* Blume and *Rhizophora mucronata* Lam. Barks

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ABSTRACT

Natural product has been increasingly popular among the consumers, people starts to look for alternative from these natural products than continuing using the synthetic products especially for medicinal or domestic appliances. The objectives of this study were, first to determine the amount of extractives present in the bark of *R. apiculata* and *R. mucronata* using methanol as the solvent, and second was to determine the toxicity of the bark extracts against pathogenic microorganisms through series of bioassay tests at different concentration. For the study on the antifungal properties, bioassay was performed on *Fusarium oxysporum* and *Aspergillus niger* using agar dilution method while paper disc diffusion method was performed on *Candida albicans*. The minimal inhibition concentration for the tested fungi was 1 % for agar dilution method and for *C. albicans*, 0.01 % of the extracted crude. Screening on the antibacterial properties of the methanol extract, paper disc diffusion method was used on *E. coli* and *S. aureus* at different concentration. Among the two extracts, *R. mucronata* showed higher potential with antifungal and antibacterial than compared with bark extract from *R. apiculata* however after performing a two way ANOVA, it was revealed that there was no significant different on the antibacterial properties between the two studied species.

Key words: bark extract, methanol, natural compounds, *R. apiculata, R. mucronata,* toxicity

ABSTRAK

Produk asli merupakan alternatif yang dicari oleh pengguna untuk kegunaan perubatan atau kegunaan harian disamping mereka cuba untuk mengurangkan kebergantungan terhadap produk-produk buatan. Dalam kajian ini, objektif yang pertama, adalah untuk menentukan jumlah ekstraktif yang ada dalam kulit kayu spesies *R. apiculata* dan *R. mucronata* menggunakan metanol sebagai pelarut, dan objectif yang kedua pula adalah untuk mengenal pasti ketoksidan kulit kayu tersebut terhadap mikroorganisma-mikroorganisma yang berpotensi menyebabkan penyakit melalui ujian bioasei pada kepekatan yang berbeza. Satu siri ujikaji biologi telah dijalankan keatas *Fusarium oxysporum* dan *Aspergillus niger* menggunakan teknik pelarutan agar manakala untuk *Candida albicans* teknik cakera kertas telah digunakan untuk mengkaji sifat anti-kulat dalam esktrak kulit kayu yang diperolehi. Takat minimal kepekatan untuk menghalang tumbesaran kulat-kulat yang diuji ialah 1 % untuk teknik agar dan untuk *C. albicans*, 0.01% diperlukan. Teknik cakera kertas juga digunakan untuk penyaringan antibacteria pada kepekatan yang berbeza di atas bacteria *E. coli* dan *S. aureus.* Sifat antikulat dan antibacteria bagi *R. mucronata* lebih tinggi berbanding *R. apiculata* tetapi setelah menjalankan analisis ANOVA dua hala, perbezaan diantara kedua-dua species terlalu kecil untuk memberi apa-apa signifikan diatas sifat antibacteria ekstrak yang diuji.

Kata kunci: ekstrak kulit kayu, methanol, *R. apiculata, R. mucronata,* ketoksidan

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LIST OF ABBREVIATIONS

A. niger	*Aspergillus niger*
anti-HIV	anti-human immunodeficiency virus
C. albicans	*Candida albicans*
DCM	dichloromethane
E. coli	*Escherichia coli*
F. oxysporum	*Fusarium oxysporum*
HE	hexane
MeOH	methanol
MIC	Minimum Inhibition Concentration
OM	outer membrane
R. apiculata	*Rhizophora apiculata*
R. mucronata	*Rhizophora mucronata*
S. aureus	*Staphylococcus aureus*
UTI	urinary tract infectious

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CHAPTER ONE

INTRODUCTION

1.1 General Background

In nature, the higher plants are capable of producing diverse chemical compounds in their secondary metabolic pathways in order to ensure their survival in their ecology. These chemical compounds act as a defense mechanism to protect these plants against herbivores, insects, fungal or disease infection while some of these secondary metabolic products act as attractant for the pollinators. Some of the secondary products give out allelopathy effect, inhibiting competitors from the same ecology, reducing interspecific competitions (Vince & Zoltan, 2011). Commercially these secondary metabolic products have been used to increase the natural durability of wood and some of them served as for medicinal purposes for human kind.

