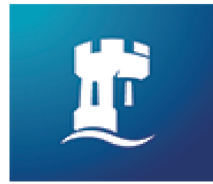

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ANTIBACTERIAL STUDY OF 5 PRIMARY RAINFOREST PLANTS FROM MALAYSIA AND ISOLATION OF SELECTIVE ANTIBACTERIAL CONSTITUENTS FROM *BURKILLANTHUS MALACCENSIS* (RIDL.) SWINGLE

Thesis submitted to the University of Nottingham for the degree of MPhil

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ACKNOWLEDGEMENT

I would like to express my profound and sincere gratitude to my supervisor, Dr. Christophe Wiart, for his invaluable guidance, encouragement and above everything, persistence and flexibility throughout this research. His methodical and thorough planning provided a structured, facilitated and quick-fix oriented research experience to which I have acquired a prodigious experience throughout the journey. His analytical guidance, his impartiality and his openness have alleviated the complexities of this research project, of which I am indebted. Besides, I would wish to thank my co-supervisor, Dr. Teng Jin Khoo, and collaborators, Dr. Chandramathi (University of Malaya), Dr. Mark Butler (NMR), Siti Nurfashiha (NMR, University Malaysia Pahang), Nor Hafizah Zainal Abedin (NMR, University Malaysia Pahang), Dr Khozirah (GC/MS, University Pahang Malaysia), Dr Sammer Yousuf (Antileishmanial activity, University of Karachi). I am also very grateful to all the pleasant and experienced laboratory technicians for their technical assistance., as well my colleagues and friends for their constant support, tolerance, and willingness to help and share the laboratory equipments. Last but not the least, special thanks to my family for their tremendous support that gave me strength and determination when I most needed them. It would never be possible without their love, care, and appreciation of my every circumstance. I genuinely appreciate their kind thoughts and caring behaviour from the core.

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LIST OF ABBREVIATIONS

LH	Leaves-Hexane (Leaves extracted using hexane)
LC	Leaves-Chloroform (Leaves extracted using chloroform)
LM	Leaves-Methanol (Leaves extracted using methanol)
BH	Bark-Hexane (Bark extracted using hexane)
BC	Bark-Chloroform (Bark extracted using chloroform)
BM	Bark-Methanol (Bark extracted using methanol)
WH	Wood-Hexane (Wood extracted using hexane)
WC	Wood-Chloroform (Wood extracted using chloroform)
WM	Wood-Methanol (Wood extracted using methanol)
SH	Seed-Hexane (Seed extracted using hexane)
SC	Seed-Chloroform (Seed extracted using chloroform)
SM	Seed-Methanol (Seed extracted using methanol)
$F^{1}H$	Fruit-Hexane (Fruit extracted using hexane)
$F^{1}C$	Fruit-Chloroform (Fruit extracted using chloroform)
$F^{1}M$	Fruit-Methanol (Fruit extracted using methanol)
FH	Flesh-Hexane (Flesh extracted using hexane)
FC	Flesh-Chloroform (Flesh extracted using chloroform)
FM	Flesh-Methanol (Flesh extracted using methanol)
PH	Peel-Hexane (Peel extracted using hexane)
PC	Peel-Chloroform (Peel extracted using chloroform)
PM	Peel-Methanol (Peel extracted using methanol)
MIC	Minimal Inhibitory Concentration
MBC	Minimal Bactericidal Concentration
TLC	Thin layer Chromatography
PTLC	Preparative Thin Layer Chromatography
NMR	Nuclear Magnetic Resonance Spectroscopy
DMSO	Dimethylsulphoxide
LCMS	Liquid Chromatography Mass Spetrometry
MRSA	Methicillin Resistant *S. aureus*
CA-MRSA	Community-Acquired Methicillin Resistant *S. aureus*
HA-MRSA	Hospital -Acquired Methicillin Resistant *S. aureus*

MDR	Multi Drug Resistance
TLC	Thin layer Chromatography
FRIM	Forest Research Institute of Malaysia
MHA	Mueller-Hinton Agar
MHB	Mueller-Hinton Broth
g	Gram
mg	Microgram
µL	Microlitre
UV	ultraviolet
OD	Optical density
UNMC	University of Nottingham Malaysia Campus
WHO	World Health Organisation
PBP	Peptidoglycan Synthase
CREs	Carbapenem-Resistant *Enterobacteriaceae*
AFAs	Antibacterial Fatty Acids
LPS	Lipopolysaccharides
CDC	Centres for Disease Control and Prevention
FDA	Food and Drug Administration
GLASS	Global Antimicrobial Resistance Surveillance System
SD	Standard Deviation
ZOI	Zone of Inhibition
P. aeruginosa	*Pseudomonas aeruginosa*
P. putida	*Pseudomonas putida*
E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*
B. cereus	*Bacillus cereus*
S. aureus	*Staphylococcus aureus*
C. difficile	*Clostridium difficile*
B. cereus	*Bacillus cereus*
L. spathacea	*Litsea spathacea*
K. maingayi	*Kibatalia maingayi*
B. malaccensis	*Burkillanthus malaccensis*
D. hasseltii	*Diospyros hasseltii*

K. retusa	*Knema retusa*
K. pneumoniae	*Klebsiella pneumoniae*
UTI	Urinary Tract Disease
MBL	Metallo-Beta-Lactamase
CRE	Carbapenem-Resistant *Enterobacteriaceae*
S. pneumoniae	*Streptoococcus pneumoniae*
S. Shigella	*Salmonella Shigella*
N. gonorrhoeae	*Neisseria gonorrhoeae*
A. baumanii	*Acinetobacter baumanii*
LC-MS	Liquid Chromatography – Mass Spectrometry
GC-MS	Gas Chromatography – Mass Spectrometry
ATCC	American Type Culture Collection
CLSI	Clinical and Laboratory Standards Institute
NCCLS	National Committee for Clinical Laboratory Standards
TCC	Total compounds chromatogram
TMS	Tetramethyl Silane
CCl_4	Carbon Tetrachloride
$CDCl_3$	Deuterochloroform
CNMR	Carbon Nuclear Magnetic Resonance Spectroscopy
HNMR	Proton Nuclear Magnetic Resonance Spectroscopy

ABSTRACT

It has now become the necessity to search for novel and more effective antibacterial compounds due to the constant progression of bacterial resistance to currently available antibiotics. Attainment in this regard has focused on tropical plants because of their ability to produce a potential spectrum of biologically active molecules. The tropical rainforest of Malaysia delivers extensive unexplored biodiversity of natural resources which is contemplated as one of the most developed ecologies worldwide. Aim of this project is to search for at least one new antibacterial pure compound from rare medicinal plants and to elucidate their mechanism of action specifically against Gram-negative bacteria.

The research was conducted to assess the *in vitro* antibacterial properties of various rare medicinal plants. Tropical plants with diverse chemical complexities are undoubtedly the most important natural resources used in pharmaceutical applications and healthcare. In this study, antibacterial properties of different rare medicinal plants *Burkillanthus malaccensis, Knema retusa, Litsea spathacea, Kibatalia maingayi,* and *Diospyros hasseltii* have been studied from the family named Rutaceae, Myristicaceae, Lauraceae, Apocynaceae, and Ebenaceae respectively against Gram-positive and Gram-negative bacteria. The objective of this research was to isolate an antibacterial compound which can penetrate the impermeable cell wall of Gram-negative bacteria, hence both classes of bacteria have been tested as a comparison.

Different plant parts of these plants were extracted consecutively using hexane, chloroform, and methanol. All the extracted samples have experimented for antibacterial assays. Samples got shortlisted eventually based on the activity against Gram-negative bacteria, adequate quantity, and time limitations for this project. The prepared crude extracts (48 samples) were subjected to qualitative antibacterial experiments (Kirby-Bauer disc diffusion assay) to examine the antibacterial activities against ATCC bacterial strains. The antibacterial effects of selected extracts (48 samples) of these plants were then tested against the

selected bacteria using broth microdilution (susceptibility testing of bacteria) and minimal bactericidal concentration (MBC) quantitative tests. In the paper disc test, the hexane extract of seed (SH) and chloroform extract of flesh (FC) specifically inhibited the growth of *P. aeruginosa* with the inhibition zone diameters of 11.30±1.50 mm and 23.30±2.00 mm, respectively. In the microdilution broth assay, both these extracts selectively inhibited the growth of *P. aeruginosa* with the MIC value of 1000 ug/ml. Based on these antibacterial results against Gram-negative bacteria attained and because of sufficient yield, these extracts were further analyzed by chromatographic methods (TLC, PTLC) resulting in the isolation into 11 fractions (9 fractions from FC and 2 fractions from SH) which were tested against Gram-negative ATCC bacterial strains. Out of these fractions, fraction S1 from the hexane extracted seed (SH) and the fraction F1 from the chloroform extracted flesh (FC) inhibited the growth of *P. aeruginosa* and presented spots on TLC. NMR analysis of these compounds and contrast of spectral data with existing literature allowed the identification of S1 and F1 as dihydroxy acidissiminol and werneria chromene.

The alkaloid dihydroxy acidissiminol and werneria chromene were bactericidal against *Pseudomonas spp.* Over the past few decades, it has been a dramatic decrease in the number of new antibiotics approved by the FDA despite increasing bacterial resistance globally. The result of our study provides evidence that the rainforest of Malaysia comprises plants with high potential for the development of plant-based material to improve the current treatment strategies for bacterial infections. *In vivo* studies on the antibacterial activities of dihydroxy acidissiminol and werneria chromene against *P. aeruginosa* are warranted.

CHAPTER I: INTRODUCTION

1.1 Background

Antibacterials have served a fundamental role in minimizing mortality rates due to infectious diseases. Antibacterials have been always used in the prevention of infections and the treatment of infections in humans and animals. Nevertheless, infectious diseases tend to be the leading cause of death in the world, and bacteria have become more resistant to modern antibiotics in recent years. The widespread use of these compounds is considered to further promote the production of antibacterial resistance (Yao, Fiona, Ten-Jin, & Christophe, 2014). The number of resistant pathogenic bacteria around the world is increasing dramatically, and the search for new antibacterial agents from medicinal plants to counter these pathogens has become critical preventing incurable infectious diseases (Yao et al., 2014).

Robert Koch developed a general relation between a single, isolated microbe, especially bacterium and disease, in late in the nineteenth century. He found the bacteria responsible for causing subsequent anthrax, cholera, and tuberculosis (Shakoor, Hussain, Abbas, Muhammad, & Kashif, 2014) etc. Initially, bacteriology was developed to construct a linear-causal model of infectious disease aetiology from bacteria that had significant public health implications and medical practices that target pathogenic bacteria in the inhibition and treatment of various deadly infectious diseases (Mohamed Senouci Bereksi, Hafida Hassaïne, 2018)

Since ancient times, plants have been extensively used as a therapeutic origin for the treatment of distinct human ailments (Shakoor, Hussain, Abbas, Muhammad, & Kashif, 2014). The World Health Organisation (WHO) claims that about 80% of people in the third world still rely on contemporary medicinal products for their community health requirements (Bereksi, Hassaïne, Bekhechi & Abdelouahid, 2018) and the use of active ingredients of the natural products or their active constituents are indeed a major part of traditional treatment (Yuan, Ma, Ye, & Piao, 2016). Along with, around 25% - 50% of existing

pharmaceutical products are mostly derived from the natural products, implying the relevance and efficacy of plant species as a vital therapeutic tactic (Wijesekera, 2017). Throughout the past several years, involvement in herbal remedies has gained the momentum tremendously. This resurgence might be credited to several accelerating variables including population growth, inadequate availability of medicines in certain regions of the world, unaffordable treatment costs for conventional ailments, consequences of several allopathic medicines in existing use and as well as resistance to widely used conventional medicines (Elisha, Botha, McGaw & Eloff, 2017). Several studies have noted that antibacterial activity is accountable for alkaloids, saponins, phenolics and flavonoids in various crops. The main and significant phase of isolating the bioactive compounds from plant resources and natural sources is the extraction, pharmacological testing, isolation and characterization of bioactive compounds, toxicological assessment, and clinical assessment (Bereksi, Hassaïne, Bekhechi & Abdelouahid, 2018).

It was found that the rate of antibiotic-resistant infections was strongly correlated with the level of antibiotic use. The widespread use of antibiotics by a human was not impossible to justify. For example, antibiotics are found at many places to be administered to patients on their demand without the medical requirements and while some countries would permit antibiotics to be sold without prescriptions from healthcare professionals which worsen antibiotic violence such as antibiotic resistance. It is vitally important to understand that antibiotics are commonly used in food for animals for marketing and prophylactic growth purposes, which have significantly increased antibiotic resistance to the bacteria (Aslam et al., 2018) as well.

Nonetheless, there is nothing to explain how widespread antibiotic use could contribute to the outbreak of multidrug-resistant bacterial strain and potentially bring us back to the pre-antibiotic age that was the time of public health catastrophe. As the predictable risk becomes robust, the World Health Organization (WHO) has released the first-ever list of "priority pathogens" resistant to antibiotics that present the greatest risk to human health as a warning to raise awareness among the public (Subramani, Narayanasamy, & Feussner,

2017). The first crucial priority pathogen includes *P. aeruginosa* which was resistant to carbapenem, while the second high-priority pathogen includes *S. aureus* and that was resistant to methicillin. All the pathogenic bacteria identified as priorities are not only resistant to several antibiotics, but also capable of transmitting genetic material to other bacteria which induce them to become drug-resistant (Subramani et al., 2017).

To tackle the widespread imminent major public health problem, there will be an immediate demand for new types of antibiotics. Many diverse antibiotic choices are needed to give clinicians greater autonomy to provide patients with effective treatments. Lately, conventional medicines, especially botanical sources, have been expected to be a possible source in the combating multidrug resistance challenges as they have been shown to be useful in the management of infectious diseases in traditional systems (P. D. Gupta & Birdi, 2017).

Scientists are therefore now making great endeavours to investigate the evidence-based effectiveness of herbal plants and standardize their use to allow them to be used safely in mainstream medicine used by humans. Most of the antibiotics available today are heavily dependent on microbial sources and are found to be limited in the effective life spans of all available antibiotics (Podolsky, 2018). Since then plants have been proven to be a natural potential source that could be relied upon to produce new antibiotics. Most plants have been documented with a vast range of biological activities, such as antibacterial, antifungal, antidiabetic, and anticancer activities, and therefore there has been that work to isolate, purify and classify the active compounds from the plants recently. It has been found that most bioactive compounds are simply secondary metabolites or their subsequent derivatives rather than plant-based primary metabolites (Guerriero et al., 2018). Secondary metabolites refer to the compounds generated from the primary metabolism to support plant growth. However, some of the plant's secondary metabolites are even found to be capable of affecting cell signalling to interfere with the pathogenic process caused by pathogens (P. D. Gupta & Birdi, 2017). The study has been conducted to properly assess *in vitro* antibacterial properties from different rare medicinal

plants. Tropical plants with various chemical complexities are without hesitation the most important natural resources used in pharmaceutical and healthcare applications.

In this project different rare medicinal plants and their plant parts have been used to examine the antibacterial characteristics from the families named Rutaceae, Myristicaceae, Lauraceae, Ebenaceae, and Apocynaceae against Gram-positive and negative bacteria. Nosocomial Gram-negative bacteria are causing huge health threat globally in hospital sittings (Durani, 2012). In the treatment of patients in ICUs infected with multidrug-resistant to *P. aeruginosa* has become more and more problematic and there is a critical need for new antibacterial agents against these types of bacteria (Durani, 2012). Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria because of their structure. Gram-negative bacteria have a cell wall composed of a thin layer of peptidoglycan surrounded by an outer membrane. This outer membrane of Gram-negative bacteria contains a unique component, lipopolysaccharide, in addition to proteins and phospholipids. The unique structure of the outer membrane of Gram-negative bacteria prevents certain drugs and antibiotics from entering the cell, which means these bacteria have increased resistance to drugs and are more dangerous as disease-causing organisms (Ghai & Ghai, 2018). In brief, these bacteria are more resistant to antibiotics.

To date, most antibiotics are targeted at intracellular processes and must be able to penetrate the bacterial cell envelope. Notably, the outer membrane of Gram-negative bacteria provides a formidable barrier that must be overcome. Even though the objective of this research was to search for an antibacterial compound which has selective activity against Gram-negative bacteria, hence both classes of bacteria have been tested as a collation.

These plant species were selected because: (i) they have not been studied before for their antibacterial effects and their chemical constituents. (ii) They belong to the families known to produce antibacterial agents. (iii) They were presenting fruits or flowers for botanical identification, and (iv) sufficient amount of raw material was available in the fields. For instance, the family Rutaceae has been found to contain many secondary metabolites such as alkaloids, coumarins, terpenoids with a large spectrum of biological activities (List of plants in the family Rutaceae', 2020).

