UNIVERSITI PUTRA MALAYSIA

COMPARATIVE ANALYSES OF ANTIMICROBIAL ACTIVITIES OF Bauhinia purpurea L., Dicranopteris linearis (Burm.f.) Underw., Melastoma malabathricum L. AND Muntingia calabura L. METHANOLIC EXTRACTS

CHUAH EE LEY

FPSK (M) 2014 7

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By

CHUAH EE LEY

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

August 2014

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DEDICATION

This thesis is dedicated to the following individuals who have accompanied me through thick and thin in completing this study:

To my parents - Thank you for encouraging me all the way.

To my brother - Thank you for your support.

To my supervisor and co-supervisors - Thank you for believing in me.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirement for the Degree of Master of Science

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August 2014

Chairman: Assoc. Prof. Mohd. Nasir bin Mohd. Desa, PhD
Faculty: Medicine and Health Sciences

Microbial infections are common issues that happen in the society. However, the emergence of multidrug-resistant microbials have caused complications in diagnosing the effective treatments for patients to overcome the infections. The efficacy of antimicrobial agents available in the market against such resistant isolates have been compromised, aside from the side effects to human health caused by prolonged use of these drugs. The vast usage of traditional medicines in folklore era has triggered interest to seek for alternatives from plant sources in battling against these increasing multidrug- resistant microbials. This research aimed to compare a few assays in determining the antimicrobial activities of plant extracts. This study utilised disc diffusion assay, broth microdilution assay (visual turbidity inspection and spectrophotometric analysis) and colorimetric resazurin microtiter assay (REMA) to analyse the antimicrobial activities of methanolic leaf extracts of *Bauhinia purpurea* (BPME), *Dicranopteris linearis* (DLME), *Melastoma malabathricum* (MMME) and *Muntingia calabura* (MCME) against four American Type Culture Collection (ATCC®) bacterial strains, which were *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™, *Staphylococcus aureus* ATCC® 25923™ and *Staphylococcus aureus* ATCC® 700699™. Comparative analyses showed that MMME and MCME elicited greater antimicrobial activities compared to BPME and DLME, with Gram-positive strains showing greater susceptibility patterns. Interestingly, the methicillin-resistant *Staphylococcus aureus*/vancomycin-intermediate *S. aureus* (MRSA/VISA) strain employed in this study showed the greatest susceptibility pattern among the tested bacterial strains. Comparative analyses revealed that REMA would be a more accurate method to determine the minimum inhibitory concentration (MIC) values as the absence of colour change of resazurin may not signify the non-viability of bacterial cells, but rather the bacteriostatic phase of cells due to inhibitory effect of antimicrobial agents (plant extracts). On the other hand, conventional plating method on solid growth media and observation of bacterial growth after overnight incubation would be a more precise way to determine the minimum bactericidal concentration (MBC) values due to the bacterial growth can be observed easily by observing any presence of single colonies on the surface of solid media. Growth indicator which is usually

employed in determining the MBC values may not be the most accurate way to determine the MBC values. This is so as it was observed that the bacterial suspension treated with methanolic leaf extract which changed the colour of resazurin from blue to purple did not harbour any bacterial growth upon plated on solid growth media. This may be due to the toxicity of antimicrobial agents which might have impaired the cell's viability and its ability to proliferate. This probably resulted the reduced capability of the cell to reduce resazurin (blue pigments) to resorufin (pink pigments). Disc diffusion assay can be employed as a preliminary screening for antimicrobial activities of potential antimicrobial agents before further tests are carried out, whereas spectrophotometric analysis can be employed as a supplementive measurement to observe the susceptibility pattern of microbials when treated with antimicrobial agents.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Sarjana Sains.

ANALISIS PERBANDINGAN AKTIVITI ANTIMIKROB EKSTRAK METANOL *Bauhinia purpurea* L., *Dicranopteris linearis* (Burm.f.) Underw., *Melastoma malabathricum* L. DAN *Muntingia calabura* L.