In Cuba, the locals have been using *Rhizophora mangle*, the red mangrove from the same studied genus, as medicine due to its curative properties (Lázaro *et al.*, 2011). *Rhizophora apiculata*, one of the selected species for this study has been reported to be traditionally used as a sterilizing agent, deodorizer and growth promoting agents with the bark part shows high antioxidant ability (Mingzhe & Hongbin, 2012). Another studied species, *Rhizophora mucronata* has also been used clinically for treating astringent, angina, hemorrhage, diabetes, diarrhea, dysentery and hematuria (Naidu & Vadlapudi, 2010).

Previous study done Lim, Darah and Jain (2006) had discovered the antimicrobial properties from the crude plant extracts and bioassay fractionation of the *R. apiculata* on few selected microorganisms and yeast. Therefore in this study, we focused more on the

methanol extracts from the bark of *R. apiculata* and *R. mucronata* on their toxicity properties against few selected microorganism and fungi. The main focus of the targeted compounds in the extractive is the polar compounds properties, extracted using methanol.

The part selected for this study is bark mainly because bark is considered as an unused part in charcoal industry in countries like Thailand, Peninsular Malaysia, Sumatra, Myanmar and Southern Vietnam, where they had been using *Rhizophora* spp. as the main resource (FAO, 2005). The harvested logs are being debarked before being transferred into the processing area. According to Lim, *et al.* (2006), the process of scrapping off the bark to get the log in the charcoal making process is considered as a waste because the lack of knowledge on the bark's properties. Therefore the main objective in this study is to find out the possibility of the methanol extracts from the bark able to inhibit the tested microorganism.

The hypothesis was that there are certain chemical compounds presence within the bark extract of *R. apiculata* and *R. mucronata* whether it was from tannin, or phenolic compound, or alkaloids, or glycosides, or essential oil or other organic compounds involved in inhibiting the growth of the tested microorganisms. These microorganisms chosen are pathogenic microorganisms that have been the cause of many diseases, basically to test whether the extracted compound is antibacterial and antifungal. A series of bioassay tests were done through agar dilution method and disc diffusion method with the MeOH crude extract being tested at different concentrations, in order to determine the maximum inhibitory concentration with three replicates on each of the tested treatments.

1.2 Objectives

The objectives of this research are first, to determine the extractive content in the bark of *R. apiculata* and *R. mucronata* using methanol as the solvent. Second, to determine the toxicity of the methanol bark extract from *R. apiculata* and *R. mucronata* as an antibacterial and antifungal agent in inhibiting the growth of the pathogenic tested microorganism at different concentrations. Third, to observe the maximum inhibitory concentration required of the methanol extract in inhibiting the tested bacteria and fungi growth.

CHAPTER TWO

LITERATURE REVIEWS

2.1 The Mangrove Forests Ecological Roles and Commercial Values

Rhizophora apiculata Blume and *Rhizophora mucronata* L. are dicotyledonous trees, under the family of Rhizophoraceae that is also known as 'the mangrove family'. The mangroves are only found in tropical climate, in the intertidal zones, between regions 30° North and South where the land interacting with the sea. According to Tan (2001), mangroves with the highest diversity are found in region from Malaysia to New Guinea, majority in Indo-Pacific, while some in East Africa, West Africa, Caribbean and South America. The unique ability of the mangrove tree is that most of the trees in the mangrove forest are designed to be halophytic or salt resistance, they are physiologically designed to survive inundation by seawater and poor soil oxygen and stabilize even under poor soil structure condition (Bandaranayake, 2002). They have effective ultra filtration system at the root level that block more than necessary salt from entering the cells. Even if the salt gets through, it would be stored either in leaves then being shed off or stored within the barks (Tan, 2001).

For the past years, mangroves have been providing us direct and indirect services whether we realize it or not. From an ecological aspect, the mangrove forest has been playing a very important role in maintaining the ecosystem balance by providing protection for the costal land, controls the coastal erosion while stabilizing the sediments

around the coastal area. In the sense of preserving the marine biotic, the mangrove provides favorable nesting ground for most of the marine life (Bandaranayake, 2002).

The mangroves have also been widely used in constructions of dwellings, making furniture, rafts, boats, fences and even as a dying agent from the extracted tannin. In charcoal and firewood industries, the mangrove twigs are highly appreciated as the main fuel resource as they produce more heat with less smoke compared to the Indian coal (Patra & Thatoi, 2011). In the aspect of food productions, mangrove has been the main providers in shrimp fisheries, crabs, lobsters, oysters and fishes.