Plants from the family Myristicaceae have emerged as a credible source of new antimicrobials. It also has evaluated the antibacterial efficacy of aqueous (cold and hot water) extracts of *Myristica fragrans* which is commonly known as nutmeg. Nutmeg is one of the very common spices which is also traditionally used as medicine for treating various infectious diseases. Several studies showed that *Myristica fragrans* have antibacterial activity against both Gram-positive and negative organisms (Sylvester, Douye V, 2018).

Lauraceae is a world-wide distributed family of flowering plants and several species are used for cosmetics and gastronomic purposes, but the medicinal property of most of the species against infections is not completely explained. *Laurus nobilis L.* is an evergreen plant belonging to this family, native to Southern Europe and the Mediterranean area. Studies demonstrated antibacterial activity against some of the highly susceptible strains of pathogenic bacteria (Fidan et al., 2018) of this species.

Persimmon, a deciduous tree of the family Ebenaceae, is found throughout south-east Asia and contains high levels of tannins. This class of natural compounds exhibit favourable toxicity profiles along with the bactericidal activity. Consistent with these observations, persimmon leaves show antibacterial activity. About 130 species have been studied so far and compounds such as terpenoids coumarins and naphthoquinones have been isolated from Ebenaceae. Antibacterial activity was evaluated based on the minimal inhibition concentration (MIC) (Arakawa, Takasaki, Tajima, Fukamachi & Igarashi, 2014).

Apocynaceae has been chosen as a group to be evaluated in this study because of its popular use of chemosystematic correlations and pharmacognostic interest. Alkaloids were detected together with triterpenes, and the presence of alkaloids may be responsible for the antibacterial activity observed in the crude organic extract from *T. angulate* ('Apocynaceae: Characters, Distribution and Types', 2019).

The research goal was to isolate antibacterial compounds from the plant parts of the families mentioned above which are specifically active against Gram-negative bacteria with bactericidal activity. Notably, the outer membrane of Gram-negative bacteria provides a formidable barrier that needs to be overcome by the antibacterial compound (Ghai & Ghai, 2018). Both classes of bacteria have been studied here throughout the antibacterial qualitative and quantitative experiments as a comparison. *E. coli, P. aeruginosa and P. putida* were experimented as ATCC Gram-negative bacteria whereas *S. aureus, B. cereus, B. subtilis* have experimented as a contrast from ATCC Gram-positive bacteria. Choosing of bacteria varied through the experiments due to availability.

Numerous plant elements of these plants were extracted and subsequently analyzed the antibacterial activities against various ATCC bacterial strains through qualitative Kirby-Bauer disc diffusion assay. The selected extracts of these plants were then tested against the selected bacteria broth microdilution (susceptibility testing of bacteria) and minimal bactericidal concentration (MBC) method depending on the adequate yields and antibacterial activity against Gram-negative bacteria. Based on the results from antibacterial assays, continued with shortlisted samples for further isolation and structural elucidation via thin-layer chromatography (TLC), preparative thin-layer chromatography (PTLC), gas chromatography-mass spectrometry (GC-MS), and nuclear magnetic resonance (NMR). A comprehensive structural elucidation of these plants can, therefore, guide to the identification of significant biologically active molecules that might be of enormous benefit to the existing antibiotic treatment strategies.

CHAPTER II: LITERATURE REVIEW

2.1 History of antibiotics development

Antibiotics are categorized as chemical substances that can be used to treat bacterial infections within the body. It generally refers to a compound which is produced by one microorganism that selectively inhibits the growth of another or even kills them (Antimicrobial resistance remains a threat, 2017). Prior to the golden antibiotic era, the first chemical compound that was available in the market as antibiotics were arsephenamine, an arsenic derivative active against *treponema pallidum*. It was branded by naming Salvarsan® and then Mapharsen® in the market in 1911. It was then followed by sulphanilamide synthesized by Paul Gelmo. It was branded under the name Prontosil® in 1935. While the famous antibiotic, penicillin, was only available in the market in 1940 from industrial production using *Penicillium chrysogenum* (Brown & Wright, 2016).

During golden antibiotic era (1940-1970), It was estimated about near a million of soil microorganisms have been screened during that time and this had contributed to a great number of microbial metabolites being discovered (Brown & Wright, 2016). During that time, Selman Waksman performed systematic research of the antimicrobial activity of soil bacteria for the first time, particularly on streptomycetes. With several cultural techniques and strategies developed by him, some major antibiotics have been discovered such as streptomycin. With the inspirational work done by Selman Waksman, it has been reported that more than 20 classes of antibiotics were discovered from different bacterial species and fungi during that golden era (Afouda et al., 2019).

After the long period of the golden antibiotic era, the search for new classes of antibiotics was stagnant since 1980 with the ongoing strategy of discovering new microbial metabolites from the soil microorganisms as there were greater difficulties of cultivating the more fastidious microbes under laboratory conditions. The research strategy has then switched to the *in-vitro* synthesis of new classes of antibiotics with the understanding mechanism of

actions from the discovered classes antibiotics. However, only a few successful classes of drugs have been made such as oxazolidinones in 1987 (Lewis, 2015).

2.2 Bacteria and bacterial structure

It is very important to understand the constitutional elements of these single organisms that are crucial for antibiotic development for antibacterial treatment (Bulgarelli, Schlaeppi, Spaepen, van Themaat & Schulze-Lefert, 2013). A cell membrane of bacteria is made of peptidoglycan. Many bacteria need a cell wall (only present in prokaryotes) in order to survive which helps to make a perfect drug target. This cell wall gives structure to bacteria and protects in opposition to the external environment (Wang, Wilksch, Lithgow, Strugnell, & Gee, 2013). The structural composition of bacteria categorizes Gram-positive and Gram-negative bacteria. This configuration is liable for the discrepancies among bacteria and their susceptibility. Gram-positive bacteria consist of a dense cell wall that already exists in an enormous mass in the human body (Fischetti, 2019). It shows violet stain under a microscope because it has a thick cell wall that helps to trap the crystal violet-iodine complex.

Cell walls of Gram-negative bacteria are less compact and comprise of the double membrane, which transcends the rigidity of Gram-positive bacteria (Bulgarelli, Schlaeppi, Spaepen, van Themaat & Schulze-Lefert, 2013). It cannot hold the stain of a violet-iodine complex because it has thin cell walls but can hold the stain of Safranin which makes it visible as red. It is very essential to observe the strains of bacteria that have been impacted when discovering the latest antibiotics. Usually, when evaluating the activity of new antibacterial agents, the results portrayed by Gram-negative bacteria are considered more important since Gram-negative bacteria are even more repellent of antibiotics owing to the special characteristics of the cell wall. Exceptionally Gram-negative strains are a bit different from Gram-positive regarding defence mechanism because there are alarming barriers that should be overwhelmed (Demetrio L.ValleJr. 2015). There is an unusual lipid on the outer membrane bilayer which is known as lipopolysaccharide (LPS) rather than the usual

glycerophospho lipid that found in most other biological membranes (Zhang, Meredith, & Kahne, 2013).

Gram-negative bacteria have complex envelop that holds various protein channels involved in transporting nutrients (sugars, amino acids, salts, metals, etc.) inside and out from the bacterial cell wall, known as influx and efflux system. There are some definite energy-dependent efflux pumps among these transporters (Clifton et al., 2014) and functions of these energy-dependent efflux pumps are to identify destructive agents that have penetrated the protective cell walls of the organisms and reached the periplasm or cytoplasm, and immediately expel them before the agents reached their intended targets.

Due to large impermeable cell well, Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria. Gram-positive bacteria have a thicker layer of peptidoglycan that makes up the cell wall and thus stain purple in a gram stain test. As discussed at the beginning of this chapter the outer membrane of Gram-negative bacteria contains a unique component, lipopolysaccharide (LPS). The special characteristics of LPS are that the fatty acid chains are all saturated and this characteristic significantly reduces the lipid fluidity by making it turgid and firm which severely obstructed the entry of antibiotics, which permits some resistance towards any antibacterial agents. The exclusive structure of the outer membrane of Gram-negative bacteria prevents certain drugs and antibiotics from entering the cell, which means these bacteria have increased resistance to drugs (Ghai & Ghai, 2018).

Extracts of plants (especially nonpolar or liposoluble substance) tend to disrupt the membranes of bacteria. Liposoluble substances might be able to penetrate the outer membrane of Gram-negative bacteria (Ghai & Ghai, 2018). Disruption of membrane permeability increases the loss of cellular constituents leading to the death of bacteria. Plants extracts (polar, especially when containing phenolics) are also known to inhibit the enzymatic activity of bacteria and at high concentration to coagulate the bacterial cytoplasm. Other extracts (containing planar constituents like for instance isoquinolines) can inhibit the replication of bacterial DNA by inhibition of topoisomerase (Ghai & Ghai,

2018). Other extracts (containing tannins) can chelate iron in the medium depriving the bacteria of iron. Thus, the extracts might penetrate the outer membrane of Gram-negative bacteria and disturbed cellular function, metabolism, and loss of cellular constituents, leading their death.

To date, most antibiotics are targeted at intracellular processes and must be able to penetrate the bacterial cell envelope. Even though the objective of this research was to search for an antibacterial compound which is able to penetrate the impermeable cell wall of Gram-negative bacteria, but studies have been done on both classes of bacteria as a comparison (Ghai & Ghai, 2018). *E. coli, P. aeruginosa and P. putida* have experimented from ATCC Gram-negative bacteria whereas *S. aureus, B. cereus, B. subtilis* have experimented as a contradiction from ATCC Gram-positive bacteria. Both Gram-negative and Gram-positive bacteria used in this project are discussed below.

2.2.1 Gram-negative bacteria

E. coli is a bacterium that is rod-shaped, non-spore forming and usually motile by peritrichous flagella. This bacterium belongs from the Enterobacteriaceae family, which often involves nosocomial infections and threatens to generate susceptible species to all commercially accessible agents (Clifton et al., 2014). Furthermore, it is frequently observed in human and animal intestines, which helps in food digestion. Besides, some strains can occasionally cause severe infections including Shiga toxin-producing *E. coli* (STEC) and toxigenic *E. coli.* Different transmission methods can lead to *E. coli* infections, generally by consuming contaminated food or water, such as eating uncooked meat which may be contaminated with *E. coli* during the slaughtering process, raw fruits and vegetables or maybe by consuming unpasteurised milk, from an infected person, food processing, improper food handling (Ilangovan, Connery & Waksman, 2015). As reported by the San Francisco University of California, *E. coli* accounts for approximately 90% of urinary tract diseases (UTIs) and other bacterial diseases such as diarrhoea, meningitis, intestinal diseases, and pneumonia (Opal, 2016). Therefore, in microbiology research,

antibacterial testing against the Enterobacteriaceae family (*E. coli*) is crucial to avoid this form of bacteria from developing and spreading.

Another rod-shaped ATCC Gram-negative bacterial strain used in this research is *P. aeruginosa* which is an opportunistic pathogen and most vulnerable to patients who are immune-compromised and critically ill. Besides, infections of this bacterium are usually associated with health care and cause a total of 51,000 health care infections in the United States per year. In recent years, carbapenemase enzymes which major belongs to Metallo-beta-Lactamases (MBLs) have been found in *P. aeruginosa* and *E. coli* strains (Duedu, Offei, Codjoe, & Donkor, 2017).

The bacteria are commonly found in the environment, for example in soil, water, and plants. They generally do not trigger healthy people to become infected. If a healthy individual is infected by this bacterium, it is usually mild. More serious infections happen in individuals already in hospital with another disease or condition, or individuals with a weak immune system. Infections obtained in a hospital environment, involve pseudomonades as relatively prevalent pathogens (Opal, 2016). A pathogen is a disease-causing microorganism. Hospital-acquired infections are called nosocomial infections. Infections can occur in any part of the body. Symptoms rely on which part of the body is infected. Antibiotics are used to treat infections. *Pseudomonas* infection could be deadly in people who are already very ill (Hauser, Mecsas & Moir, 2016).

Previously *P. putida* was perceived as a pathogen with low virulence and was regarded as an unusual reason for bacteremia. However, *P. putida* isolates, which are multidrug-resistant (MDR) and carbapenem-resistant, have recently emerged, causing nosocomial infections to severely ill patients (Hauser, Mecsas & Moir, 2016). The consequence of *P. putida* bacteremia is still unpredictable, which is multidrug-resistant and carbapenem-resistant *P. putida* from the fluorescent class of *Pseudomonas* species, as well as other nonfermenting Gram-negative organisms that are commonly available in the ecosystem (e.g., *Acinetobacter, Burkholderia,* and *Stenotrophomonas* species), were previously

thought to be of low pathogenicity (Edwin MD, 2018). Nevertheless, these have been gradually identified as significant human pathogens over the past three decades. Since *P. putida* can colonize moist, wet and inanimate surfaces in hospitals, it can cause nosocomial infections, especially in patients with immunocompromise and patients mainly with catheters and attached with various other medical devices.

Although this organism induces health-related infections, there is a deficit of trial data on *P. putida* infectious diseases due to the relatively lower virulence, and higher antimicrobial susceptibility of *P. putida* compared to other species of *Pseudomonas*, especially *P. aeruginos* (Kayış, Er & İpek, 2017). Recently, however, the necessity of multi-drug-resistant (MDR) and carbapenem-resistant *P. putida* has now become a matter of concern. There has been no internal investigation into the outcome of MDR *P. putida* bacteremia. *P. putida* infections are usually rare and are relatively limited to patients with immune-compromised or patients with intrusive medical devices. Clinical isolates of *P. putida* demonstrated high sensitivity to different antibiotics in previous reports (Thomas, Okamoto, Bankowski & Seto, 2013). For instance, it has been reported that 100% susceptibility to ciprofloxacin and tobramycin from *P. putida* clinical isolates and 87% to imipenem and piperacillin/tazobactam.

2.2.2 Gram-positive bacteria

S. aureus is a Gram-positive, coccus and facultative anaerobic bacteria. In the last few years, *S. aureus* the most ill-famed bacteria in Gram-positive organism has an intense association with mankind that generally found at low numbers in normal human flora such as skin, mucous membrane and the environment. This bacterium often causes skin diseases but can spread through the bloodstreams and infected organs. Infections of *S. aureus* usually associated with hospital-acquired and transmitted through direct contact with infected patients (Aminu, Usman-Sani & Usman, 2014). However, one of the major worldwide concerns is strains of *S. aureus* with evolving resistance to methicillin (MRSA). In brief, treatment of *S. aureus* especially multi-drug

resistant strains remains challenging to manage and even increasing prevalent in hospital and emerging in the community. MRSA is very contagious and can spread to an individual who has the infection through direct contact. Infections with MRSA typically happen when anyone's skin has a cut or break. Although infection with MRSA can be severe, it can be handled with certain antibiotics efficiently. MRSA infections are categorized as either hospital-acquired (HA-MRSA) or community-acquired (CA-MRSA) (Aminu, Usman-Sani & Usman, 2014).

B. subtilis is also one of the organisms used in this research, is a spore-forming bacterium capable of growing at different temperatures and pH levels that add to many foodborne diseases and poisoning (El-Arabi et al., 2013). The protective endospores allow them to stay dormant for prolonged periods in reaction to harsh circumstances and to become fully functioning bacteria when circumstances are more favourable (Lone et al., 2016). This bacterium usually isolated from raw meat, processed meat, soil, and plant materials. Besides, *B. subtilis* produces a potent β-lactamase as well conferring marked resistance to β-lactam antibiotics yet still susceptible to treatment with certain antibiotics (chloramphenicol, gentamicin, vancomycin, and erythromycin) (Brem et al., 2014).

2.3 Antibiotics

Antibiotics are drugs that treat bacterial diseases by killing bacteria or inhibiting progression by restricting the multiplication of bacteria (Stanton, 2013). Antibiotics in medical management are the most strategic and prescribed medications worldwide. Antibiotics gave quite a lot of therapeutic recommendations by minimizing the risk of infection after surgery or other hazards, such as after organ transplantation, which was the reason for elevated mortality rates before antibiotics were discovered. So, it is high time to rescue this miraculous drug, which can save millions of our lives. Antibiotics are divided conforming to their spectrum activity such as Broad spectrum (effective opposed to both Gram '+', '-' bacteria) and narrow-spectrum antibiotics

(effective opposed to only Gram '+' bacteria) (Xu, Peng, Wang, Fan & Li, 2014).

