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ANTIBACTERIAL ACTIVITY AND CHEMICAL ANALYSIS OF METHANOLIC EXTRACTS OF Piper sarmentosum Roxb.

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By

SHARIFAH FARHANA BINTI SYED AB RAHMAN

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

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Father, mother and beloved family

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

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January 2015

Chairman: Associate Professor Kamaruzaman bin Sijam, PhD

Faculty: Agriculture

Piper sarmentosum Roxb. is a cultivated plant that is also found wild in South East Asia. It is well known due to its medicinal properties and a variety of active chemical constituents. The potential of the plant extract against bacterial leaf blight and sheath brown rot of rice caused by *Xanthomonas oryzae* pv. *oryzae* and *Pseudomonas fuscovaginae*, respectively, were evaluated in this study. The leaf and fruit of *P. sarmentosum* were extracted using aqueous methanol 80% (v/v) and the yield percentage of crude extracts obtained was 7.5% and 7.1% for the fruit and leaf extract, respectively. The antibacterial activities of the methanolic leaf extract of *P. sarmentosum* were screened for potential use as a biological control agent against rice pathogenic bacteria by agar well diffusion assay. The concentrations of the extract tested were 25, 50, 100 and 200 mg/mL. The zone of inhibition produced by the extract against the strains was measured and compared with standard antibiotic streptomycin sulfate (30 µg/mL). The leaf extract showed antibacterial activity against *X. oryzae pv. oryzae* and *P. fuscovaginae* with the diameter of the inhibition zone ranging from 9.00±0.00 to 19.33±1.53 mm. Similarly, the fruit extract also showed positive inhibition against the tested bacteria with the diameter ranging from 9.33±0.58 to 19.33±1.15 mm. Positive control, streptomycin sulfate, showed inhibition against all the tested bacterial isolates with the range of 17.67±0.58 to 18.00±1.73 mm for the leaf extract and 15.67±5.13 to 21.00±3.46 mm for the fruit extract. Negative control, aqueous methanol 80% (v/v), showed no inhibition zone against any microorganism for both the leaf and fruit extracts. The Minimal Inhibition Concentration (MIC) and Minimal Bactericidal Concentration (MBC) were then determined. The MIC was obtained at 12.5 mg/mL for both tested bacteria while for MBC; it was at 12.5 mg/mL for *X. oryzea* and 25.0 mg/mL for *P. fuscovaginae*. For the fruit extract, the MBC value obtained for both *P. fuscovaginae* and *X. oryzae* were at 25.0 mg/mL. The phenolic compounds present in the leaf and fruit extracts of *P. sarmentosum* were further identified using HPLC analysis. Five standards were used in the analysis – caffeic acid, tannic acid, gallic acid, quercetin and naringin. The identification of each compound was based on a combination of retention time and spectral matching. The phenolic compounds were separated and

quantified in the analysis to obtain the amount of the phenolic compounds present in each of the samples. The fraction of the elute containing the purified compound was collected and further tested for antibacterial activity against the pathogenic bacteria. All of the collected fractions, which were detected as the phenolic compounds based on the standard used, showed positive inhibition against the tested bacteria. The results obtained from this study suggest that the leaf and fruit extracts of *P. sarmentosum* have potential to be developed as a novel bactericide.

Abstrak thesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

AKTIVITI ANTIBAKTERIA DAN ANALISIS KIMIA EKSTRAK METANOL *Piper sarmentosum* Roxb.