The chosen genus for the study is *Rhizophora*, from the family Rhizophoraceae that has another 14 genera under this family, one of the many families present in the mangroves (Sambamurty, 2005). This genus is highly salt-tolerant, possess the ability to live in salt water and specially adapt their morphology and physiological mechanism to the harsh environment living in a mangrove ecosystem. The genus lives as trees or shrubs and conspicuously viviparous (bears seeds). The species under this genus shares unique characteristic as an adaptation features for them to be able to tolerate the harsh environment of their habitat. One of these modifications involved having stilt roots, a root system that develop aerial or above-ground. The lenticels, tiny pores on the root surface to allow air get through the root into the trees. Water or salt could not get through it. An air reservoir made by the aerenchymatous tissues at the root, helps to keep the tree survived during tidal (Tan, 2001).

Even the genus name, *Rhizo-phora* is derived from a Greek word that was taken from the characteristic itself. *Rhizo* from the word *rhiza* means root while *phora* from the word *phoros* means bearing (Wiart, 2006). The flowering part of this genus bears 4

calyxes with pointed lobes, and stamens 6-16, with separate fruiting body, seeds propagule (Duke, 2006).

The distinguishable traits between *R. apiculata* and *R. mucronata* are: 1) the abaxial leaf (undersurface) for *R. mucronata* has small corky spots while *R. apiculata* is without any of the corky spots; 2) the peduncle length longer than the petiole length for *R.mucronata*; 3) the distinguishable features of the flower, bracts for *R. apiculata* is corky brown while the flower for *R. mucronata* has short style with length less than 2.5mm (Duke, 2006). Other distinguishable traits between these two species are the shape of the leaves. The leaves for *R. mucronata* seem to have broader leaves than compared to *R. apiculata*. From a rough observation, the tannin in bark for *R. apiculata* seemed to be more, the bark sample for *R. apiculata* was cover with a more oil-like appearance while the bark sample from *R. mucronata* was in a lot drier state compared with these two.

2.2 Medicinal values of the *R. apiculata* and *R. mucronata*

In term of ethnomedicine, mangroves have been used traditionally in curing diseases by the local folks. For instance, people from Pitchavaram are highly depending on the mangroves as their medicinal source (Prabhakaran & Kavitha, 2012). The studied species, *R. apiculata* has been traditionally used in treating diarrhea, nausea, vomiting, typhoid, hepatitis, antiseptic, insecticide, while *R. mucronata* has also been traditionally used in treating diabetes, hemorrhage, hepatitis and ulcer (Patra & Thatoi, 2011).

There had been discovery that *R. mucronata* contains sugar, protein, phenolic group, alkaloid, triterpenes, flavonoids, catachin, tannin and anthroquinone in its bark extracts while *R. apiculata* contains protein, phenolic group and tannin (Ravikumar *et al.*,

2010). Tannin is an astringent where the substance has the tendency to bind rapidly, it exists in the form of condensed tannin and hydrolysable tannin. Condensed tannin is the product of large polymers of flavonoids attaching together while hydrolysable tannin is the polymers composed of monosaccharide added with catechin derivatives attached to it (Bernhoff, 2010). Phenolic compound has been properly studied by Sansei (1994) to prove on its properties as enzyme inhibitors, anti-tumor promoting activity and also as an inhibitor for metabolic activity that produced on the superoxide. These secondary metabolic products are what contribute to their uses as traditional medicinal uses.

In the study that was done by Ravikumar *et al.* (2010), they had chosen five different mangrove plants species which were *Rhizophora apiculata, Rhizophora mucronata, Bruguiera cylindrical, Ceriops decandra* and *Avicennia marina*. They run a series of tests on four different parts of the studied tree species; the bark, hypocotyl, flower and collar. The bioassay test was done against the urinary tract infectious (UTI) bacterial pathogens. From their result, the statistical data showed that the bark produced the second highest percentage of antibacterial sensitivity toward UTI bacterial pathogens while the highest came from the hypocotyl.

For a long time, bark has been considered as a waste wood product from sawmilling, plywood production and particleboard productions. Although it has been known that bark possess some useful byproducts, the development of this utilization has yet to be perfected or commercialized to the public since this discovery is still under development and research. Bark of the Indiana's birch has been efficiently utilized from making canoes to cloth production for the people living in the South Pacific. While some had utilized the bark in making cork, dyes, gums, or for its tannins, resin, latex, arrow

poisons, and some other has been utilizing the bark for its medicinal purposes (Tariq, 2013).