During this golden antibiotic period (1940-1970), it was predicted that roughly one million soil microorganisms were tested and a significant number of microbial metabolites were discovered (Wohlleben, Mast, Stegmann, & Ziemert, 2016). Some major antibiotics such as streptomycin have been discovered with several cultural techniques and strategies developed by him. With Selman Waksman's inspiring work, who was the first person to investigate with soil bacteria reported that more than 20 groups of antibiotics were discovered during this golden era from different bacterial species and fungi (E. D. Brown & Wright, 2016).

Despite the long period of the golden antibiotic era, the discovery of new groups of antibiotics has stagnated since 1980 with the ongoing tactic of discovering new microbial metabolites from soil microorganisms as there have been greater complications in cultivating the more costly microbes under laboratory conditions (Monciardini, Iorio, Maffioli, Sosio & Donadio, 2014). The work approach then changed to the *in-vitro* synthesis of new antibiotic groups with the understanding mechanism of actions from the classes of antibiotics discovered. Just a few effective groups of drugs such as oxazolidinones were developed in 1987. At around the same time, modification of the groups of antibiotics discovered tends to be the standard of antibiotics published on the market as Plazomicin and that was a semi-synthetic antibiotic of the aminoglycoside group in 2018 (Afouda et al., 2019).

2.4 Antibiotic resistance

Most of the major discovery has ever happened to the excellent researchers that took place over and above their aspirations till today or if that happened by luck or coincidence. The first-ever antibiotic 'Penicillin' was unintentionally identified in 1928 through the contamination of an agar plate in 1928 by Alexander Fleming (Ventola, 2015). This resistant property of bacteria is one of the healthcare system's most extreme threats. Resistant bacteria

infections are now also life-threatening. Some human pathogens even rebel against certain antibiotic categories (Ventola, 2015).

Since bacteria are living organisms, in a competitive environment they struggle to survive bacteria's genetic materials can provide themselves with the basic survival needs by encoding the genes while random mutations can occur in the bacterial genetic materials, leading to the appearance of a mutated gene that could alter the survival ability. The level and pattern of antibiotic use may impose evolutionary pressure on the environment, culminating in a selected environment that is advantageous to bacterial strains with mutated genes that could induce antibiotic resistance and disrupt the balance of strains of bacteria living in the area. The bacteria's resistant strains will then overpower the non-resistant strains to keep living and replicating, which may lead in the antibiotic being used becoming ineffective. As the process repeats with the implementation of the new antibiotics, there will be bacterial strains containing various mutated resistant genes and becoming the dominant strains (P. D. Gupta & Birdi, 2017).

Because the spontaneous mutation rate for the genes in bacteria is around 10^8-10^9 and bacteria are growing rapidly, a resistant strain of the bacterial population is generated only for a short period. Furthermore, the golden antibiotic age has deteriorated with the change to rational drug design strategy becoming uncompromising and more demanding, pharmaceutical companies turning their focus away from antibiotic production. All of this leads to the pace of development in stagnating new antibiotics.

Nonetheless, there are more outbreaks for bacteria's multidrug-resistant strains, and they have been proven to cause a variety of hospital infections. Resistant rates are expected to continue to rise, resulting in 10 million annual deaths with an economic burden of 100 trillion USD by 2050 (Spellberg, 2012). When bacteria evolve and acculturate no longer respond to antibacterial drugs means antibiotics already achieved resistant to bacteria. According to the Centres for Disease Control and Prevention (CDC), resistance occurs mainly when antibiotics are not effectively and efficiently prescribed, when bacteria are

allowed access to antibiotic sub-therapeutic doses and when resistance genes are transferred (Prevel, Berdaï & Boyer, 2019).

The naturally accompanying developed resistant characteristic of bacteria has distorted both (preventive and therapeutic) efficacies of antibiotics. The resistance that is known by today in their cells to resist the effect of the antibiotics which are; (i) Enzymatic inactivation of the antibiotic – was an established cellular enzyme in bacteria has been modified to manage antibiotics in such a way that it is no longer effective against bacteria, for example, β-lactamase enzymes, which hydrolyse the most extensively used antibiotics such as, β-lactamase (penicillin and cephalosporin) (Gonzalez-Bello, 2017); (ii) Drug elimination by activation of efflux pumps– one or more of these efflux pumps avoid the accumulation of antibacterial agents intracellularly, thereby reducing the concentration of antibacterial substances in cells. (Rahman, Yarnall & Doyle, 2017). (iii) Changes in the outer membrane permeability decrease the uptake- these alterations interfere efficacy of the antibiotic; (iv) Modifications on the drug target– the drug target undergoes modification which distorts the active site of the drug and hence limits its potency (Gonzalez-Bello, 2017).

In the United States, each year almost 2 million people achieve serious antibacterial infections which are already resistant to one or more than one antibiotic that is designed to treat those infections. Minimum 23,000 people die per year and because of these infections. Many other people die from some convoluted complexities occurred by resistance and in addition to that, each year almost 250,000 people require treatment for *C. difficile* infections whereas around 14,000 people die due to *C. difficile* infections (Surawicz et al., 2013). In treating these life-threatening bacteria, antibiotics contribute a major role here in these complexities which can even prevent many infections. Antibiotic-resistant infections add on considerable costs which already overburdened U.S. healthcare system with roughly estimated almost 20 billion USD in direct healthcare costs (Ventola, 2015). In the case of susceptible patients, the risk of development of life-threatening complexities increases and it has been really difficult to treat simple diseases for this kind of patients (Llor & Bjerrum, 2014). Several classes of antibiotics are given below (Table 1).

Table 1: Classes of antibiotics – (taken from Andy Brunning's, 'A Brief Overview of Classes of Antibiotics' Compound Interest, 2014)

	Year	Examples…	Bacterial targets
β-lactamase	1920s	Penicillins, Cephalosporins, Carbapenems	Cell wall
Sulfonamides	1930s	Sulfasalazine, Sulfisoxazole, Zonisamide	Folate
Aminoglycosides	1940s	Streptomycin, Neomycin, Gentamicin	Ribosomes
Tetracyclines	1940s	Doxycycline, Minocycline, Metacycline	Ribosomes
Macrolides	1950s	Azithromycin, Erythromycin, Clarithromycin	Ribosomes
Glycopeptides	1950s	Vancomycin, Teicoplanin, Oritavancin	Cell wall
Quinolones	1960s	Ciprofloxacin, Levofloxacin, Enoxacin	DNA
Oxazolidinones	1970s	Linezolid, Tedizolid	Ribosomes

World Health Organisation (WHO) has categorised globally concerned seven bacteria those are: *E. coli, K. pneumoniae*, *S. aureus*, *S. pneumoniae* and Non-typhoidal *S. shigella* and *N. gonorrhoeae.* As reported by several surveys, the resistance of *K. pneumonia* to their last line of antibiotic 'carbapenem' is more than 50% and the same thing goes for. Gram-negative bacteria are improving day-to-day resistance and becoming a significant problem across Europe. The resistance of MRSA and *S. pneumoniae* had decreased or endured constant since 2011-2014 and resistant rate reported 25% and above.

According to CDC take the risk factors of this resistance should be taken more seriously with the note of primary concern as it is now a matter of life threat (Health & Services, 2013). These most spreading and dangerous bacteria (such as *C. difficile*, carbapenem-resistant *Enterobacteriaceae* (CREs) and drug-resistant *N. gonorrhoeae)* required some aggressive action. Resistance develops hastily right after developing a new antibiotic (Rolain, Abat, Jimeno, Fournier, & Raoult, 2016). The first line defence antibiotics are rendered ineffective once bacteria develop no longer effectiveness towards those first-line chosen drugs. In that case, an alternative that left only is drugs that usually consist of higher toxicity profile with lower therapeutical benefit and at greater expense.

Antibiotic resistance can develop from two possible routes: 1) metamorphosis to the existing genes in bacteria where hereditary information of these resistant genes can be transmitted between bacterial cells through three (03) basic mechanisms known as- transformation, transduction and conjugation (Munita & Arias, 2016). Then bacterium with this resistant gene will collect more genes and ultimately it will weaken the activity of antibiotics and develops resistance afterwards. 2) alteration to the antibiotic molecule decreased penetration/increased efflux of the drug and 3) changes in the target site of the drug (Munita & Arias, 2016). For further research, understanding the physiological and biochemical mechanisms behind resistance towards antibiotics is crucial and important as well.

2.5 Action against antibiotic resistance

Governments around the globe are spreading public awareness about antibiotic resistance and its risk factors. Many nations are now implementing new initiatives and establishing initiatives to demonstrate antibiotic efficacy and decrease resistance risk. Antibiotic governance promotes multiple interventions and impactful policies aimed at making the public understand the optimal use of antibiotics in the patient's best interests. WHO has already developed Global Antimicrobial Resistance Surveillance System (GLASS), which aims to collect data around the world and to present an in-depth review of antibiotic resistance (Organization, 2018).

The CDC encourages four (04) key approaches to prevent antibiotic resistance from progressing. Tackling and tracking resistant bacteria and not prescribing antibiotics for diseases known to be resistant is now high time. As stated already antibiotics are now not prescribing and taking appropriately and thus fail to achieve their optimum use.

Four core actions might combat to fight with these deadly bacterial infections:

- To prevent bacterial infections and to prevent the spreading of resistance
- To track resistant bacteria
- To improve the use of current conventional antibiotics with proper guidelines provided by the healthcare systems.
- To promote the development and discovery of new antibiotics and to develop new diagnostic tests for resistant bacteria

Bacteria will inevitably discover methods to resist antibiotics that are being created, and that is why aggressive action is needed on a prior basis.

2.6 Uses of plants for antibiotics

Besides discovering microorganism antibiotics, researchers also found that most of the latest discovered antibiotics came from plants. Since centuries, nature has supplied people with fundamental requirements to maintain life in terms of food, shelter, clothing, transportation, fertilizer, paper, fragrance and last but not the least, medicines (Karunamoorthi, Jegajeevanram, Vijayalakshmi, & Mengistie, 2013). Since ancient times, plant-derived medications have been used in folk remedies to treat multiple diseases such as skin-related issues, fever, and flu, wound healing, stomachache, nausea, vomiting, and urinary issues associated with most of these bacterial diseases.

The assertion of long-established medicine and the discovery of bacterial resistance to traditional antibiotics which guided researchers in the second half of the 20th century to find out about the antimicrobial activities in medicinal plants (Renwick, Simpkin, & Mossialos, 2016). Since this is a plant-derived compound known to have fewer side effects, no toxicity or minimal toxicity to

the cells of the proprietor are considered prospective competitors for future antibiotics (Renwick, Brogan, & Mossialos, 2016).

Upon estimation, there are around 500,000 plant species found on Earth, including the unexplained ones. There was evidence that medicinal plants are a key source of drugs that people used to cure various infectious diseases in ancient times (Subramani et al., 2017). However, most developing countries also had records of plant use in the treatment of many diseases as they are easily accessible and have more economical options compared to developed medicines (Compean & Ynalvez, 2014). While records have been documented in traditional medicine systems indicating that herbal plants could be a valuable resource for people to treat diseases, many of these herbal medicines do not fit into the modern medicinal structure available as the current medicine standard.

Phytoconstituents such as flavonoids, alkaloids, tannins, and triterpenoids have established interesting activities and potentials that could be incorporated in modern medicines in combating broad-spectrum microorganisms which is present in plants, (Bilal et al., 2017). Reported by World Health Organization (WHO), almost 20,000 medicinal plants exist in 91 countries including 12 mega biodiversity countries. Malaysia is quite enriched with natural resources of flora and fauna along with miscellaneous remedial efficacy. It has been reported to several studies that, alkaloids, saponins, phenolics and flavonoids in plants are responsible for antibacterial activity (Weng & Huang, 2014).

There are a few benefits in the system of healthcare of using natural products. In addition to a diverse range of signs of disease such as anti-cancer, anti-infective, anti-diabetic, anti-bacterial, anti-viral, etc., plants also exhibit an exemplary range of chemical structures (Piasecka, Jedrzejczak-Rey & Bednarek, 2015). Upon analysing the chemical properties of naturally derived drugs has revealed that almost 50% of them were in conformity (Lipinski's Rule#5) for orally available medicines and the remaining had higher molecular weights, more rotatable bonds, and with more stereogenic centres, they still exhibited relative low log p values (Walters, 2012).

According to some studies, most bioactive compounds are simply secondary metabolites or their respective derivatives rather than plant-based primary metabolites. Primary metabolites refer to the essential macromolecules required for growth and metabolism, such as carbohydrates and proteins. Secondary metabolites refer to the compounds generated from the primary metabolism to support plant growth (Compean & Ynalvez, 2014). Such secondary metabolites were found to have a significant effect on plants ability to adapt under abiotic and biotic environmental conditions as well as to have defensive mechanisms to defend plants from external attacks by herbivores and pathogens.

With regard to the defensive mechanisms, it is noticed that they include the potential to conflict with the molecular targets in the pathogens including such linking to some of the protein domains to block some of the protein-protein interactions and even change the protein domains to prevent any more associations. However, some of the secondary metabolites are even found to be capable of affecting cell signalling to interfere with the pathogenic process caused by pathogens. Therefore, the secondary metabolites can execute enhanced antimicrobial activities due to multiple cellular and organic targets in the pathogens accessible for them to function on (Vinale et al., 2014).

In addition, crude plant extracts generally contain a blend of bioactive compounds that suggest combinations of the compounds used over a single isolated bioactive compound that promote synergistic effects to slow down the bacteria's level of resistance (O'Connell et al., 2013). Antimicrobial activities have been identified from secondary metabolites, tannins, terpenoids, alkaloids, etc., which can be further examined for the possibility of human use as antibiotics. Pharmacognosy is the branch of pharmaceutical science which is concerned about the discovery of drugs that have been derived from natural origins. Medicines derived naturally have always been used to treat minor ailments long time ago but more efforts are needed to be devoted into to this research and making them safer as there are a lot of scopes in healthcare management due to the vast possibilities of pure pharmaceutical compounds in plants. In another hand, Ethnopharmacy is another branch of pharmaceutical

science where it is concerned about other natural sources used in various ethnic groups (Jonville et al., 2013). As the rate of new developing antibiotics is decreasing and due to adopting resistance towards existing antibiotics, the needs of developing new antibiotics are dire.

So here in this project, *B. malaccensis* from the family Rutaceae and *K. retusa* from the family Myristaceae, *L. spathacea* from family Lauraceae and *D. hasseltii* from family Ebenaceae, and *K. maingayi* from family Apocynacea have been chosen for antibacterial studies. Even though these selected rare plants do not have any record regarding antibacterial activity and they are never studied before. But the families they belong from does have antibacterial activities which have been discussed in chapter I. The descriptions of the selected plants are given below.

2.6.1 *B. malaccensis* (Ridl.) Swingle plant description

B. malaccensis is a monotypic genus of flowering plants from the family of citrus, Rutaceae; which contains the single species *B. malaccensis*. This species is very scarce and has never been studied before. This species is now almost extinct in Peninsular Malaysia. It is native to Sumatra in Indonesia. Its common name is ‘Malay Ghost Lime’. This species is a part of the same subfamily (Aurantioideae), tribe (Citreae), and subtribe (Citrinae), and genus Citrus. It is technically known as a citrus fruit tree (Schwartz, Nylinder, Ramadugu, Antonelli & Pfeil, 2016).

B. malaccensis grows either single or in small groups in primary and secondary forest habitat. Trees from this species are mostly of about 15 m high and 12 cm in diameter, has a spiny brown trunk with green branches are green; leaves are a dark glossy green colour. Dark green leaves that were oval-shaped and consisted of multiple veins. Lamina was smooth in texture, with smooth edges and pointed tip (Figure 1). Fruits are fleshy, and yellow in colour, cylindrical pulp vesicles and numerous seeds. Lime-like green fruit that was round in shape (Figure 2). Outer peel was thin and rough and the flesh (cortex) was thin in a layer. It had medium-sized, pale green coloured seeds that were

dewdrop shaped and each was separated from the other by being placed onto separate seed cavities. There were 74 seeds on average, in each fruit. These seeds had a sticky pale juicy secretion around it, making them very sticky (Aatiani, 2016).

The family Rutaceae has been found containing many secondary metabolites such as alkaloids, coumarins, terpenoids with a large spectrum of biological activities. Flavonoids are among the active chemical components of Rutaceae species as already reported (Aatiani, 2016).