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Jangkitan mikrob adalah isu biasa yang berlaku di kalangan masyarakat. Namun begitu, kemunculan mikrob tahan ubat telah mengakibatkan kerumitan dalam mengdiagnosis rawatan yang berkesan untuk pesakit bagi mengatasi jangkitan tersebut. Keberkesanan agen antimikrob yang terdapat di pasaran terhadap isolat tahan ubat sebegini telah dikompromi, selain daripada kesan-kesan sampingan pada kesihatan manusia akibat daripada penggunaan ubat-ubatan dalam jangka masa yang panjang. Penggunaan ubat-ubatan tradisional yang meluas pada zaman dahulu telah mencetuskan minat untuk mencari alternatif daripada sumber-sumber tumbuhan dalam memerangi mikrob penentang-multiubatan yang semakin bertambah. Penyelidikan ini bertujuan untuk membandingkan beberapa kaedah dalam menentukan aktiviti antimikrob ekstrak tumbuhan. Kajian ini menggunakan ujian difusi cakera, ujian mikropencairan 'broth' (pemeriksaan kekeruhan secara pemerhatian dengan mata kasar dan analisis spektrofotometrik) dan ujian kolorimetrik resazurin mikrotiter (REMA) untuk menganalisis aktiviti antimikrob ekstrak metanol daun *Bauhinia purpurea* (BPME), *Dicranopteris linearis* (DLME), *Melastoma malabathricum* (MMME) dan *Muntingia calabura* (MCME) terhadap empat 'American Type Culture Collection' (ATCC®) strain bakteria, iaitu *Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™, *Staphylococcus aureus* ATCC® 25923™ dan *Staphylococcus aureus* ATCC® 700699™. Analisis perbandingan menunjukkan bahawa MMME dan MCME menghasilkan aktiviti antimikrob yang lebih tinggi berbanding dengan BPME dan DLME, di mana strain bakteria Gram-positif menunjukkan corak kecenderungan yang lebih tinggi. Yang menariknya, strain 'methicillin-resistant *Staphylococcus aureus*/vancomycin-intermediate *S. aureus*' (MRSA/VISA) yang digunakan di dalam kajian ini menunjukkan corak kecenderungan yang tertinggi di kalangan strain-strain bakteria yang diuji. Analisis perbandingan menunjukkan bahawa REMA adalah kaedah yang lebih tepat untuk menentukan nilai kepekatan minimum perencatan (MIC) kerana ketiadaan penukaran warna resazurin tidak bererti sel-sel bakteria telah mati, sebaliknya sel-sel berkemungkinan berada di fasa bakteriostatik disebabkan oleh kesan perencatan oleh agen antimikrob (ekstrak tumbuhan). Di samping itu, kaedah 'plating' secara konvensional pada media pertumbuhan pepejal

dan pemerhatian pertumbuhan bakteria selepas inkubasi semalaman adalah kaedah yang lebih tepat untuk menentukan nilai kepekatan minimum 'bactericidal' (MBC) kerana pertumbuhan bakteria boleh diperhatikan dengan lebih mudah dengan pemerhatian terhadap sebarang pertumbuhan koloni bakteria pada permukaan media pepejal. Penanda pertumbuhan yang biasa digunakan untuk menentukan nilai MBC mungkin bukan kaedah yang paling tepat dalam penentuan nilai MBC. Hal ini yang demikian kerana pemerhatian mendapati tiada pertumbuhan koloni bakteria apabila suspensi bakteria yang dirawat dengan ekstrak metanol daun yang menukarkan warna resazurin dari biru ke ungu diselaputkan pada permukaan media pertumbuhan pepejal. Ini mungkin disebabkan oleh ketoksikan agen antimikrob yang berkemungkinan telah menjejaskan keaktifan sel dan kebolehannya untuk tumbuh. Hal ini mungkin telah mengurangkan keupayaan sel untuk menukarkan resazurin (pigmen biru) kepada resorufin (pigmen merah jambu). Ujian difusi cakera boleh digunakan sebagai pemeriksaan asas untuk aktiviti antimikrob bagi agen antimikrob yang berpotensi sebelum ujian yang lebih lanjut dilaksanakan, manakala analisis spektrofotometrik boleh digunakan sebagai pengukuran tambahan untuk memerhati corak kecenderungan mikrob apabila dirawat dengan agen antimikrob.

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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 Master of Science. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
ATCC	American Type Culture Collection
BPME	*B. purpurea* methanolic extract
CFU	Colony-forming units
CFU/mL	Colony-forming unit per mililitre
CLSI	Clinical nad Laboratory Standards Institute
COAD	Chronic obstructive airways disease
DAEC	Diffusely adherent *E. coli*
DLME	*D. linearis* methanolic extract
DMSO	Dimethyl sulfoxide
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
IBS	Institute of Bioscience
MBC	Minimum bactericidal concentration
MCME	*M. calabura* methanolic extract
MDR	Multidrug resistance
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
mg	milligram