A review by Patra and Thatoi (2011) showed the list of mangroves that have diverse metabolic products and the potential of these products to be or currently use as. There have been few researches published on the bioassay tests done on *Rhizophora apiculata* to prove for its anti-HIV, antibacterial and anti-yeast activities. While there were also a few reported on *Rhizophora mucronata* for its antiviral and anti-HIV properties (Biber, 2006; Lim *et al.*, 2006; Premanathan, *et al.*, 1999). A research had reported that the extraction of the heartwood with methylene dichloride has the potential to be boll weevil antifeedant, antifungal and antimicrobial activity (Kokpol *et al.*, 1993). In the article, they explained the process of isolating three compounds that are 2,6-dimethoxy-p-benzoquinone, syringaldehyde and sitosterol 3-glucoside from the extracted heartwood and further tested for its respected functions.

2.3 Bioactivity studies done on *Rhizophora apiculata* and *Rhizophora mucronata*

A research that had been done by Pimpliskar *et al.* (2012) had comparatively studied on *R. apiculata* and *R. mucronata* on four selected structures of the tree; the leaf, pneumatophore, seed and fruit. They had compared the result that they had obtained from screening test on the tested microorganisms. The variables that they used in their research were manipulating the extract as hot and cold, to test for the thermostability of the chemical components present in the extract on polar and non-polar extractions. The tested organisms involved were the gram positive and negative bacteria along with a representative from the fungi community, *Candida albicans.* In the study, it was reported

that *R. mucronata* posses more thermostable chemical components present in the extract than *R. apiculata* possess more thermolabile components (Pimpliskar *et al.*, 2012).

Bioassay, stands for biological assay or assessment, is set of standard preparation procedures that are used to determine the potential or concentration of a substance by testing its effect on growth on living organism or tissue or cell. While conducting bioassay, a few parameters to be considered are the relevant targeted organisms, reliability of the obtained result, sensitivity of the tested organism, fast, simple and standardized detection method (Sjögren, 2005). Antifungal bioassay simply means that conducting a biological assessment on fungi growth to determine the potential and find out the maximum inhibitory concentration of the crude that has significant impact on the growth.

Antifungal agent is drug that can selectively killed fungal pathogens from a host without causing any harm on the host. It produces minimal amount of toxicity to eliminate the fungi pathogen with no effect on disrupting the host metabolic process (Dixon & Walsh, 1996). Studying for the antifungal effect is a lot more difficult than studying for the antibacterial effect on the collected crude because fungi are eukaryotic organism, having well-defined nucleus, in multicellur forms except for yeast and have networks of hyphae with its wall is made up from chitin. Due to their complexity, their growth rates are a lot slower than bacteria. Agar dilution technique is a standard technique commonly used in for susceptibility test for fungal, where the antifungal substance is incorporated into the nutrient agar at different level of concentrations, thus the output will be observed on the growth of the fungal on the media (Wiegand *et al.*,

2008). Another few methods commonly used in bioassay test are agar Kirby-Bauer antibiotic testing (paper disc diffusion method) and broth diffusion method.

Antibacterial bioassay involved bacteria being isolated for clinical trials, two commonly used methods for AST are disc diffusion and agar dilution (Wheat, 2001). Studying on the antibacterial is used to kill and prevent growth on the growth bacteria, while finding out the minimum inhibitory concentration (MIC) of the tested crude in disturbing the bacterial metabolic activities. Bioassay on the antibacterial properties is a lot simpler method than compared to antifungal because bacteria are prokaryotic organism, unicellular and reproduce through cell replication, and thus the result can be collected only after 16 hours of incubating.

The method chosen in the study for bioassay test was done through disc diffusion assay but with modification (Pimpliskar *et al.*, 2012). Disc diffusion assay is one of the common techniques that have been universally used in testing the susceptibility of the antimicrobial studied substance against the tested organisms, the studied antimicrobial substance is to be impregnated into a piece of disc and placed on top of an agar while allowing the chemical to be absorbed by the agar and infecting the tested organism. The responding output from this method would be the size of the inhibition zone around the paper disc on the agar plate (Pimpliskar *et al.*, 2012). However this technique may provide the biggest flaw if the chemical impregnated on the disc could not diffuse into the agar due to various factors that may occur throughout the experiment. Therefore, the agar dilution technique is proposed in this study.