Figure 1: Leaf sample of *B. malaccensis* from family rutacea

Figure 2: Fruit sample of *B. malaccensis* from family rutacea

2.6.2 *K. retusa* (King) Warb. plant description

K. retusa belongs to the family Myristaceae, distinct species which is only restricted to Peninsular, Malaysia. This genus consists of an estimated around 60 species dispersed around other tropical counties such as Australia, Asia, Africa and can be found in Singapore, Thailand other than Malaysia. This species is very rare and unexplored just like *B. malaccensis.* Trees are around 6-18 m tall, comprises of dark brown rough bark with leaves of this species are quite large, dark glossy green in colour, ranging from 35-60 cm (Figure 3) (Callmander, Schatz, & Lowry, 2015). Secondary nerves of the leaves have been observed roughly within 16-24 pairs with 2-4 cm gaps in between each nerve. Both male and female flowers are observed. It has been known that this species is commonly used in treating various sores, cancers, and other serious illness. Plants from the family Myristicaceae have emerged as a credible source of new antimicrobials. It also has evaluated the antibacterial efficacy of aqueous (cold and hot water) extracts of *Myristica fragrans* which is commonly known as nutmeg. Several studies showed that *Myristica fragrans* have antibacterial activity against both Gram-positive and negative organisms. As such, it could be used for broad-spectrum antibiotics.

Figure 3: Leaf sample of *K. retusa* from family Myristicaceae

2.6.3 *L. spathacea* Gamble plant description

L. spathacea comes from the family of Lauraceae endemic to Peninsular Malaysia (N. Salleh, Azeman, Kiew, Kamin, & Kong, 2017). It can be found growing in lowland and hilly areas. The family of Lauraceae is in a major group of angiosperms (flowering plants) and most of them are evergreen trees in the habit (Joppa, Visconti, Jenkins, & Pimm, 2013). It is a tree or shrub which is 4.5-15 m tall; the leaves are in an alternate pattern. The leaf-blade upper surface is glossy, dark green, the lower surface is green with yellowish midrib (Figure 4). Fruits globose to elliptic 15-20 x 11-14 mm, smooth, red, or purplish when mature, cupule shallow, 13-15 mm in diameter, 6-7.2 mm high, margin entire or undulate, surface smooth. Many species of Lauraceae contain high concentrations of essential oils which are beneficial for perfumes and spices, as well as some of the species, are valued in medicinal material. However, research on the antimicrobial activity of this species was never thoroughly been investigated.

Lauraceae is a world-wide distributed family of flowering plants which most of are aromatic. Due to this characteristic, several species are used for cosmetics and gastronomic purposes. *Laurus nobilis* L. is an evergreen plant belonging to the Lauraceae family, native to Southern Europe and the Mediterranean area. The study demonstrated antibacterial activity against some of the highly susceptible strains of pathogenic and spoilage bacteria and yeasts (Fidan et al., 2018).

Figure 4: Leaf sample of *L. spathac* from family Lauraceae

2.6.4 *D. hasseltii* Zoll. plant description

The distribution of *D. hasseltii* mostly can be found in Southeast Asia, Thailand, Malaysia, and Indonesia from a member of the Ebenaceae family. This plant has its own vernacular names such as in Malaysia, known as merangat, Indonesia as semak or kesamak and in Thailand was called tako (Delfianti, Yuniastuti, & Cahyani, 2019). *D. hasseltii* is a species of plants that produce edible seeds and fruits, whereas recognised their cultivation status as wild (Milow, Malek, Edo, & Ong, 2014). The juice of the fruits is used as a varnish, yet medicinal uses of this plant remained unknown. The ecology of this plant can be found from coastal sandy or rocky shores up to lower montane mossy forests, including limestone hills and peat swamps. They are absent from mangrove forests. The tree can reach its maximum growth from 10 to 30 metres tall. Due to its edible seed, the tree is usually harvested from the wild and dioecious (very rarely monoecious) trees without latex.

The outer part of the bark is thin, black, hard, and finely longitudinally cracked or fissured. The leaves are simple, alternate, entire, exstipulate, petiolate, drying yellowish, dark brown or black on one or both sides (Figure 5). Shapes of the leaves can be oblongoid to elliptic with blade measurement of 12-35 cm height and 4-15 cm width. Besides, this plant has the shape of a fruit of a globose with a size of 1.5-3.5 cm in diameter. The flowers are unisexual (sporadically bisexual), articulated at the base, radially symmetrical, The fruits looked like a berry, pericarp fibrous or fleshy (Figure 5). The seeds are 1-16, arranged in a single whorl around a central axis, with thin coriaceous testa and thick horny endosperm, wedge-shaped seeds.

A few studied on several types of *Diospyros* species showed different biological activities among them. One of the most recognisable in this species is *Diospyros kaki*, which commonly known as persimmon. Persimmon, a deciduous tree of the family Ebenaceae, is found throughout East Asia and contains high levels of tannins. This class of natural compounds exhibit bactericidal activity suggesting potential medical applications. Consistent with these observations, persimmon leaves show antibacterial activity. About 130

species have been studied so far and compounds such as terpenoids coumarins and naphthoquinones have been isolated from Ebenaceae. Antibacterial activity was evaluated based on the minimal inhibition concentration (MIC). As stated by (Nordin & Zakaria, 2016), persimmon fruit has great medicinal qualities and widely acknowledged used to treat diarrhoea. Thus, in-depth research on *D. hasseltii a*pplication as well as in cosmetic and dermatological products.

Figure 5: Fruit and leaf sample of *D. hasseltii* from family Ebenaceae

2.6.5 *K. maingayi* (Hook.f.) Woodson plant description

According to (I. Norhayati et al., 2013), the distribution of *K. maingayi* can be found in Thailand, Sumatra, Peninsular Malaysia, Borneo, and the Philippines (Mindanao). The ecology of this plant can be found low land and lower montane forests. The tree can grow up to 40 m tall, to 120 cm diameter, sometimes with buttresses with white latex in all parts. Texturewise the barks are smooth or rough, pale brown, dark grey or whitish, inner bark pale yellow, less often brown. The leaves are coriaceous, glabrous; blade narrowly to broadly elliptic, base cuneate or decurrent onto petiole, apex acuminate or subcaudate; domatia absent or present, if present, with or without hairs; petiole 2-10 mm long (Figure 6). For flower parts, the pedicels are 7-12(-15) mm long, sepals acute or acuminate at apex, 1.5-3 x 1-2 mm, glabrous or rarely, corolla white or pale yellow (Figure 6). The fruits are very narrowly cylindrical, 8-50 x 0.4-0.6 cm. Seed grains 20-35 x 1.5-3 mm, bealglabrous for 5-10 mm, bearing an apical come for 0-65 mm: coma hairs 10-80 mm long.

The fundamental botanical characteristics of plants in this family are the excretion of abundant milky latex; easy exstipulate, opposite or whorled leaves; and showy, often pure white and moderately aromatic flowers with contorted lobes, with paired fruits. The exudation of milky latex may be noted during the processing of the bark of *Kibatalia* when the bark is removed from the forest. The features that make this plant remarkable are its capacity to produce a sequence of monoterpenoid indole alkaloids, including vinblastine and vincristine obtained from Catharanthusroseus G, in particular.

Figure 6: Leaf and flower sample of *K. maingayi* from family Apocynaceae.

Other secondary metabolites found in the Apocynaceae family, besides vinblastine and vincristine are steroidal alkaloids. There are 80 Asia-Pacific Apocynaceae species reported to have medicinal value, some of which are *Tabernaemontana sp*, *Amalocalyx sp*., *Melodinus sp*., and *Kibatalia sp.* Most of these plants use latex to treat gastrointestinal diseases, alleviate fever and pain, and treat diabetes and infectious illnesses (eastern medicinal plants). In this research, however, Kibatalia's leaves and barks were tested for their antimicrobial characteristics (Turner, Low, Rodda, Wong & Middleton, 2018).

2.7 Objectives

Malaysia's tropical rainforest is considered one of the world's most evolved and dynamic ecosystems serving an unexploited abundance of reserves (Tan, 2015). With the objective of evaluating native medicinal plants for bioactive compounds, this distinct natural heritage brought a resurgence of interest. Therefore, the goals of this research were:

i) To collect some rare medicinal plants possibly which has never studied before from Manong, the primary rainforest of Malaysia.

ii) To evaluate *in vitro* qualitative (Kirby-Bauer disc diffusion assay) antibacterial activity of *B. malaccensis* (Rutaceae), *K. retusa* (Myristicacea), *L. spathacea* (Lauraceae), *D. hasseltii* (Ebenaceae), and *K. maingayi* (Apocynaceae).

iii) To evaluate *in vitro* quantitative (Minimum inhibitory concentration-MIC and minimal bactericidal concentration-MBC) antibacterial assay of the selected extracts.

iv) To isolate and characterized at least one antibacterial constituent from the extracts with antibacterial activity specifically against Gram-negative bacteria.

v) To determine the mechanism of action (bactericidal or bacteriostatic) of the isolated compounds based on the calculation of MIC and MBC ratio.

CHAPTER III: PLANT COLLECTION, EXTRACTION AND YIELD

3.1 Collection of plants

The plants were collected from the rainforest of Perak, Peninsular Malaysia, in mid of February 2018. The samples for this project were sent to the Forest Research Institute of Malaysia (FRIM), Kepong, Selangor to identify the species (Table 2). The voucher specimens were stored in FRIM.

Table 2: Basic information on the studied plants

No.	Voucher Number	Family	Genus	Local name
1.	PID 120218-12	Rutaceae	*Burkillanthus*	Limau hantu
2.	PID 130218-13	Myristicacea	*Knema*	Penarahan
3	PID 351117-22	Lauraceae	*Litsea*	Medang
4	PID 361117-22	Ebenaceae	*Diospyros*	Kayu Arang
5	PID 341117-22	Apocynacea	*Kibatalia*	Jelutong Pipit

3.2 Separating and processing plant parts

After collecting leaves, barks, woods, seeds, flesh, and peels of fruits for this project then the plant parts were separated and air-dried at room temperature for almost 2 weeks. During the processing of the plant parts, the conditions of the plant parts were observed and recorded such as the production of latex, smell, texture, and colour of the plant parts. The dried plant materials were then finely fragmentized by grinding using aluminium collection blender (Philips, China) and the powders obtained from grinding were weighted with top loading balance (Sartorius AG, Germany). The powders were weighed and labelled with appropriate indications and stored under room temperature before they were been extracted.

Table 3: Basic information on the studied plants collected in Manong primary rainforest

Family, genus species and authority	Voucher N° Traditional uses	Date of collection[a]	Location	Common name	Part collected	Traditional uses
APOCYNACEAE						
Kibatalia maingayi (Hook. f.) Woodson	NB0328	23/3/2017	4 º.69’, 100 º.82’	Jelutong pipit	Leaves, bark	None
EBENACEAE						
Diospyros hasseltii Zoll.	NB0245	12/3/2017	4.73 º, 100. º 81’	Merangat	Leaves, bark, fruits	Seeds eaten
LAURACEAE						
Litsea spathacea Gamble	NB0523	5/3/2017	4.70 º, 100. º 83’	Derahan	Leaves, bark	Fever
MYRISTICACEAE						
Knema retusa (King) Warb.	NB0314	25/3/2017	4 º.69’, 100 º.82’	Pala hutan	Leaves, bark	None
RUTACEAE						
Burkillanthus malaccensis (Ridl.) Swingle	NB0541	10/3/2017	4 º.61’, 100 º.80’	Limau hantu	Leaves, barks, fruits,	None

[a]Date of collection: day/month/year

3.3 Microscopic analysis

Microscopic analysis was carried out to identify a structural characteristic of species (*K. retusa* and *B. malaccensis*) and the optical microscope is an excellent instrument for identifying plants and examining objects. The dried leaf powder required boiling to soften the material and bleaching for disintegration and isolation of tissue. A few grams of the powdered sample was required for disintegration and isolation of tissue. Different clear observations of tissue objects were captured with different magnification by using a microscope.

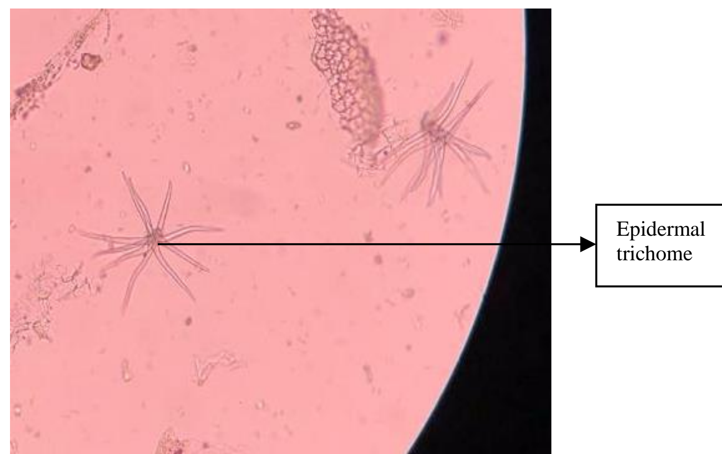

Figure 7: Microscopy view of *K. retusa* leaf with 10x magnification

Epidermal trichomes are observed in *K. retusa* leaves (Figure 7) as groups of unicellular hairs or small papillose outgrowths. The clothing hairs are characteristic to this species.

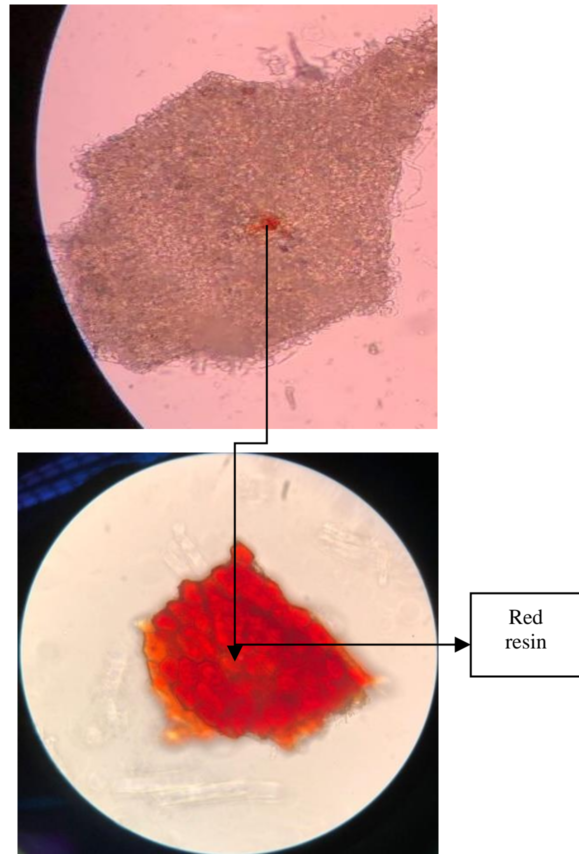

Figure 8: Microscopy view of *K. retusa* bark with 10x magnification

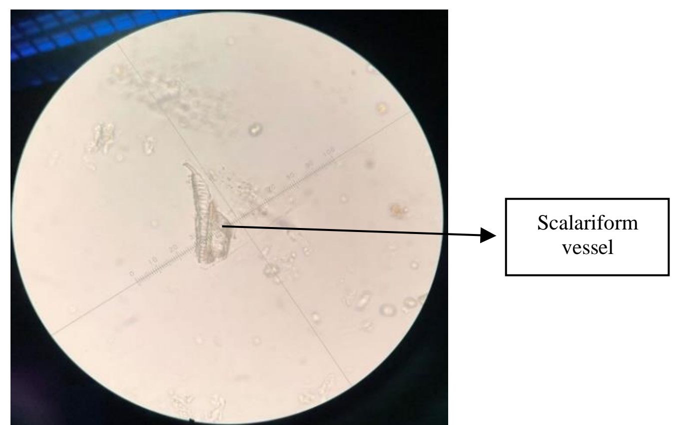

Figure 9: Microscopy view of *K. retusa* bark with 4x magnification

K. retusa is characteristic for its red resin (W. M. N. H. W. Salleh & Ahmad, 2017) which is usually found in the wood and bark of the plant. A small sample of this can be seen (Figure 8). In the image at 10x magnification, the vibrant red colours concentrated clustered cells forming the red sap has been observed. The last image displays a scalariform vessel (xylem component) located mostly on the inner layer of the bark.