mg/disc	milligram per disc
mg/L	milligram per litre
mg/mL	milligram per millilitre
mm	millimeter
MMME	*M. malabathricum* methanolic extract
MNEC	Meningitis-associated *E. coli*
MRSA	Methicillin-resistant *S. aureus*
MRSA/VISA	Methicillin-resistant/Vancomycin-intermediate *S. aureus*
MSSA	Methicillin-sensitive *S. aureus*
nm	nanometer
NNIS	National Nosocomial Infections Surveillance
PBP2a	Penicillin-binding protein 2a
REMA	Resazurin microtiter assay
SPSS	Scientific Package of Social Science
UPEC	Uropathogenic *E. coli*
UPM	Universiti Putra Malaysia
UTIs	Urinary tract infections
VISA	Vancomycin-intermediate *S. aureus*
VRSA	Vancomycin-resistant *S. aureus*
v/v	volume per volume
w/v	weight per volume
ZOI	Zone of inhibition
°C	degree Celsius
µg	microgram
µL	microlitre
µm	micrometer

CHAPTER 1

INTRODUCTION

1.1 Introduction

The discovery of microorganisms by Robert Hooke and Antonie van Leeuwenhoek back in the 17th century (Gest, 2004) has since opened the window to the world of microbiology. Microorganisms have been present by means of commensalism to human beings if not causing diseases which were then controllable with the use of traditional medicines. Over thousands of years, human have been depending on the nature as a medicinal source. The importance of traditional medicines in treating human diseases and as preventive measures is inarguable back in our ancestry era and has since been passed down from one generation to another by means of theories and practices. For decades to centuries, natural products have been derived from different sources ranging from terrestrial plants, microorganisms, vertebrates and invertebrates to pelagic organisms in search of cure against diseases (Newman *et al.*, 1999).

The use of plants in sophisticated traditional medicines by all ethnics and cultures has been acknowledged (Baquar, 1995) and is gaining popularity globally. They have been used for primary health care of the poor in developing countries as well as in countries where conventional medicines is the predominant diagnostic tools. Despite their existence and long history of usage in folkloric medicinal practices as well as their medicinal significances, the lack of attention received by traditional medicines from modern researchers and drug developments is undeniable (Tadeg *et al.*, 2005). Limited effort has been put in to promote the importance of ethnomedicines in advanced countries which focus more on synthetic drug developments. Although there have been documentations of the use of folk medicines in treating various illness and infectious diseases, the birth of antibiotics and their capability in reducing infectious diseases cases have successfully overwritten the essentiality of traditional medicines.

However, the use of antibiotics as the first line of defence against the spread of diseases have been compromised these days due to the emergence of antibiotic resistant pathogenic strains. Such is an evolutionary process of microorganisms acquiring the ability to resist the lethal action of antibiotics (Ahmad and Dar, 2011). Incidence of multi-drug resistant strains have been increasingly documented in recent years. An example of this is the emergence of methicillin-resistant *Staphylococcus aureus* in the 1960s which has since caused the increase of nosocomial infections caused by this particular strain (Abramson and Sexton, 1999). The rise of the multi-drug resistant strains is due to the genetic mutations within the microbial populations over the years. Besides that, the misuse of antimicrobial agents in drug prescriptions, lack of quality in drug developments and non-obsequiousness of patients in drug administration have caused the emergence of antibiotic resistant strains which presence has resulted many complicacies in human health (Mwambete, 2009). Bacterial resistance to most of the available antibacterial agents has been reported (Tanaka *et al.*, 2006) and this has raised the concern of public health care worldwide. Pharmaceutical and biotechnology companies have since battling with time and

intensify their efforts in discovering novel antibacterial agents which are mandatory in the attempt to overcome this serious matter.

Administration of synthetic antimicrobial agents has been reported to affect the natural microflora in the human body. Antibiotics are capable of reducing the popularity of intestinal microbiota which plays essential role in general gut health, failing of which may cause acute diseases and chronic health problems (Dethlefsen *et al.*, 2008). A study found that antibiotic-associated diarrhoea (AAD) is caused by altered functionality of gut microflora by antibiotics (Beaugerie and Petit, 2004). As such, synthetically derived drugs have been withdrawn from the market years after their commercialisations due to adverse side effects to human health (Choudhury *et al.*, 2011).

Since the use of synthetically derived antimicrobial agents have caused so much mishaps to mankind, attempts to go back to the nature in lieu of searching for natural product from plants sources capable of overcoming the spread of diseases caused by these infectious and dangerous strains has been done. Plants produce bioactive compounds, whereby most of which serve as their defence mechanisms against pesticides, herbivores and microorganisms, hence their potential as sources of antimicrobial agents (Cowan, 1999, Mithraja *et al.*, 2012). The remarkably vast diversity of plants with an estimation of 250,000 to 500,000 species on Earth (Borris, 1996) increase the possibility of finding novel antimicrobial agents to subdue the arising antimicrobial resistance problem.

