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DEVELOPMENT OF Acacia spp. NANOBACTERICIDES THROUGH NANOEMULSIONS FOR CONTROLLING Xanthomonas oryzae pv. oryzae IN RICE

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By

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October 2017

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Bacterial leaf blight caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), the main bacterial disease of paddy, causing serious yield loss during infection. Copper-based chemical fungicides and antibiotics have been applied to control this disease. Plant extracts have also shown potential to control *Xoo* since they may contain numerous active compounds. *Acacia* species are among the plants that have shown potential as antibacterial agents against numerous tested pathogens, especially the clinical isolates. In Peninsular Malaysia, *Acacia auriculiformis* and *Acacia mangium* are the two most common *Acacia* species. This study was conducted to determine the antibacterial activity of *Acacia* leaf extracts against *Xoo*, identify and quantify the phytochemical compounds content present in the extracts, suppress *Xoo* biofilm formation, and develop a nanoemulsion formulation using the *Acacia* leaf extract. Ethyl acetate and methanol leaf extracts from both species demonstrated inhibition of *Xoo in vitro*, while n-hexane and chloroform extracts failed to inhibit *Xoo.* The ethyl acetate and methanol leaf extracts demonstrated bactericidal and bacteriostatic effects based on their MIC index that was 2 (AAEA) and 8 (AMMH). However, methanol leaf extract showed better inhibition against *Xoo*. Scanning electron microscope (SEM) observation showed that the untreated *Xoo* cells were in healthy form, while the treated cells were deformed.

The GC-MS analysis for AAEA and AMMH leaf extracts revealed that most of the active compounds belong to the phenolic acids, terpenoids, esters, fatty alcohols and other volatile compounds. The LC-MS/MS analysis gave equal numbers of identified compounds for both leaf extracts. The compounds belong to the flavonoids and phenolic compounds groups. Quantification of targeted active compounds demonstrated that rutin present abundantly in AAEA at 9.19 ng/mL and in AMMH at 48.80 ng/mL. The suppression of *Xoo* biofilm formation using AMMH leaf extract at various concentrations showed that the highest

concentration of 12.5 mg/mL inhibited 81.25% of *Xoo* biofilm. Increased in the concentration of the extract had increased the loss of aggregation of the *Xoo* cells, as seen under confocal laser scanning microscopy (CLSM). The stable P2 AMMH nanoemulsion formulation possessed the mean size and zeta potential of 13.14 nm and -8.32 mV respectively. The nanoemulsion formulation was stable at both high (54±2°C) and low temperatures (0±2°C) at specified times. Glasshouse application demonstrated that P2 AMMH nanoemulsion formulation at a concentration of 6.25 mg/mL was able to control *Xoo.* The AUDPC value and protection index (PI) demonstrated by this concentration were almost similar to the treatment using streptomycin sulfate at concentrations of 0.2 mg/mL. This study confirmed that *Acacia* spp. leaf extract possess antibacterial activity against *X. oryzae* pv. *oryzae.*

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBENTUKAN NANOBAKTERISIDA *Acacia* spp. MELALUI NANOEMULSI UNTUK KAWALAN *Xanthomonas oryzae pv. oryzae* PADA PADI

Oleh

SITI NUR SARAH BINTI SHAFIEI

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Bakteria hawar daun disebabkan oleh *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), dianggap sebagai penyakit utama bakteria padi yang menyebabkan kehilangan hasil yang serius semasa jangkitan. Racun kulat kimia berasaskan kuprum dan antibiotik merupakan antara rawatan yang telah digunapakai untuk mengawal penyakit ini. Ekstrak tumbuhan telah menunjukkan potensi yang baik untuk mengawal *Xoo* berikutan ia mengandungi pelbagai sebatian aktif. Spesies *Acacia* adalah antara tumbuhan yang telah menunjukkan potensi sebagai agen antibakteria terhadap pelbagai patogen yang diuji terutama isolat klinikal. Di Semenanjung Malaysia, *Acacia auriculiformis* dan *Acacia mangium* adalah dua spesis *Acacia* paling biasa didapati. Kajian ini dijalankan untuk menentukan aktiviti antibakteria ekstrak daun *Acacia* terhadap *Xoo*, mengenalpasti dan menentukan kandungan sebatian fitokimia di dalam ekstrak, menyekat pembentukan biofilm *Xoo* dan membentuk formulasi nanoemulsi menggunakan ekstrak daun *Acacia.* Hasil kajian menunjukkan bahawa ekstrat daun etil asetat dan metanol daripada kedua-dua spesies telah menunjukkan perencatan terhadap *Xoo* secara *in vitro* berbanding dengan ekstrak n-heksana dan kloroform yang gagal merencatkan *Xoo*. Esktrak daun etil asetat dan metanol menunjukkan kesan bakterisidal dan bacteriostatik berdasarkan indeks MIC masing-masing iaitu 2 (AAEA) dan 8 (AMMH). Walau bagaimanapun, ekstrak daun metanol menunjukkan perencatan yang lebih baik terhadap *Xoo*. Imej mikroskop imbasan elektron (SEM) menunjukkan bahawa sel-sel *Xoo* yang tidak dirawat berada dalam bentuk yang normal manakala sel-sel yang dirawat menjadi terencat.