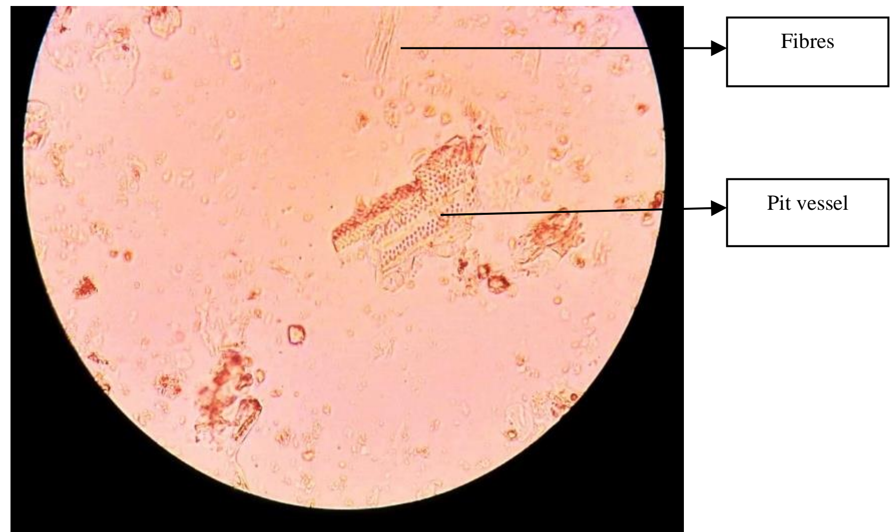

Figure 10: Microscopy view of *B. malaccensis* bark with 4x magnification

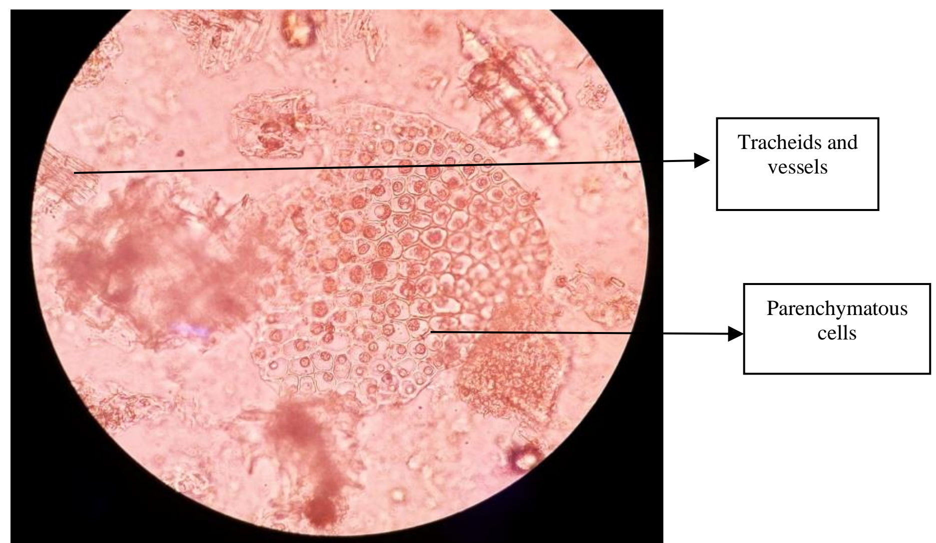

Figure 11: Microscopy view of *B. malaccensis* bark with 10x magnifications

B. malaccensis bark shows phloem parenchyma, fibres, elongated cork cells, parenchymatous cells and brown resin masses, tracheids and vessels along with medullary rays, pitted vessel, xylem fibre showing lumen and group of fibres and so on.

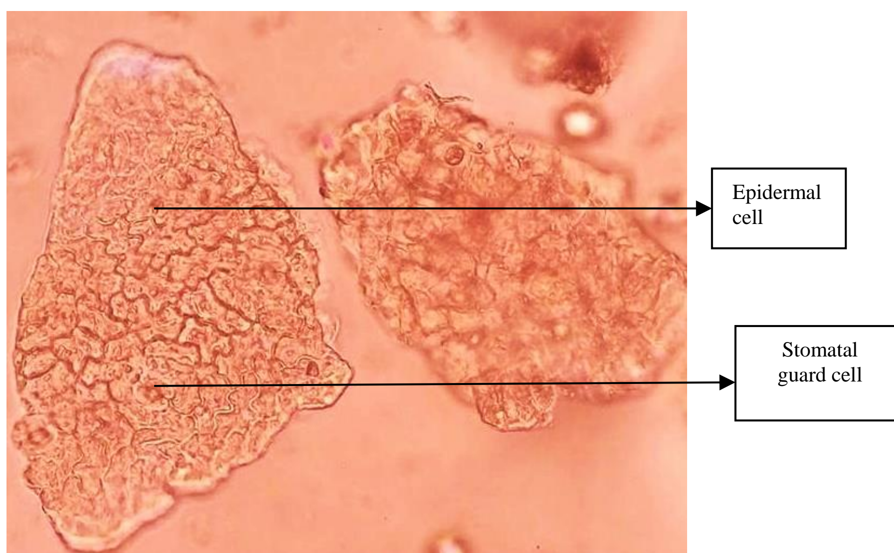

Figure 12: Microscopy view of *B. malaccensis* leaves with 10x magnification

While viewing under the microscope at 10x magnification, it was possible to observe the epidermal cells and that tend to be irregular. In addition to the epidermal cells the leaf spores (stomata) in between the epidermal cells were visible as well (Figure 12).

3.4 Extraction of plant materials

The plant powders (200g) were mixed sequentially at room temperature with various organic solvents of different increasing polarity starting with hexane (Friendemann Schmidt, Australia), chloroform, and methanol (Friendemann Schmidt, Australia). Each extraction was performed by maceration of plant powder-to-solvent ratio of 1:5 (w/v) for 3 days at room temperature (Hossain, Al-Hdhrami, Weli, Al-Riyami, & Al-Sabahi, 2014). The respective solvents were then eventually filtered through qualitative filter papers (Whatman International Ltd., England) and the filtrates were concentrated afterwards to dryness under reduced pressure at 40°C using a rotary evaporator

(Buchi Labortechnik AG, Switzerland). The dry extracts obtained were weighed with an analytical balance (Sartorius AG, Germany).

Solvents are evaporated through heat and vacuum by using rotary evaporator for fast and effective evaporation (Hossain et al., 2014). The filtrate obtained through soxhlet extractor was transferred into the round bottom flask (immerged halfway into the water bath) of rotary evaporator up to no more than halfway. Then water bath was turned up to be heated 50°C and the vacuum and condenser were switched on, rotation dial was turned on to an optimal (3-5) speed, allowing the flask to spin in the water. Hexane or other respective solvents which were condensed in the condenser was collected in a different bottle for recycling. The solution was again poured into the flask and continued this process until the entire filtrate has been extracted collected and stored into small glass vials. The final weight of the extracts has been recorded before and after drying.

3.5 Yield of extracts

Dried plant parts were eventually extracted with hexane, chloroform, and methanol to obtain lipophilic (non-polar), amphiphilic (mid-polar), and hydrophilic (polar) extracts, respectively. The reason behind choosing three types of solvents in extractions was to determine at which fraction the antibacterial activity from the crude extracts give the most activities. Since there are a variety of different solutes or chemical compound exist in their parts, thus, it is difficult to predict their solubility in a particular solvent. To resolve this, their chemical compounds can be separated by considering their solubility by using the concept of polarity (Kumoro, Hasan, & Singh, 2012). Therefore, different types of solvents with different polarity were used to extract the different chemical constituents from the plants.

$$\text{Yield (\%)} = \frac{\text{Weight of dried crude extract (g)}}{\text{Weight of dried plant material (g)}} \times 100$$

Table 4: Percentage yields of all the studied plants

Genus species	Part extracted	Percentage Yield (%)		
		Hexane	Chloroform	Methanol
K. retusa	Leaves	0.27	1.09	0.39
	Barks	0.59	0.29	1.03
	Woods	0.51	5.62	6.53
B. malaccensis	Leaves	18.24	33.23	109.23
	Barks	3.80	5.42	12.92
	Woods	1.04	3.46	4.21
	Epicarp (Peel)	5.10	29.60	21.74
	Flesh (Endocarp)	-	9.59	10.57
	Seed	15.50	5.17	30.66
L. spathacea	Leaves	1.59	4.98	3.00
	Barks	0.72	3.27	2.24
D. hasseltii	Leaves	3.93	2.98	2.42
	Barks	0.73	0.91	1.75
	Fruits	3.60	4.90	5.90
K. maingayi	Leaves	1.57	3.05	3.17
	Barks	1.15	1.67	1.73
Average yields		3.89	7.20	13.44

Here the order of solvents used was from non-polar, mid polar to polar which was from hexane, chloroform, and methanol. The polarity characteristics of solvents used in this study are as follows; hexane is non-polar, so it will remove all the fatty and lipophilic compounds from the plants; chloroform is intermediate, so it will extract all the components which have an intermediate polarity, and methanol is very polar, so it will extract all polar compounds from the plants. Yields of leaves, barks and woods extracted with hexane chloroform and methanol from *K. retusa* were (0.27, 1.09, and 0.39) %, (0.59, 0.29, and 1.03) % and (0.51, 5.62, and 6.53) % respectively. Yields of leaves, barks, woods, peel, flesh and seed extracted with hexane chloroform and methanol from *B. malaccensis* were (18.24, 33.23, 109.23) %, (3.80, 5.42, and 12.92) %,

(1.04, 3.46, and 4.21) %, (5.10, 29.60, and 21.74) %, (0, 9.59, and 10.57) % and (15.50, 5.17, and 30.66) % accordingly. Yields of leaves and barks extracted with hexane chloroform and methanol from *L. spathacea* were (1.59, 4.98, and 3.00) % and (0.72, 3.27, and 2.24) % respectively. Yields of leaves, barks and fruits extracted with hexane chloroform and methanol from *D. hasseltii* were (3.93, 2.98, and 2.42) %, (0.73, 0.91, and 1.75) % and (3.60, 4.90, and 5.90) % consecutively. Yields of leaves and barks extracted with hexane chloroform and methanol from *K. maingayi* were (1.57, 3.05, and 3.17) % and (1.15, 1.67, and 1.73) % accordingly. The differences in antimicrobial activities in plants extract strongly related to the solubility of active compounds in plant materials according to the polarity of solvents which were used during extraction (Teka et al., 2015). Hence, the variation in the antibacterial activities in this study could have caused by the extraction solvent. In this experiment, the mass of powder used was 200g.

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CHAPTER IV: *IN VITRO* ASSAY (PAPER DISC DIFFUSION)

This project started with qualitative antibacterial assay (paper disc diffusion) on 48 crude extracts from 5 rare selected medicinal plants. The aim was to isolate an antibacterial pure compound specifically against Gram-negative bacteria but both bacterial strains were tested here as a comparison. Here in this chapter, selected 5 plants were tested against 2 Gram-negative (*E. coli* and *P. aeruginosa*) and 2 Gram-positive bacteria (*S. aureus* and *B. subtilis*). Samples with positive antibacterial activities are only mentioned here in this chapter (Table 5 and 6) and (Figure 13-21) and both positive and negative results are given in the appendix (Table A.1 and A.2). Based on the results observed in this experiment, further quantitative experiment and isolation were carried out with selected samples. Selection of bacteria varied in both antibacterial experiments due to the availability of bacteria in the laboratory at that moment. Paper disc assay was carried out in the microbiology laboratory of the University of Nottingham. Gram-positive bacteria were used here as a comparison but focus, discussion and conclusion of the results largely got against Gram-negative bacteria. This chapter is composed of overview (4.1), protocols and methods (4.2), results and discussion (4.3), (Table 5 and 6) and (Figure 13-21) and conclusion (4.4) of the experiment of paper disc assay.

4.1 Overview of paper disc assay

Antimicrobial susceptibility testing of these plants using the disc diffusion method also known as Kirby-Bauer test has been introduced in 1940 and one of the classic microbiology techniques and still commonly used in most clinical microbiological laboratories (Gerbig, Aubihl & Engohang-Ndong, 2013). The traditional technique of assessing antimicrobial susceptibility was focused on methods of broth dilution, which are time-consuming to execute, although it is still at the high standard today. This led to the development of a disc diffusion process to determine antimicrobial susceptibility of bacteria. This method was chosen mainly owing to its simplicity, efficiency, and convenience for determining the antimicrobial properties of the studied extract. The aim of the paper disc diffusion assay is to assess the susceptibility or tolerance of

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pathogenic aerobic and facultative anaerobic bacteria to different antimicrobial (Nagy, Boyanova & Justesen, 2018).

The bacteria cell is developed on Mueller-Hinton agar in the presence of specific antimicrobial impregnated filter paper discs. The presence or absence of inhibition around the discs is an indirect measurement of the capability of that compound to inhibit that bacteria. In this test, the diameter zone of growth inhibition around the disc impregnated with extracts of *B. malaccensis*, *K. retusa*, *D. hasseltii*, *L. spathacea,* and *K. maingayi* are measured and indicates to susceptibility to that plant extracts.

4.2 Protocols and methods of paper disc assay

The antibacterial activities of the selected crude extracts of various medicinal plants were assessed against four Gram-positive and Gram-negative bacteria using paper disc assay method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) formerly known as National Committee for Clinical Laboratory Standards (NCCLS) (Nagy, Boyanova & Justesen, 2018).

4.2.1 Preparation of agar plates

Mueller-Hinton agar (MHA) (Difco Laboratories, USA) was prepared according to the manufacturer's instructions. The whole procedure was carried out in a microbiology lab under a biohazard safety cabinet to ensure sterility. To prepare agar medium, 38 g of Mueller-Hinton agar (MHA) was weighed accurately and poured into an autoclaved empty Schott bottle and after that distilled water was poured into the bottle up to 1 litre and then mixed together until it dissolves completely. The mixture was then sent for autoclaving. The agar medium was maintained at a temperature around 60°C (need to be warm and liquid) immediately after autoclaving and poured into the petri dishes approximately up to halfway in a horizontal surface. The dishes were left to cool down and set for about 15 minutes, and then were covered with the lids and stored in the refrigerator.

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4.2.2 Preparation of impregnated filter paper discs

Qualitative filter paper No. 1 (Whatman International Ltd., England) was used to prepare paper discs for antibacterial testing. The paper was hole-punched, to a width of 6 mm, and autoclaved to ensure sterility. 5 mg/1 mL solutions were prepared for each extract and 20 µL of this was used to soak each paper disc using a volumetric pipette.

Discs were placed on glass discs to dry; these discs were being dried under the biohazard safety cabinet. Negative control discs were also prepared. The paper discs were then left to dry for 24 hours before being placed onto the agar medium. The antibiotic chloramphenicol and ampicillin were used as positive controls and DMSO (R & M Chemicals, UK) used as the negative controls which were pre-prepared discs (20 µL in each disc).

4.2.3 Growing the bacteria

MHB was required for the bacterial growth and this 21 g of MHB was weighed accurately and added to an autoclaved Schott bottle with 1 L of distilled water. The mixture then sent for autoclaving to ensure sterility. 10 mL of the broth (autoclaved) was measured into glass vials. Four different Gram-positive and Gram-negative bacteria were used for *B. malaccensis, K. retusa* and in this experiment and those were *B. subtilis (*ATCC 6633), *S. aureus* (ATCC 11632), *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 10145). A total of four bacterial strains including both Gram-negative bacteria was used for *L. spathacea* and *D. hasseltii* which consist of *E. coli* (ATCC 25218), *P. aeruginosa* (ATCC 10145) and Gram-positive bacteria which consist of *S. aureus* (ATCC 29213) and *B. subtilis* (ATCC 6633) were selected to assess the susceptibility patterns against the plant extracts and maintained in the microbiology department of University of Nottingham. Bacteria selection fluctuated during antibacterial experiments due to the availability of bacteria at that moment.

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By using a sterilized inoculating loop, bacterial colonies were taken and mixed into the MHB contained glass vials, then placed into an incubator shaker for 60-90 minutes depending on bacteria which allowed the bacteria to grow in the broth. The UV absorbance was measured at an optical density to ensure the bacteria had colonised enough or not (Nahi, Othman & Omar, 2016). A sample from each vial placed in a cuvette and its UV absorbance (600 nm) was tested, to ensure bacterial optimum density ranged within 0.08 to 0.1.

$$\text{Standard deviation} = \sqrt{(\text{first result} - \text{mean})^2 + \frac{(\text{last result} - \text{mean})^2}{2}}$$

4.2.4 Disc assay

To perform the disc assay diffusion a sterile cotton swab was immersed into the glass vial containing bacterial suspension and swabbing it from different angles on the agar plate ensuring the entire surface of the agar plate should have inoculated by bacterial solution or ‘L’ spreader can also be used instead of a cotton swab. 10 µLof bacterial suspension should be taken out by pipette and put on the agar plate. A glass ‘L’ spreader (heated each time before use by Bunsen burner to ensure sterility) can be used to spread the suspension all over the agar plate. Sterile forceps were then used to place pre-soaked paper discs firmly onto the agar plates. Plates were divided evenly into either five or six sections and labelled accordingly for the discs. As well as the extract discs, the negative and positive controls were also placed on the agar plates. The petri dishes were lastly closed with their lids then sealed by parafilm. They were placed in an incubator at 37^0 C and left for 24 hours (Nahi, Othman & Omar, 2016).