Oleh

SHARIFAH FARHANA BINTI SYED AB RAHMAN

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Piper sarmentosum Roxb. adalah tanaman yang ditanam, dijumpai tumbuh meliar di Asia Tenggara. Pokok ini terkenal dengan pelbagai khasiat perubatan dan mengandungi pelbagai bahan kimia aktif. Potensi ekstrak pokok ini terhadap penyakit hawar daun dan seludang reput perang yang di sebabkan oleh *Xanthomonas oryzae pv. oryzae* dan *Pseudomonas fuscovaginae*, telah dikaji dalam kajian ini. Daun dan buah dari pokok *P. sarmentosum* telah diekstrak menggunakan metanol 80% (v/v). Peratusan hasil ekstrak mentah yang di perolehi untuk setiap ekstrak adalah 7.5% bagi ekstrak buah dan 7.1% bagi ekstrak daun. Aktiviti antibakteria ekstrak methanol daun dan buah *P. sarmentosum* telah disaring untuk mengenalpasti potensinya sebagai agen kawalan biologi terhadap bakteria pathogen padi menggunakan kaedah penyebaran agar perigi. Kepekatan ekstrak yang diuji adalah 25, 50, 100 dan 200 mg/mL. Zon perencatan pertumbuhan yang di hasilkan oleh ekstrak terhadap bakteria pathogen telah diukur dan dibandingkan dengan antibiotik streptomycin sulfate (30 µg/mL). Ekstrak daun menunjukkan aktiviti antibakteria terhadap semua bakteria yang diuji dengan zon perencatan pertumbuhan dengan diameter di antara 9.00±0.00 mm hingga 19.33±1.53 mm. Manakala, ekstrak buah juga menunjukkan perencatan pertumbuhan yang positif terhadap bakteria pathogen dengan diameter di antara 9.33±0.58 mm hingga 19.33±1.15 mm. Kawalan positif oleh streptomycin sulfate menunjukkan perencatan terhadap semua bakteria yang di uji dengan julat di antara 17.67±0.58 mm hingga 18.00±1.73 mm bagi ekstrak daun dan 15.67±5.13 mm hingga 21.00±3.46 mm bagi ekstrak buah. Kawalan negatif, methanol 80% (v/v) tidak menunjukkan sebarang zon perencatan terhadap semua bakteria yang diuji bagi kedua-dua ekstrak. Kepekatan perencatan minimum (MIC) dan kepekatan minimum bakterisida (MBC) juga telah ditentukan. MIC yang berjaya merencat pertumbuhan bakteria patogen di perolehi pada kepekatan 12.5 mg/mL bagi kedua-dua bakteria. Bagi MBC, kepekatan terendah bagi ekstrak daun yang menunjukkan kesan bakterisidal adalah pada kepekatan 12.5 mg/mL bagi *X. oryzae* dan 25.0 mg/mL bagi *P. fuscovaginae*. Bagi ekstrak buah, nilai MBC bagi kedua-dua bakteria *P. fuscovaginae* dan *X. oryzae* adalah 25.0 mg/mL. Sebatian fenolik yang hadir dalam ekstrak daun dan buah *P. sarmentosum* seterusnya diuji dengan analisis HPLC. 5 piawaian digunakan dalam analisis ini iaitu caffeic acid, tannic acid, gallic

acid, quercetin dan naringin. Setiap komponen dikenalpasti berdasarkan kombinasi pengekalan masa dan penyesuaian spektra. Sebatian fenolik diasingkan dan kuantitinya dikira bagi mendapatkan jumlah fenolik asid yang terdapat di dalam setiap sampel. Semua piawaian yang di gunakan telah di kesan di dalam kedua-dua ekstrak daun dan buah *P. sarmentosum.* Pecahan bagi cecair yang mengandungi sebatian bersih telah dikumpulkan dan diuji bagi aktiviti antibakteria terhadap bakteria patogen. Kesemua pecahan yang di kumpulkan yang di kesan sebagai sebatian fenolik berdasarkan standard yang digunakan, menunjukkan perencatan positif terhadap bakteria yang diuji. Keputusan kajian menunjukkan ekstrak daun dan buah dari pokok ini mempunyai potensi untuk diformulasikan sebagai bakterisid.

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I certify that a Thesis Examination Committee has met on 23 January 2015 to conduct the final examination of Sharifah Farhana binti Syed Ab Rahman on her thesis entitled "Antibacterial Activity and Chemical Analysis of Methanolic Extracts of *Piper sarmentosum* Roxb" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U. (A) 106] 15 March 1998. The Committee recommends that the student be awarded the relevant degree of Master of Science.

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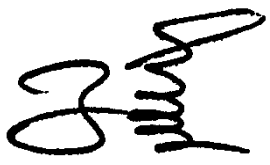

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BLB	Bacterial leaf blight
GC-MS	Gas chromatography-Mass spectrometry
HCL	Hydrochloric acid
HPLC	High pressure liquid chromatography
JMP	Jump software
LCL	Lowest calibration level
LC-MS	Liquid chromatography-mass spectrometry
mAU	milliabsorbance units
mg/ml	Milligram/milliliter
MIC	Minimum inhibition concentration
MHA	Mueller Hinton agar
MBC	Minimum bactericidal concentration
MHB	Mueller Hinton broth
MRSA	Methillin-resistant *Staphylococcus aureus*
MS	Mass spectra
NA	Nutrient agar
OD	Optical density
ppm	Part per million
RTD	Rice tungro disease
SD	Standard deviation
TLC	Thin layer chromatography
TTC	Triphenyltetrazolium chloride