The pharmacotherapeutic agents in some local plants in Malaysia have been previously reported to elicit antimicrobial activities by either one of the two most commonly used screening method in determining the antimicrobial susceptibility level, disc or agar well diffusion assay and the broth dilution assay. Whilst most studies focus on the antimicrobial activity of one species of plant against a few bacterial strains or vice versa, or utilized single assay to determine the antimicrobial activity level, this study attempts to evaluate the antimicrobial activities of four species of plants (*Bauhinia purpurea*, *Dicranopteris linearis*, *Melastoma malabathricum* and *Muntingia calabura*) by means of comparing their activity levels using three antimicrobial assays (the disc diffusion assay, broth microdilution assay and colorimetric resazurin microtiter assay (REMA)) against four American Type Culture Collection (ATCC®) strains (*Escherichia coli* ATCC® 25922™, *Pseudomonas aeruginosa* ATCC® 27853™, *Staphylococcus aureus* ATCC® 25923™ and *Staphylococcus aureus* ATCC® 700699™).

A research by Zakaria *et al*. (2010) has found that methanolic extract elicited highest antimicrobial activity compared to other extracts extracted with other solvents, namely aqueous and chloroform. A separate study by Yao *et al.* (2004) also proved that methanol is capable of drawing out bioactive compounds from the plants at significantly higher level compared to water, chloroform and ethyl acetate. Another study reported that most of the antibacterial activity were portrayed by methanolic extracts of plants in the respective study (Rabe and Staden, 1997). Hence, methanol will be employed in the plant extraction process in this study and the outcome among the different plant extracts subjected to the various antimicrobial assays will be compared.

The antimicrobial activities of crude methanolic leaf extracts instead of isolated pure compounds will be evaluated to look at the synergistic effect of the constituents combined. It was investigated that single bioactive compound is capable of changing its properties when other compounds are present (Barnabas and Nagarajan, 1988).

Many studies have shown that methanolic plant extracts exhibited higher antimicrobial activities compared to aqueous plant extracts (Doughari, 2006, Zakaria *et al.*, 2007b, Zakaria *et al.*, 2010b). Hence, this study will be focusing on methanol extraction to yield plant extracts with more bioactive compounds.

1.2 Problem Statement

Most of the previous antimicrobial activity studies focused on one single bacterial strain against one or more plant extracts, or a single plant extract against one or more bacterial strains. Besides that, most of the previous studies employed only one or two antimicrobial assays, which are either the disc diffusion and broth dilution or broth dilution and colorimetric assay.

This study aims to evaluate the antimicrobial activities of four plant extracts against four bacterial strains in a single study, unlike previous study which only looked at the antimicrobial activity of a plant extract at one time. On top of that, all three antimicrobial assays commonly used in antimicrobial activity studies will be employed in this study to enhance the accuracy of the results as most of the previous studies only used one antimicrobial assay per study. Four instead of one ATCC® strains is employed in this study to look at the susceptibility pattern of the microorganisms comparatively when tested against the plant extracts and this will directly determine which of the four plant extracts has the highest efficacy against the bacterial strains. With similar methodology settings, the results obtained in this study can be compared between one another to determine the plant extract which elicit the greatest antimicrobial activity.

1.3 Objectives

The objectives of this study are as listed:

1.3.1 General Objective

To compare the antimicrobial activities of methanolic leaf extracts of *B. purpurea*, *D. linearis*, *M. malabathricum* and *M. calabura* against *S. aureus* ATCC® 25923™, *S. aureus* ATCC® 700699™, *E. coli* ATCC® 25922™ and *P. aeruginosa* ATCC® 27853™ through different antimicrobial assays.

1.3.2 Specific Objectives

1. To screen for antimicrobial activities of methanolic leaf extracts against the ATCC® strains using the disc diffusion assay.
2. To determine the minimum inhibitory concentration (MIC) values and minimum bactericidal concentration (MBC) values using broth microdilution assay and REMA.

3. To compare the association of broth microdilution assay and REMA in determining the MIC and MBC values.

1.4 Research Hypothesis

Four methanolic leaf extracts of *B. purpurea*, *D. linearis*, *M. malabathricum* and *M. calabura* possess antimicrobial activities.

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