4.2.5 Reading plates and interpreting results

The paper disc diffusion method was applied to test the antibacterial activity of the crude plant extracts in opposition to different Gram-positive and Gram-negative bacteria cells. After 16-18 hours of incubation, the diameters of

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the zones of inhibition were measured by the unaided eyes. Zones were measured using sliding callipers or a ruler, which was held on the back of the inverted petri plates. Readings from 6mm indicated negative results, as this is the width of the paper disc, so zones of inhibition were above 7 mm, denotes activity. The experiments were repeated in triplicates for being accurate and reproducible.

4.3 Result and Discussion of paper disc assays

Plants are indeed an unexploited source of medicinal products with high therapeutic values. The consequences of medicinal plant extracts for the growth of antibacterial medicinal products seem challenging as they can assuage the adverse effects often associated with synthetic antibiotics (Jahanshahi-Anbuhi et al., 2017). Kirby-Bauer Disc Diffusion Assay was implemented in the current research to assess antibacterial actions of the crude extract against ATCC bacterial strains. This qualitative method is broadly used in antibiotic susceptibility tests in which filter paper discs are usually impregnated with antibacterial agents (here crude extracts) are transferred to the inoculated agar plate (Abdallah, 2016). The effectiveness of these samples can then be terminated by evaluating the diameter of the inhibition regions that result from their diffusion into the agar medium around the discs (Jahanshahi-Anbuhi et al., 2017). The paper disc test is a well recognised and established test with a standardised method. In correlation to other methods, it is a consistent test which is quite advantageous and reliable. This is a qualitative experiment which can proof the bacteria as resistant, intermediate and susceptible (Balouiri, Sadiki, & Ibnsouda, 2016). This experiment was terminated with both Gram-positive and Gram-negative for contrariety despite the fact that the aim was only to observe activity against Gram-negative bacteria.

Gram-negative bacteria are commonly more resistant to plant-based antibacterial agents as opposed to Gram-positive bacteria (Chandra et al., 2017). The variations in susceptibility of these two bacteria groups can be ascribed to their outer structures (Singariya, Mourya, & Kumar, 2012). As discussed earlier,

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the cell structures of Gram-negative and Gram-positive bacteria in the previous chapter (Chapter III). Due to an outer membrane layer, Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria. The outer membrane of Gram-negative bacteria contains some proteinic channels called porins (also aquaporins) (Galdiero et al., 2012). These porins will only al1ow the penetration of small (low molecular weight) xenobiotics (antibiotics) natural products from plants and therefore high molecular weight xenobiotics will not be able to have antibacterial effects against Gram-negative bacteria (Medhi, Goyal, Semwal & Prakash, 2019). For instance, aminoglycosides antibiotics are not able to inhibit the growth of Gram-negative bacteria because they cannot penetrate porins (Ghai & Ghai, 2018). Gram-positive bacteria do not have an outer membrane and their peptidoglycan wall is directly accessible to antibacterials compounds.

To date, most antibiotics are addressed at intracellular processes and must be able to penetrate the bacterial cell envelope. Notably, the outer membrane of Gram-negative bacteria provides a formidable barrier that must be overcome (Ghai & Ghai, 2018). This experiment was used for the processing of crude extracts in this research and was then tested for antibacterial activity. The Kirby-Bauer standardized paper disc diffusion method was applied to detect the antibacterial activity of the crude extracts of *B. malaccensis, K. retusa, L. spathacea, D. hasseltii,* and *K. maingayi* against both Gram-positive and negative bacteria. As the width of the paper disc is 6 mm, so zones of inhibition which were 7 mm above denote activity. In this experiment, a total of 48 extracts of 5 different plants was extracted with 4 different solvents and tested against 2 Gram-positive and 2 Gram-negative bacteria. Results only which implied positive antibacterial activity are included in the tables (Table 5 and 6), readable clear figures (Figure 13-21) and both positive and negative results are given in the appendix (Table A.1 and A.2).

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4.3.1 Antibacterial activity assessment with disc diffusion assay by *B. malaccensis*

All the tests were performed in triplicates here. Inhibition zones developed by the extracts of *B. malaccensis* LC, LM, BH, WH, and WC (100.00 µg/disc) showed activity against *S. aureus* and on the other hand, LM, BH, WH, WC (100.00 µg/disc) also illustrated similar activity against another Gram-positive bacteria *B. subtilis* (Figure 13, 14 and 15). In terms of Gram-negative bacteria only FC (Figure 16), FM and SH (Figure 17) (100.00 µg/disc) were active against *P. aeruginosa* and no activity against *E. coli.* Out of 14 extracts of *B. malaccensis* against Gram-positive bacteria cells (*S. aureus* and *B. subtilis*), 8 extracts showed zones of inhibitions. In LM the ZOI was (7.60 ± 0.30) mm, means 0.30 mm deviation away from a mean of 7.60 mm whereas BH, WH and WC showed 2.80 mm, 0 mm and 0.90 mm deviation away from a mean of 10.60 mm, 8.00 mm and 8.50 mm respectively against *S. aureus* (Table 5). In correlation with the positive control, antibiotic chloramphenicol, ampicillin and cephalexin have been used and showed inhibition zone diameters of (27.00 ± 3.00) mm, (10.00 ± 2.00) mm and (29.29 ± 0.20) mm. So, the antibiotic control of chloramphenicol here was showing a promisingly significant zone of inhibition measurement. On the other hand, LM, WH (Figure 13, 15) and WC showed an almost similar result in compare with ampicillin which is very positive.

In consideration of the inhibition developed by the extracts against of *B. subtilis,* LM, BH, WH and WC with inhibition zone diameters of (7.20 ± 0.80) mm, (8.30 ± 1.50) mm, (9.50 ± 1.80) mm and (8.30 ± 2.30) mm respectively. Here chloramphenicol, ampicillin and cephalexin were used as a positive control and according to the comparison they showed ZOI diameters of (27.00 ± 3.00) mm, (20.00 ± 4.00) mm and (30.00 ± 0.50) mm respectively (Table 5), which means extracts of *B. malaccensis* against *B. subtilis* showing less activity towards *S. aureus* with a similar result as ampicillin showed.

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Interestingly only FC, FM and SH displayed ZOI diameters of (11.30 ± 1.50) mm, (20.30 ± 1.20) mm and (23.30 ± 2.00) mm respectively against *P. aeruginosa.* FC, FM, and SH did not portray any antibacterial activity against any other bacteria. So, FC, FM and SH caught attention here. Here is a positive control, chloramphenicol manifested (28.00 ± 5.00) mm and (30.00 ± 2.00) mm of a zone of inhibition diameters against both the Gram-negative bacteria, whereas ampicillin showed (16.50 ± 3.00) mm against *E. coli* and no activity against *P. aeruginosa* (Table 5). Cephalexin showed (22.40 ± 0.60) mm and (10.00 ± 0.50) mm against *E. coli* and *P. aeruginosa.* Results which did not imply any positive response are not included in the (Table 5), in addition to only clear and understandable figures are attached in this chapter.

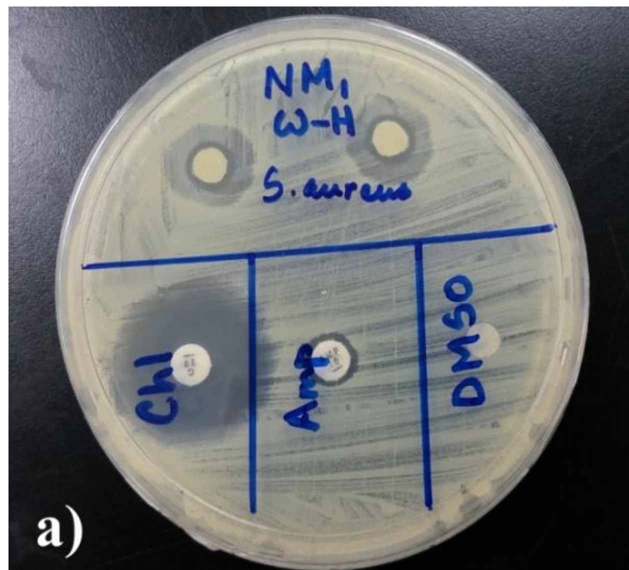

Figure 13: Inhibition zone developed by hexane extract (100 µg/disc) of wood of *B. malaccensis* against *S. aureus*

Note: Pictures only with a good zone of inhibitions are included here

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Figure 14: Inhibition zone developed by hexane extract (100 µg/disc) of the bark of *B. malaccensis* against *B. subtilis*

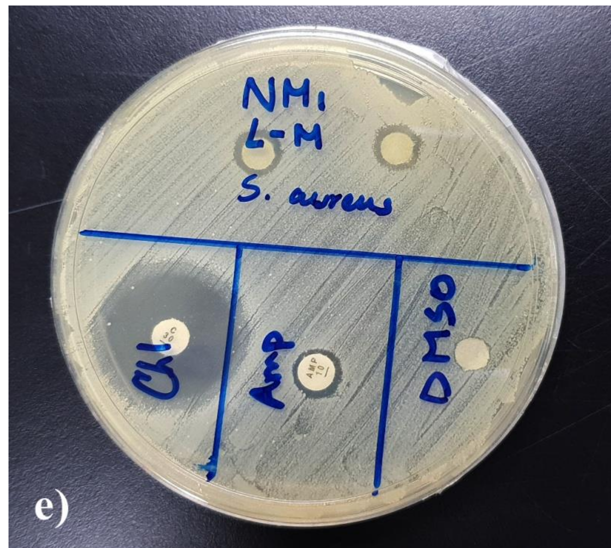

Figure 15: Inhibition zone developed by methanolic extract (100 µg/disc) of leaves of *B. malaccensis* against *S. aureus*

Note: Pictures only with a good zone of inhibitions are included here

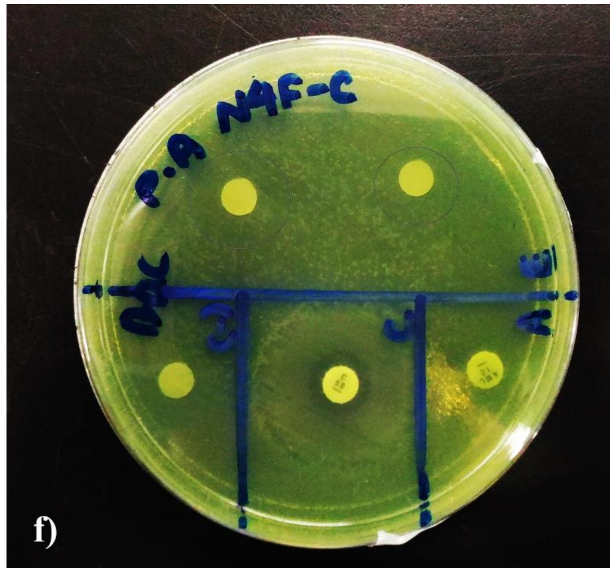

Figure 16: Inhibition zone developed by chloroform extract (200 µg/disc) of the flesh of *B. malaccensis* against *P. aeruginosa*

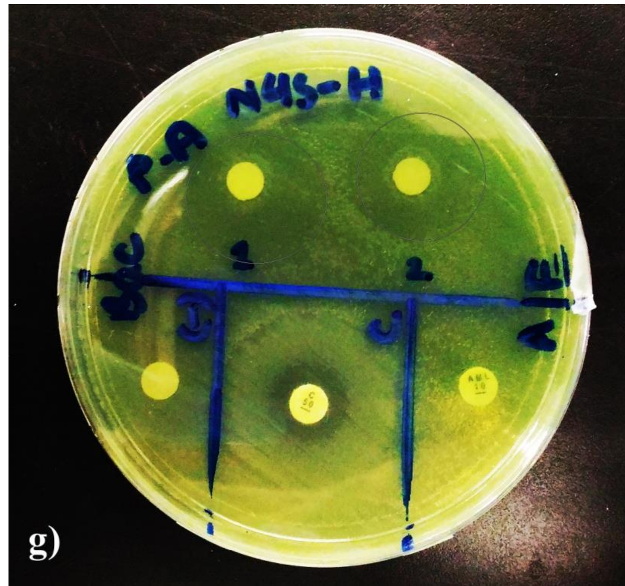

Figure 17: Inhibition zone developed by hexane extract (200 µg/disc) of the seed of *B. malaccensis* against *P. aeruginosa*

Note: Pictures only with a good zone of inhibitions are included here

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4.3.2 Antibacterial activity assessment with disc diffusion assay by *K. retusa*

Inhibition zones established by the extracts of *K. retusa,* (LM, BH, BC, WH, WC (100.00 µg/disc) demonstrated activity against *S. aureus* and BH, WH and WC showed zones of inhibition against *B. subtilis*. None of the extracts tested was active against the Gram-negative bacteria tested except WC which showed activity against *E. coli.* So, in comparison, extracts from both the plants did not show any significant activity towards Gram-negative bacteria but with Gram-positive bacteria (Figure 18 and 19) and (Table 5). LM, BH, BC, WH, WC showed ZOI diameters of (8.50 ± 0.50) mm, (8.00 ± 0.90) mm, (7.80 ± 0.30) mm, (7.80 ± 0.30) mm and (8.20 ± 0.30) mm against *S. aureus* and BH, WH and WC showed ZOI diameters of (7.20 ± 0.30) mm, (7.80 ± 1.00) mm and (8.00 ± 1.70) mm against *B. subtilis*. Positive controls such as chloramphenicol, ampicillin and cephalexin showed ZOI diameters of (27.00 ± 3.00) mm, (10.00 ± 2.00) mm and (29.29 ± 0.20) mm against *S. aureus* respectively; (27.00 ± 3.30) mm, (20.00 ± 4.00) mm and (30.00 ± 0.50) mm zone of inhibition diameters against *B. subtilis* (Table 5). This alludes antibacterial activity against Gram-positive bacteria, however supposing comparison with the antibiotic result, it is not strongly significant. Although as it demonstrated good standard deviation which particularly will be ongoing in the following experiments. In subject to Gram-negative bacteria any effective antibacterial activity is not obtained but only W-C showed ZOI diameters of (7.30 ± 0.60) mm of the mean against *E. coli*. Only affirmative antibacterial results are embodied in (Table 5) and readable figures are given at the end of this chapter and all the tests were performed in triplicates here.

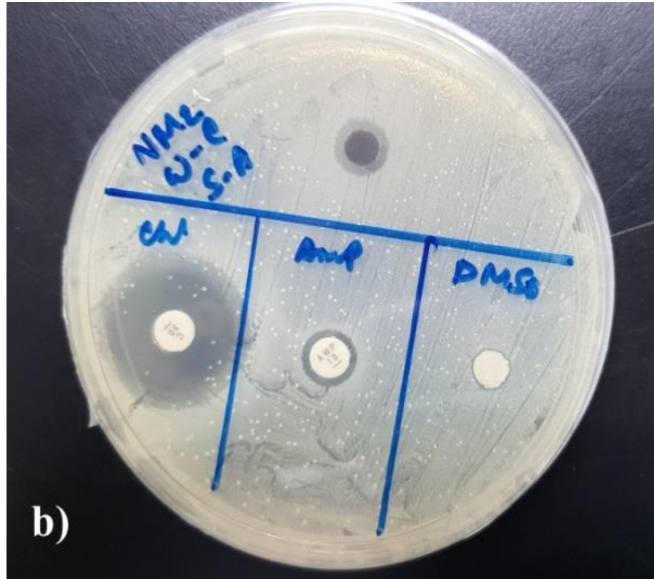

Figure 18: Inhibition zone developed by chloroform extract (100 µg/disc) of wood of *K. retusa* against *S. aureus*

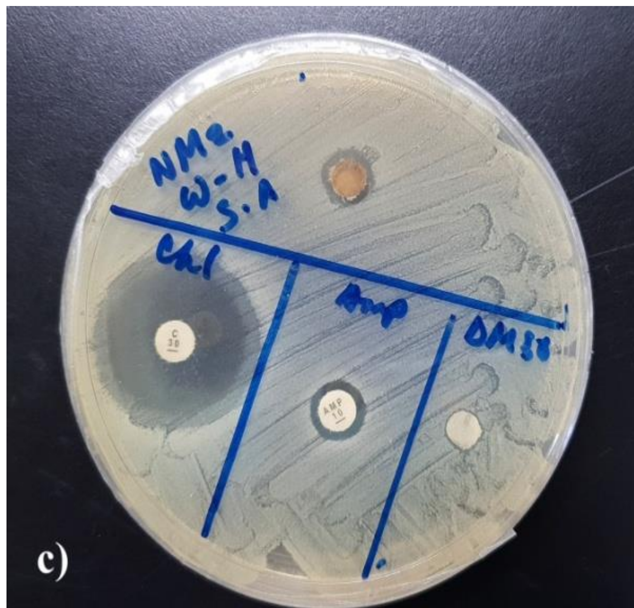

Figure 19: Inhibition zone developed by hexane extract (100 µg/disc) of wood of *K. retusa* against *S. aureus*

Note: Pictures only with a good zone of inhibitions are included here

Table 5: Zones of inhibitions against 4 bacteria cells by *K. retusa* (c100.00 µg/disc), *B. malaccensis;* (c100.00 µg/disc)

	Zone of Inhibition (mm)			
Genus species Part/Solvent	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*
K. retusa				
LC	6.66 ± 0.30	-	-	-
LM	8.50 ± 0.50	-	-	-
BH	8.00 ± 0.90	7.20 ± 0.30	-	-
BC	7.80 ± 0.30	7.00 ± 0.50	-	-
WH	7.80 ± 0.30	7.80 ± 1.00	-	-
WC	8.20 ± 0.30	8.00 ± 1.70	7.30 ± 0.60	-
B. malaccensis				
LH	6.50 ± 0.50	6.50	-	6.60 ± 0.30
LC	7.00 ± 0.50	6.50	-	
LM	7.60 ± 0.30	7.20 ± 0.80	6.60 ± 0.30	-
BH	10.60 ± 2.80	8.30 ± 1.50	-	-
BC	7.00	6.50	-	-
BM	6.80 ± 0.30	6.60 ± 0.30	-	-
WH	8.00	9.50 ± 1.80	-	6.80 ± 0.30
WC	8.50 ± 0.90	8.30 ± 2.30	-	-
WM	-	-	-	7.00
FC	-	-	-	11.30±1.50
FM	-	-	-	20.30±1.20
SH	-	-	-	23.30±2.00
Standard Antibiotics				
Chloramphenicol	27.00 ± 3.00	27.00 ± 3.00	28.00 ± 5.00	30.00 ± 2.00
Ampicillin (10 ug)	10.00 ± 2.00	20.00 ± 4.00	16.50 ± 3.00	-
Cephalexin (30 ug)	29.29 ± 0.20	30.00 ± 0.50	22.40 ± 0.60	10.00 ± 0.50

Note: samples with no activity are not included here in this table.
The tests were performed in triplicates.