2.4 Background on tested microorganisms: *Fusarium oxysporum, Aspergillus niger, Candida albicans, Staphylococcus aureus* and *Escherichia coli*

Fungus species tested: *Fusarium oxysporum,* **Figure 1.**

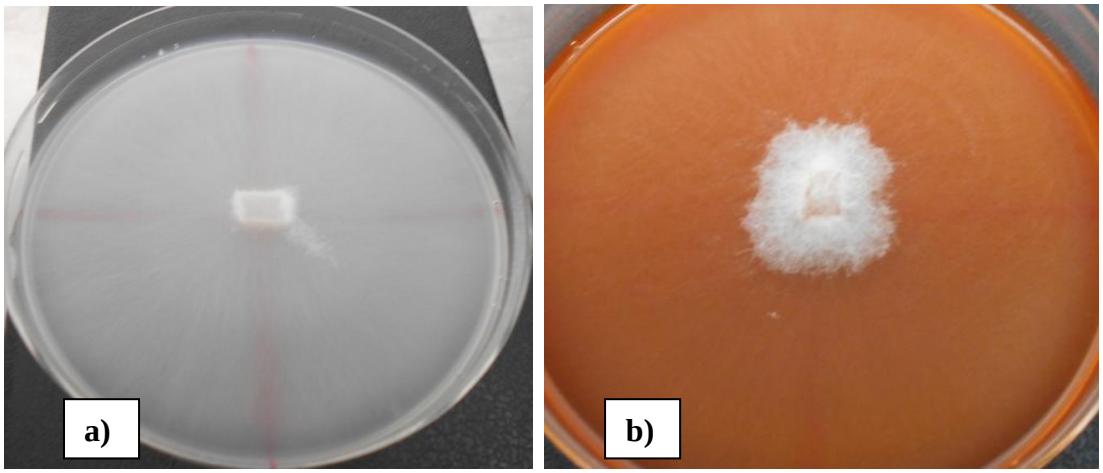

Figure 1 a) *Fusarium oxysporum* stock culture, cultured through hyphal tipping
b) Chlamydospores stage

F. oxysporum is a soil-borne fungus that causes to a crop disease which is called as Fusarium wilt. This disease infected most of the solanaceous crop plants such a tomato, potato, pepper and egg-plant, few of the major crop that are commercialized in plantation. It can exist in three states: macroconidia, microconidia and chlamydospores. Macroconidia general morphology as short to medium length with its straight to slightly curved, relatively slender thin wall, it has at least 3 septate presence. Microconidia take the shape of an oval, elliptical or kidney shape, with their aerial mycelium present false heads. They usually found abundant in the aerial mycelia, while chlamydospores formed after 2-4 weeks, located either terminal or intercalary in aerial (Leslie & Summerell, 2006).

Fungus species tested: *Aspergillus niger*

A. niger is a pathogenic fungus that causes black mould on infected fruits and vegetables, it is a common type of fungus that is found in the environment even when only little nutrient available and able to colonize variety of substrates to survive (US Environmental Protection Agency, 1997; *Aspergillus: Niger, Fumigatues, Flavus, Symptoms and causes*, n.d.). Black mould formed on rotting fruits and vegetables due to this fungus had cause great losses and have been threatening the food resource (Ibatsam, *et al*., 2012). Infections that can be caused by *A. niger* on human or animal are aspergillosis, a sort of lung disease that commonly infected farmers that had been exposed to peat dust for a long time, and atomycosis, an infection on the external auditory canal due to poor hygiene, warm and moist climate. The symptoms include scaling, itching and pain (Otomycosis, n.d.).

Fungus species tested: *Candida albicans*

Candida albicans is dimorphic fungus that can grow both as yeast through blastospore and in hyphal form (filamentous cell) (Jenkinson, et. al., 2002). It can be found within the gastrointestinal and genitourinary tract (Sudbery, 2004). Under normal condition, C. albicans grows and survives as commensals, however transformation on these fungus allowed them to become pathogenic and causal to painful superficial infection such as vaginitis or severe surface infections on mouth and esophagus in HIV patients (Jenkinson, et. al., 2002; Sudbery, 2004). It is fourth of the common cause of hospital-causal infections and had been caused off the death over 10 000 deaths in US.