UV	Ultra violet
v/v	Volume/volume
µg	Microgram
µl	Microlitre

CHAPTER 1

INTRODUCTION

1.1 Background

Plants have been a great source of medicinal agents for many years and a remarkable number of natural products obtained from medicinal plants have contributed to disease control either as a crude extract or in a purified form. An extensive range of medicinal plants parts is used to extract raw drugs as they have various medicinal properties. They have been the most impressive sources for a large number of modern drugs, many based on their use in traditional folk medicine. According to the World Health Organization, medicinal plants are the best source to acquire a variety of drugs, and various medicinal plants have been used as a source of medicine in daily life to treat various types of disease all over the world (Alo *et al*., 2012). Medicinal plants have been of great interest for clinical microbiologists to screen for new therapeutics (Ashokkumar & Rajkumar, 2010). Plants produce a broad range of bioactive molecules known as secondary metabolites (Dash *et al*., 2011), thus making them a rich source of diverse kinds of medicine. The reasons can be attributed to easy accessibility and affordability of plants compared to commercial drugs. In addition, the main advantages of using these natural derived products are that they are safe to human health, do not leave any harmful effects on the environment and incur low cost in counteracting microbial infections (Al-Zubaydi *et al*., 2009).

Beyond good agronomic practices, farmers usually rely heavily on chemical pesticides to control these diseases. However, these are usually associated with a harmful effect on the ecosystems. The use of chemicals to control the bacterial blight of rice, such as Bordeaux mixture, copper and mercuric compounds are not effective for practical use to control the disease; neither are antibiotics, which have injurious effects on the rice yields (Mizukami & Wakimoto, 1969). Currently, resistant varieties are the most efficient means for controlling both diseases; however, there are some drawbacks in controlling rice disease through such methods because of the difficulty in combining genes from some resistant sources, and the breakdown of varietal resistance due to the appearance of new races, strains or pathotypes of pathogens. Furthermore, bacterium can easily overcome rice cultivars containing a single gene for resistance against the diseases (Youyong, 2004). Therefore, the search for alternatives is needed to develop safer methods for controlling these pathogens and prolonging the usage without any residue effects on the crops. This will ensure safety to consumers, farmers and the environment.

Plants are known to possess numerous secondary metabolites and compounds with antimicrobial properties (Gulluce *et al*., 2007) that have shown an inhibitory effect against the growth of pathogens (Mahesh & Satish, 2008). Efforts to search for an economic safe phytochemical, which could be developed for disease control, are still ongoing. Therefore, the screening and testing of the efficacy of plants for antibacterial activity has been carried out to discover their antibacterial activity. The use and search for natural products have attracted more attention recently. Plant

extracts are rich in a wide variety of active compounds (Al-Zubaydi *et al*., 2009) that have the potential to be developed as natural anti-bacterial agents (Bhardwaj & Laura, 2009, Dash *et al*., 2011). Plant extracts have been found *in vitro* to have antimicrobial properties (Ghosh *et al*., 2008b, Rios & Recio, 2005). Plant-based products are safer compared to synthetic chemical products and more acceptable as they are non-toxic to humans, biodegradable and non-polluting. They are less phytotoxic and possess a greater systemic effect, which have led them to be further investigated concerning their effect in controlling various types of microbe in many fields of study.

Piper sarmentosum Roxb. locally known as *Kadok*, is a tropical plant that is cultivated as well as found wild in South East Asian regions. It is well known due to its medicinal properties and a variety of active chemical constituents. *Piper sarmentosum* L. has been reported in the literature to have various biological activities. Studies have shown that this plant possesses numerous medicinal properties and has significant effect on pathogenic microbes (Zaidan *et al*., 2005, Zakaria *et al*., 2010, Fernandez *et al*., 2012). Although many bioactive compounds have been isolated from *P. sarmentosum*, none have been investigated against the antibacterial activity of plant pathogenic bacteria, especially rice pathogenic bacteria in Malaysia.

1.2 The objectives of the study

Thus, the specific objectives of the study were:

1. To extract and screen for antibacterial activities of crude fruit and leaf extracts of *P. sarmentosum* against *P. fuscovaginae* and *X. oryzae* pv. *oryzae*

2. To determine the MIC, MBC and MIC index value of crude leaf and fruit extracts of *P. sarmentosum*

3. To fractionate and quantify the amount of active phenolic compounds in *P. sarmentosum* by HPLC

4. To determine the antibacterial activity of the active phenolic compounds against *P. fuscovaginae* and *X. oryzae* pv. *oryzae*

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