Plant Parts:
L: Leave, B: Bark, W: Wood, F: Flesh, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol
S. aureus: *Staphylococcus aureus* (ATCC 11632), *B. subtilis: Bacillus subtilis* (ATCC 6633), *E. coli*: *Escherichia coli* (ATCC 8739), *P. aeruginosa*: *Pseudomonas aeruginosa* (ATCC 10145) for *K. retusa* and *B. malaccensis*
6 mm diameter paper disc and Values are given as mean of triplicate, c: concentration (100.00, 200.00 µg/disc) or (1.00 mg/disc)

4.3.3 Antibacterial activity assessment with disc diffusion assay by *L. spathacea*

S. *aureus* illustrated the most susceptible strains to the extract of *L. spathacea* where the extract concentration was increased from 100.00 µg/disc to 200.00 µg/disc. In contrast, *B. subtilis, P. aeruginosa* and *E. coli* were completely sterilised when exposed to these extracts. Regarding *L. spathacea,* LC and BH (200.00 µg/disc) showed antibacterial specific activity against *S. aureus* (Figure 20). The highest growth of inhibition has been shown by leaves chloroform extract with an average zone of inhibition diameters of (7.10 ± 0.20) mm and followed by bark hexane (7.05 ± 0.07) mm) and bark chloroform (6.90 ± 0.07) mm) (Table 6). This can be assumed, extracted compound of *L. spathacea* in this experiment simply active only against Gram-positive bacteria. The positive control, chloramphenicol and ampicillin work approximately the same as the previous assay and while the negative control showed no inhibitory effect. ZOI diameters of LC and BH of *L. spathacea* were (7.10 ± 0.20) mm and (7.00 ± 0.07) mm respectively against *S. aureus.* Positive controls were chloramphenicol and ampicillin which showed the ZOI diameters of (22.00 ± 2.00) mm and (9.00 ± 3.00) mm against *S. aureus*. Samples with no activity are not included in (Table 6).

4.3.4 Antibacterial activity assessment with disc diffusion assay by *D. hasseltii*

Inhibition zones developed by the extracts of *D. hasseltii,* LC, BC, F^1H, F^1C and F^1M (200.00 µg/disc) showed activity against *S. aureus* whilst LC and LM (200.00 µg/disc) showed antibacterial activity against *P. aeruginosa* which before showed zero activity at the concentration of 100.00 µg/disc (Table 6). This indicated a higher concentration of extract per disc is needed for this plant to exhibit their antibacterial activities and afterwards prevent the growth of the tested bacteria.

LM extract demonstrated the highest inhibition zones at concentration 200.00 µg/disc with an average of 10.10 mm against the *P. aeruginosa* and little active against S. aureus with inhibition zones of around 7.00 mm. Besides that, the chloroform extracts from leaves of *D. hasseltii* displayed similar activity performed by leaves methanol which works against the *S. aureus, B. subtilis* and *P. aeruginosa*. Based on this observation, it can be assumed that polar or semi-polar compound in the crude extract of *D. hasseltii* is in charge for the antibacterial activity considering that none of the hexane extract (predominantly dissolve nonpolar compounds) active against any tested bacteria (Table 6).

In this assay, when the sterile blank paper disc was impregnated with a concentration of 1.00 mg/disc of leaves, bark, and fruit extract of *D. hasseltii* it showed higher antibacterial activity compared to the previous assay only with BC. Based on the result in (Table 6 and 7), it can be concluded *S. aureus* was susceptible to the F^{1}C, F^{1}M, LC and LM extracts and LC were active against *P. aeruginosa* (Figure 21). F^{1}M (200.00 µg/disc) exhibited the largest inhibition zone against the *S. aureus* with an average diameter of 9.10 mm, followed by fruit chloroform (8.10 ± 0.10 mm), fruit hexane (7.00 ± 0.80) mm, leaves methanol (6.80 ± 0.10 mm) and leaves chloroform (7.00 ± 1.00) mm (Table 6).

For *D. hasseltii* the ZOI diameters of BC was (7.80 ± 0.20) mm at the concentration of 200.00 µg/disc and the ZOI diameters of BC was (8.10 ± 0.10) mm at the concentration of 1 mg/disc, whereas positive controls were chloramphenicol, ampicillin which demonstrated the ZOI diameters of (22.00 ± 2.00) mm and (9.00 ± 3.00) mm at the concentration of 200.00 mg/disc and (11.33± 2.52) mm, (19.00 ± 3.00) mm respectively at the concentration of 1.00 mg/ disc against *S. aureus*. Positive controls chloramphenicol and ampicillin showed (27.00 ± 2.30) mm and (18.00 ± 3.00) mm against *B. subtilis* at the concentration of 200.00 µg/disc. Against *P. aeruginosa* the ZOI of LM was (10.10) mm at the concentration of 200.00 µg/disc and the positive control chloramphenicol displayed the ZOI diameters of (32.00 ± 2.00) mm (Table 6 & 7). Results with no activity are not included in the tables below and all the tests are performed in triplicates.

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4.3.5 Antibacterial activity assessment with disc diffusion assay by *K. maingayi*

Leaves and bark extracts of *K. maingayi* were tested against two Gram-positive bacteria (*B. subtilis* and *S. aureus*) and two Gram-negative bacteria (*E. coli* and *P. aeruginosa*). The extracts did not exhibit any affirmative antibacterial activity. Hence the negative results are not included in the tables afterwards but the results are given in the appendix (Table A.1 and A.2). A quantitative antibacterial experiment (MIC and MBC) was conducted with the extracts of *K. maingayi* in the next chapter to further perceive the presence of antibacterial activity of the extracts of *K. maingayi.*

Table 6: Zones of inhibitions (c 200.00 µg/disc) against 3 bacteria cells by *D. hasseltii* and *L. spathacea*

	Zone of Inhibition (mm)		
Genus species Part/Solvent	*S. aureus*	*B. subtilis*	*P. aeruginosa*
D. hasseltii			
LC	7.00 ± 1.00	7.50 ± 1.50	7.50
LM	6.80 ± 0.10	-	10.10
BH	-	-	
BC	7.80 ± 0.20	-	
BM	-	-	
F^1H	7.00 ± 0.80	-	
F^1C	8.10 ± 0.10	-	
F^1M	9.10	-	
Standard Antibiotics			
Chloramphenicol	22.00 ± 2.00	26.00 ± 2.20	32.00 ± 2.00
Ampicillin	9.00 ± 3.00	20.00 ± 3.50	-
L.spathacea		-	
LC	7.10 ± 0.20		
BH	7.00 ± 0.07		
BC	6.90 ± 0.07		
Standard Antibiotics			
Chloramphenicol	22.00 ± 2.00	27.00 ± 2.30	28.00 ± 2.50
Ampicillin	9.00 ± 3.00	18.00 ± 3.00	-

Note: samples with no activity are not included here in this table.
The tests were performed in triplicates.

Table 7: Zones of inhibitions (c1.00 mg/disc) against 6 bacteria cells by *D. hasseltii*

	Zone of Inhibition (mm)	
Genus species Part/Solvent	*S. aureus*	*P. aeruginosa*
D. hasseltii		
LH	7.00	-
LC	6.80 ± 0.10	7.50
BH	7.00	-
BC	8.10 ± 0.10	-
Standard Antibiotics		
Amoxicillin	11.33 ± 2.52	-
Ceftazidine	19.00 ± 3.00	11.00 ± 2.00

Note: samples with no activity are not included here in this table.
The tests were performed in triplicates
Plant Parts:
L: Leave, B: Bark, F^1: Fruit, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol
S. aureus: *Staphylococcus aureus* (ATCC 29213), *B. subtilis: Bacillus subtilis* (ATCC11778), *E. coli*: Escherichia coli (ATCC 25218), *P. aeruginosa*: *Pseudomonas aeruginosa* (ATCC10145); for *D. hasseltii, L. spathacea* and *K. maingayi*
6 mm diameter paper disc and Values are given as mean of triplicate, c: concentration (100,200 µg/disc) or (1 mg/disc)

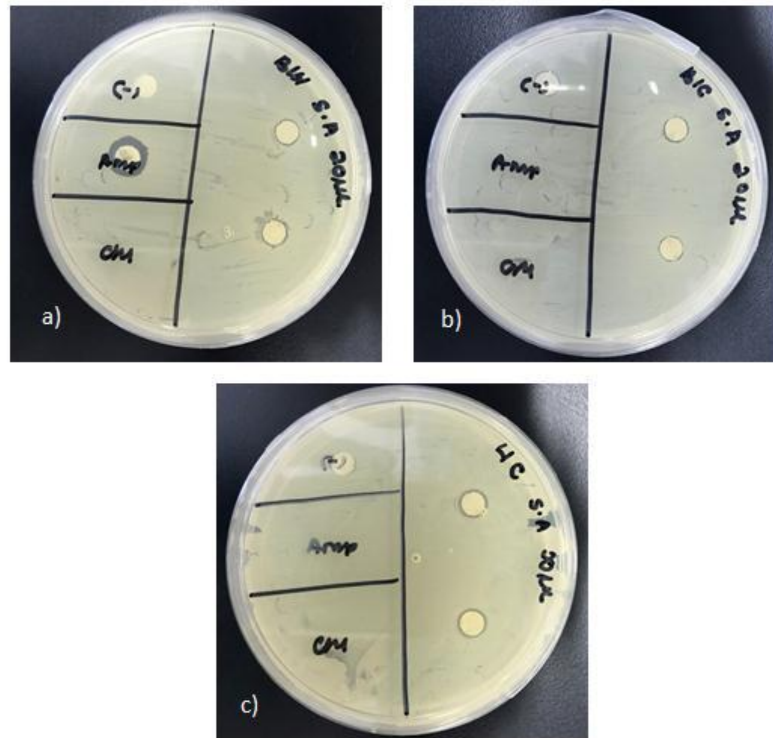

Figure 20: Zone of inhibition developed by of bark hexane (a), bark chloroform (b) and leaves chloroform (c) extracts of *L. spathacea* against *S. aureus* at conc 200 µg/disc

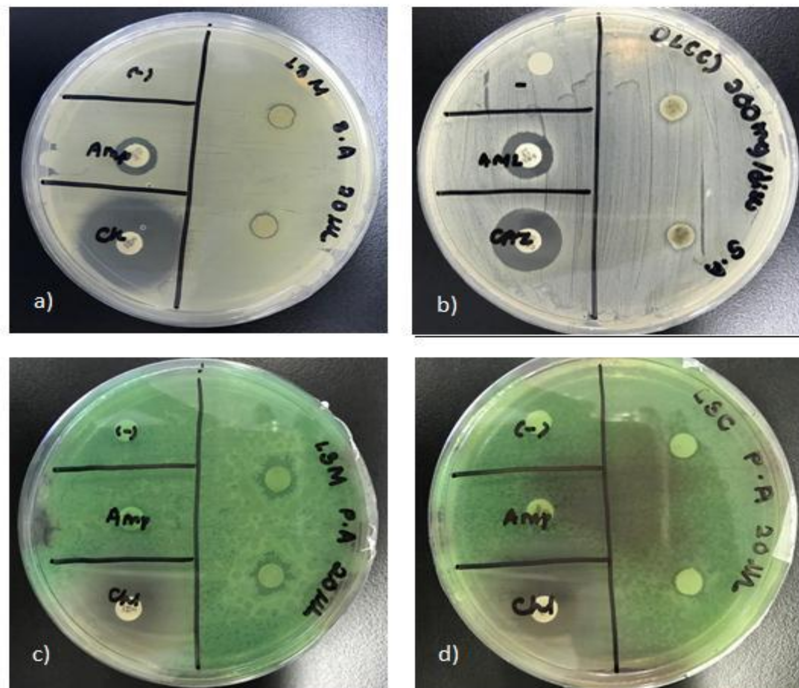

Figure 21: Zone of inhibition developed by *D. hasseltii* leaves methanol (a) & (c) extracts against *S. aureus* and leaves chloroform (b) & (d) extract against *P. aeruginosa* at a concentration 200 µg/disc.

Note: Pictures with a good zone of inhibitions are included here

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4.4 Conclusion

Out of 48 extracts tested, 8 extracts were active against *S. aureus,* 8 extracts against *B. subtilis,* 1 extract against *E. coli* and 5 extracts against *P. aeruginosa* from *B. malaccensis*. From *K. retusa*, 6 extracts were active against *S. aureus,* 4 extracts against *B. subtilis,* 1 extract against *E. coli* and none against *P. aeruginosa*. These results confirmed previous evidence that Gram-positive bacteria are more sensitive than Gram-negative bacteria to plant extracts (Manandhar, Luitel & Dahal, 2019). From *D. hasseltii* (c 200.00 µg/disc), 6 extracts were sensitive against *S. aureus,* 1 against *B. subtilis*, 2 against *P. aeruginosa,* and none against *E. coli. D. hasseltii* with (c 1mg/disc), 4 extracts were active against *S. aureus*, 1 against *P. aeruginosa* and none against E. coli and B. subtilis. None of the extracts has a potency equivalent to antibiotics. But the interesting fact is out of 48 extracts, 4 extracts (WM, FC, FM, and SH) from *B. malaccensis* were selectively sensitive against *P. aeruginosa.*

Based upon the results demonstrated above from (Table 5, 6 and 7), standard deviation distinguished of all the data above is short and limited means similarly small range of values and hence it can be said that the results are more accurate because several studies said that standard deviation with high value demonstrates that the data is widely spread which means less reliable and low standard deviation shows that the data is clustered closely around the mean area and vice versa. Assessment of the *in vitro* antibacterial activity of the above mentioned medicinal plants concluded that hexane and chloroform extracts of seed and flesh of *B. malaccensis* may be a source of novel antibacterial compounds considering their potential antibacterial activity, especially against Gram-negative bacteria.

The prospects for the development of antibacterial drugs from medicinal plants appear to be rewarding as they can mitigate the adverse effects that are often associated with synthetic antibiotics (Sharma et al. 2014). In the present study, the Kirby-Bauer disc diffusion assay was conducted to evaluate the antibacterial activities of crude extracts against ATCC Gram-positive and negative strains. This qualitative method is extensively used for antibiotic

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susceptibility testing where filter paper discs are saturated with antibacterial agents are applied on the inoculated agar plate (Hakonen et al. 2014). The potency of these agents can be determined by measuring the diameter of the zones of inhibition that resulting from their diffusion into the agar medium around the discs (Johnson et al. 2012).