Analisis GC-MS bagi ekstrak daun AAEA dan AMMH menunjukkan sebahagian besar sebatian aktif tergolong dalam kumpulan terpene, ester, phenol, alkohol dan sebatian organik mudah meruap yang lain. Analisis LC-MS/MS memberikan jumlah yang sama bagi sebatian yang dikenal pasti untuk kedua-

dua ekstrak daun *Acacia* spp. Sebatian ini terdiri daripada kumpulan flavonoid dan sebatian fenolik. Kuantifikasi sebatian aktif yang disasarkan menunjukkan bahawa rutin hadir paling banyak didalam ekstrak daun AAEA (9.19 ng/mL) dan AMMH (48.80 ng/mL). Perencatan pembentukan biofilm *Xoo* menggunakan ekstrak daun AMMH pada pelbagai kepekatan menunjukkan bahawa kepekatan tertinggi 12.5 mg/mL mampu merencatkan sebanyak 81.25% pembentukan biofilm *Xoo*. Peningkatan kepekatan ekstrak mampu meningkatkan pengurangan penggumpalan sel *Xoo*, seperti yang ditunjukkan oleh imej mikroskop pengimbasan laser konfokal (CLSM). Formulasi nanoemulsi P2 AMMH yang stabil memiliki min saiz 13.14 nm dan zeta potensi -8.32 mV. Formulasi nanoemulsi adalah stabil pada suhu tinggi (54±2°C) dan suhu rendah (0±2°C) pada tempoh waktu tertentu. Aplikasi di rumah kaca menunjukkan bahawa formulasi nanoemulsi P2 AMMH pada kepekatan 6.25 mg/mL mampu mengawal *Xoo.* Nilai AUDPC dan indeks perlindungan yang dipamerkan oleh kepekatan ini hampir sama dengan rawatan menggunakan streptomycin sulfat pada kepekatan 0.2 mg/mL. Kajian ini mengesahkan bahawa ekstrak daun *Acacia* spp. mempunyai aktiviti antibakteria terhadap *X. oryzae* pv. *oryzae.*

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

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

Xoo	*Xanthomonas oryzae* pv. *oryae*
AAEA	*Acacia auriculiformis* ethyl acetate
AAMH	*Acacia auriculiformis* methanol
AMEA	*Acacia mangium* ethyl acetate
AMMH	*Acacia mangium* methanol
EPI	Enhanced product ion
kPa	kilopascal
kcps	kilo counts per second
m/z	mass-to-charge ratio
mN/m	milliNewton per meter
mV	millivolt
MW	Molecular weight
O.D	Optical density
psi	pounds per square inch
R_f	Retention factor
R_t	Retention time
rpm	rotation per minute
v	voltage
P2 AMMH	P2 *Acacia mangium* methanol
□	Alpha
□	Beta
□	Gamma

CHAPTER 1

INTRODUCTION

Rice is staple food for over 2.7 billion people worldwide including Malaysia. Rice grows best in tropical and subtropical regions of the world (Jonit et al., 2016). Like many other food crops, rice production is affected by diseases. Rice bacteria leaf blight is widely known as one of the most prominent bacterial diseases of rice. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is a pathogen responsible for this disease (Keshavarz et al., 2011). In Malaysia, it has been reported that *Xoo* may cause serious yield losses ranging from 50-80% (Ramli, 2017). Antibiotics, chemicals, and biological controls are among control measures taken, but none of these have been truly effective in controlling *Xoo.* Plant extracts to control *Xoo* are another promising antibacterial agent to control this pathogen since plant comprises thousands of organically active secondary metabolites.

Acacia, generally known as wattle or acacias, is the second largest genus of family Leguminosae and contains an estimated 1,350 species. Australia recorded as a country with the highest concentration of *Acacia* (957 spp.) followed by America, Africa and Asia. This tree grows best in warm temperate and tropical areas of the world (Maslin et al., 2003a). *Acacia auriculiformis* and *A. mangium* are the most common *Acacia* species that can be found growing in Malaysia. It was initially planted for its woods and reforestation purposes. However, its uncontrolled breeding assisted by fast growing properties and ability to grow on degraded lands had transformed these *Acacias* into invasive plant species in Malaysia (Shariff and Bakar, 2006).

In line with increasing demand for food, the application of modern technique for manifold agriculture production is required in order to minimize the crop yield loss (Sahayaraj, 2014). Nowadays, scientific communities are eager to design rational strategies in order to develop potent antimicrobial agents. Nanobactericides have been developed and generated tremendously due to their size properties. Nanobactericides terms can be defined as antibacterial agents that are synthesized at nanoscale (Syed et al., 2016). Differing from macroparticles, these nano-sized bactericides are more active (Wang et al., 2013).

Nanobactericides using plant extracts as their base or active ingredients can be developed in order to control plant pathogens. Development of nanobactericides using plant extracts can be performed through nanoemulsion formulation. Nanoemulsion formulation ensures the effective delivery of plant extracts as active ingredients. Nanoemulsion formulation can be used on food crops as they are non-toxic and non-irritant in nature. They also offer improved physical stability, help to solubilize lipophilic drugs, provide greater absorption due to small-sized droplet, and require less energy (Jaiswal et al., 2015).

Since plant extracts can be better substitutes to synthetic pesticides and offer better solutions in terms of detaining the infection by phytopathogens and increased the biosecurity of agriculture crops, the objectives of this study were:

1. To determine the antibacterial activity of *Acacia* leaf extract against *X.* pv. *oryzae*.
2. To identify and quantify the active phytochemical contents of *Acacia* leaf extracts.
3. To suppress the *X. oryzae* pv. *oryzae* biofilm formation by *Acacia* leaf extract.
4. To develop a nanoemulsion formulation from active *Acacia* leaf extract for glasshouse application.

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