The inhibitory effects of crude extracts on the growth of ATCC Gram-positive and Gram-negative bacterial strains are depicted in (Table 5, 6 and 7), Figure (18-21) and (Appendix A1–A4). Among the crude extracts investigated, chloroform and methanol extracts of flesh, hexane extract of seed of *B. malaccensis* demonstrated potent antibacterial activities against Gram-negative bacteria with zones of inhibition ranging from 11.30±1.50 mm, 20.30±1.20 to 23.30±2.00 mm, respectively. Chloroform and methanol extracts of leaves *D. hasseltii* demonstrated potent antibacterial activities against Gram-negative bacteria (*P. aeruginosa*) with zones of inhibition ranging from 7.50±1.50 mm. However, all extracts of leaves, barks and wood were found to be less effective in inhibiting the growth of the tested Gram-negative bacteria. This was in contrary to the studies of (Tan, Bradshaw, Chu, Khoo & Wiart, 2014, Sowjanya et al. (2013), in which methanol extract of leaves (*Artabotrys uncinatus* and *Artabotrys hexapetalus*) displayed significant antibacterial activity with zones of inhibition ranging from 7 mm to 18 mm in view of their prominent inhibitory activities particularly against Gram-positive bacteria (*Artabotrys crassifolius*) and the positive control, streptomycin sulphate, created zones of inhibition ranging from 7.30±0.26 mm to 19.79±0.26 mm against all of the tested ATCC and clinical bacterial strains. Moreover in this project, the positive control chloramphenicol, ampicillin, amoxicillin and cephalexin created zones of inhibition ranging from 9.00±3.00 mm to 30.00±2.00 mm to against *P. aeruginosa.*

No inhibition was observed in the negative control. This implies that the solvent used for the restructuring of crude extracts. Considering the zones of inhibition produced by crude extracts, *P. aeruginosa* (ATCC10145) was found to be the most sensitive bacteria, followed by *E. coli* (ATCC 25218). In general, Gram-negative bacteria are more resistant to plant-based antibacterial agents

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compare to Gram-positive bacteria (Biswas et al. 2013). The susceptibility differences between these two groups of bacteria can be attributed to their distinct cell wall structures (Singariya et al. 2012). Chemical compositions and organisation of bacterial cell wall may rationalise the variations in the sensitivity of the Gram-negative and Gram-positive bacterial strains With regard to the results got here in this chapter warrants further quantitative antibacterial experiment, isolation and characterisation of the potentially active principles from the respective crude extracts.

Demonstration by wood extracts of *B. malaccensis* and *K. retusa* gave a quite good zone of inhibitions but due to inadequate yield, further experiments were not conducted on those. As the goal of this project was to isolate an antibacterial compound against Gram-negative bacteria. Hence, the focus has been on the results obtained from Gram-negative bacteria which has been compared with previous studies on both Gram-positive and Gram-negative bacteria (Tan, Bradshaw, Chu, Khoo & Wiart, 2014, Sowjanya et al. (2013). Further studies are required to isolate and characterise the biologically active compounds inside the extracts which are responsible for the observed antibacterial properties in this experiment. To get quantitative values regarding the antibacterial activity of the samples, MIC and MBC was effectuated right after disc diffusion assays were conducted. Details of MIC and MBC are explained in the next chapter (Chapter V).

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CHAPTER V: *IN VITRO* ASSAY (MIC AND MBC)

After conducting qualitative antibacterial paper disc assay (Chapter IV) on 48 crude extracts from 5 different rare medicinal plants, quantitative antibacterial assay (MIC and MBC) have been performed on the crude extracts as the next move. MIC and MBC were performed on 48 crude extracts against different Gram-negative and Gram-positive bacteria. *B. malaccensis* and *K. retusa* were experimented against 2 ATCC Gram-positive bacteria (*S. aureus* and *B. subtilis*) and 2 ATCC and clinical Gram-negative bacteria (*E. coli* and *P. aeruginosa*). *D. hasseltii, L. spathacea* and *K. maingayi* were experimented against 2 Gram-positive bacteria (MRSA and *B. subtilis*) and 4 Gram-negative bacteria (*E. coli, P. aeruginosa, A. baumannii* and *K. pneumoniae*). Samples with affirmative antibacterial activity are included in (Table 8 and 9). Samples with both positive and negative results are given in the appendix (Table A.3 and A.4). MIC and MBC of *D. hasseltii, L. spathacea* and *K. maingayi* were done in the laboratory of University of Malaya and *B. malaccensis, K. retusa* is done in the laboratory of the University of Nottingham. So, the selection of bacteria varied due to the availability of bacteria in laboratories where the experiments were carried out. Gram-positive bacteria are tested here in this experiment to compare antibacterial activity with Gram-negative bacteria.

The objective of this research was to search for an antibacterial compound and which can penetrate the impermeable cell wall of Gram-negative bacteria, hence both classes of bacteria have been tested as a comparison. This chapter is explained and designed as an overview (5.1), protocols and methods (5.2), results and discussions (5.3, Table 8 and 9) and conclusions (5.4) of MIC and MBC of the selected samples.

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5.1 Overview of MIC and MBC

The minimum inhibitory concentration (MIC) assay is a technique used to determine the lowest concentration of an antibacterial agent needed to inhibit the visible growth of bacteria after overnight incubation. By preparing solutions of the chemical *in vitro* at increasing concentrations, MIC is determined. The solutions need to be incubated with separate batches of cultured Gram-positive and Gram-negative bacteria and then measured the results by using MH agar dilution or MH broth microdilution. Results have been then graded into susceptible or often known as sensitive, intermediate, or resistant to an antibacterial compound by using a breakpoint. Breakpoints are stipulated based upon values, published in guidelines of a reference body, such as the U.S. Clinical and Laboratory Standards Institute (CLSI).

Whilst MIC is the lowest concentration of an antibacterial agent to inhibit the growth of bacteria, minimum bactericidal concentration (MBC) is the minimum concentration of an antibacterial agent that results in bacterial death on antibacterial-free media after subculture over specified time limitations such as 18 hours or 24 hours and under a certain set of conditions. MIC shows the lowest antibacterial agent level that greatly inhibits the development of bacteria, the MBC shows the lowest antimicrobial agent level which results in microbial death. MBC screening can be a successful and relatively cheap method for assessing multiple antimicrobial agents for potency simultaneously (R. C. Gupta, 2015). The MBC is complementary to the MIC. The closer the MIC is to the MBC, the more bactericidal the compounds are. Different concentrations of the compound are inoculated with cultivated bacteria and the results are calculated using agar dilution or broth dilution to determine the extent at which the MIC endpoint is formed (R. C. Gupta, 2015).

MICs were primarily used by diagnostic laboratories to confirm tolerance, but very often it is used as a reference source to determine the activity of new antibacterial compounds *in vitro*, and data from such experiments were used to establish MIC set points. This report discusses systematic methods for evaluating MICs and MBCs. The methodology provides guidance on a standard

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antibiotic, stock antibiotic solutions preparation, media, inocula preparation, incubation conditions, and reading and interpretation of the observed results.

During a product's R&D process especially while searching for the new antibacterial compound, such tests can be quite useful in determining the correct concentrations required in the final product and to verify the pathogen's susceptibility to the drugs tested for different infections. Since the concentration of the medication required to produce the effect is typically many hundred to thousand times less than the concentration contained in the finished dosage form.

5.2 Protocols and methods of MIC and MBC

5.2.1 Bacterial strains for MIC

Antibacterial sensitivity screening is an important task in the clinical microbiology practice on the selected bacterial isolates. This experiment aims to define the sensitivity of the bacterial cells to the given antimicrobial agent and to confirm the pathogen's susceptibility to the drugs of choice tested for different infections (Kłodzińska, Priemel, Rades & Nielsen, 2018).

Minimum Inhibitory Concentration (MIC) is performed in this project not only because this is one of the established methods that have been used worldwide but also to determine the resistance and susceptibility of the bacteria. Advantages in this method over other methods are the availability of the results within 24 hours instead of 48 hours like in the agar dilution method and the endpoint is easier to detect (Kłodzińska, Priemel, Rades & Nielsen, 2018). The preparation of this method was quite easy, and the results are reproducible and convenient. Computerized reports can be produced if an automated plate reader is used which can give a more quantitative and accurate reading (Choi et al., 2014). Despite all the advantages aforementioned, this method also has some disadvantages such as preparing accurate reagent solution, performing dilutions and making media, availability of bacteria. However, these problems can be avoided if one had mastered the techniques and has exquisite proficiency while

performing this experiment. The results of the MIC were observed with the naked eyes in this study due to the unavailability of an automated plate reader. However, throughout the observation, differences can be observed and contrast of the bacterial growth in the extracts can be made with the help of the negative and positive controls.

In this study, microorganisms represent pathogenic species commonly associated with nosocomial infections. Four different bacteria were used in this experiment for *B. malaccensis* and *K. retusa* are as follows *B. subtilis (*ATCC 6633), *S. aureus* (ATCC 11632), *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 10145) and for *L. spathacea* and *D. hasseltii* bacteria used were *E. coli (*ATCC 25218), *K. pneumoniae*, *A. baumanii*, *P. aeruginosa* and Gram-positive bacteria which consist of *S. aureus* (ATCC 29213) and Methicillin-Resistant *S. aureus*, MRSA. All the bacterial cells were sub-cultured from the original culture and after that which was stored at -80°C. They were freshly cultured on the Mueller Hinton Agar (MHA) and then incubated overnight at 37° C prior use. Both Gram-positive and Gram-negative bacteria have been used here in this experiment to compare the antibacterial activity. From the previous chapter (Chapter IV), the differences in activity are noticeable among Gram-positive and Gram-negative bacteria and hence this experiment was also carried for both bacteria.

Bacteria culture preparation for MIC was done by picking up a single colony from each strain using a sterile inoculating loop and then transferred into a 1 mL of Mueller Hinton Broth (MHB) which performed as the microbiological growth medium. The mixture was agitated using the vortex to obtain an even mixture of the bacterial suspension. The bacterial culture was adjusted according to 0.5 McFarland turbidity standards, which is equivalent to approximately 1.0 x 108 cfu/ mL ('The MIC and MBC of Silver Nanoparticles against Enterococcus faecalis - A Facultative Anaerobe', 2015). As stated by (Clinical and Laboratory Standards Institute (CLSI), M02-A10 and M07-A8 OD was measured at 600 nm by a spectrophotometer.

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5.2.2 Preparation of Mueller-Hinton broth

Mueller-Hinton broth (MHB) (Difco Laboratories, USA) was prepared in accordance with the manufacturer's orders from a commercially accessible dehydrated framework. The broth was placed on a tray to cool down to room temperature after 15 minutes of autoclaving at 121° C before being stored at 4°C until further use.

5.2.3 Preparation of microdilution plates

60 μL of MHB was added to 40 μL of each isolated compound (final concentrations varying from 7.8125 μg / mL to 1000 μg / mL) before inoculation in a 96-well microtiter plate (Jet Biofil, China). Chloramphenicol, tetracycline, cefotaxime, kanamycin, vancomycin, gentamycin was used as a positive control in respect of the availability and resistance to the distinct bacteria. A mixture consisted of with DMSO, purified water and respective solvent to the tested sample were served as a negative control.

5.2.4 Preparation of inoculums

Using the growth technique as stated earlier above, a standardized inoculum was prepared. The adjusted inoculum suspension was optimally diluted in broth and place that in the shaker machine at 180 rpm for about 40-90 minutes at 37° C to get the ultimate experiment concentration of approximately 5×10^5 CFU / mL in each well.

Minimum inhibitory concentration (MIC) has been described as the lowest concentration of the compound to prevent the growth of microorganism (Zeedan, Abdalhamed, Abdeen, Ottai, & Abdel-Shafy, 2014). So as mentioned above that the sterile 96-well plates were prepared by distributing 60 μL of MHB as diluents and media for bacterial growth followed by 40 μL of diluted plant extracts from the stock solution which contain the concentration of 5 mg/mL into the first row of well that make up a total of 100 μL of suspension into the first row. Another 50 μL of fresh MHB was added from the second row to the

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eighth row of the well. Serial dilutions were performed by transferring 50 µL of the mixture of extracts and MHB from the first well to the subsequent wells and the last 50 µL was discarded into a beaker.

The MIC test was done in triplicates and under the biohazard safety cabinet to ensure the sterility. After the serial dilutions, 50 µL of bacterial suspension was added into each well afterwards. The concentrations of plant extracts from the first well to the eighth well were 1000 µg/mL, 500µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, and 7.8125 µg/mL respectively. Negative controls contained the bacterial suspension and the solvent used to dilute the extracts which were diluted DMSO but without the plant extract. Chloramphenicol dilutions for *S. aureus* (ATCC 11632) and *B. subtilis (*ATCC 6633), tetracycline for *P. aeruginosa* (ATCC 10145) and *E. coli* (ATCC 8739) served as a positive control.

5.2.5 Inoculation of microdilution plates

All the 96 well-plates were covered with a lid and sealed with para-film and incubated at 37° C for 24 hours. After 24 hours, the bottom of the 96 well-plates was observed and wells that showed inhibition of bacterial growth were observed and the concentration at which it inhibits the growth of bacteria was recorded. As for the positive control, the MIC values were taken following MIC interpretive standards from the CSLI, M02-A10 and M07-A8. The amount of growth in the 96 well plate wells containing isolated compounds was compared with the amount of positive growth control wells used in each set of experiments to establish growth endpoints.

5.2.6 Incubation of microdilution plates

The inoculated microdilution plates were then incubated in an ambient air incubator chamber (Binder, Germany) within 15 minutes after the inoculum at 37 ° C for 16 hours to 20 hours. It is better to place microdilution plates single inside the incubator rather than stacking to maintain incubation temperature. The value of MIC was eventually recorded as the smallest concentration of each

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isolated compound that inhibited the development of bacteria in the reservoirs of microdilution as identified by the unaided eye.

5.2.7 Determination of MBC of plants extract against bacteria

The minimum bactericidal concentrations (MBC) of isolated selected compounds were assessed in accordance with the CLSI rules, previously known as NCCLS. On Mueller-Hinton agar (MHA) (Difco Laboratories, USA) plates, an aliquot of 10 μL was separated from each well-displaying growth inhibition and subcultured after MIC determination. The plates were incubated at 35^0 C - 37^0 C for 18 - 24 hours and examined for bacterial growth in corresponding to plant extract concentration. Eventually, the value of MBC was taken as the lowest concentration of each selected isolated compound that resulted in 99.9% killing of the final inoculum.

5.3 Result and Discussions of MIC and MBC

5.3.1 Result of MIC and MBC of *B. malaccensis* and *K. retusa*

Out of 48 extracts tested, 12 extracts were (LH, LC, LM from *B. malaccensis*; LM, WH from *K. retusa*; LC, LM from *L. spathacea*; LC, LM, BM from *K. maingayi*; and LC, BM from *D. hasseltii*) sensitive against Gram-positive bacteria. Of note, observed that 4 extracts (BC, SH, FC, and FM from *B. malaccensis*) giving selective activity against *P. aeruginosa*. In this study, five different plants species were selected from the family of rutacaea, Myristicaceae, Lauraceae, Apocynaceae and Ebenaceae. It has been observed that two types of bacteria (Gram-positive and negative) were used, from the ATCC types for the plant species of rutacea (*B. malaccensis*) and Myristicaceae (*K. retusa*). Gram-positive bacteria were also experimented here to compare with Gram-negative bacteria and hence results from both are included in (Table 8). Six different bacterial strains were used for the plant species of Lauraceae, Apocynaceae and Ebenaceae (*L. spathacea, K. maingayi* and *D. hasseltii* respectively) and three of them are clinical strains (*P. aeruginosa, A. baumanii,* and *K. pneumonia*). MIC and MBC of these plant species were done in the

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laboratory of University of Malaya and *B. malaccensis, K. retusa* is done in the laboratory of the University of Nottingham. So, the selection of bacteria varied due to the availability of bacteria in laboratories where the experiments were carried out. Gram-positive bacteria are tested here in this experiment to compare antibacterial activity with Gram-negative bacteria.

Here in this experiment, it was found that the leaves extract (LC) and (LM) from *B. malaccensis* and woods extract (WH), leaves extract (LM) from *K. retusa* exerted broad-spectrum antimicrobial agent, and this discovery has given us a new hope on this extract for development of new antibiotics. The interesting fact is that flesh and seed extracts from the plant *B. malaccensis* shows broad spectrum moderate activity against specifically Gram-negative bacteria cells.

Based on the results obtained in (Table 8) from the MIC value, 3 extracts worked on *S. aureus* (ATCC 11632) which are LH, LC, LM of *B. malaccensis* and LM, WH, from *K. retusa.* The lowest MIC obtained was from the LC (*B. malaccensis*), LM and WH (*K. retusa*) which were 0.25 mg/mL where the rest of the extracts were 0.5 mg/mL and 1 mg/mL. For *B. subtilis (*ATCC 6633), there are 4 extracts worked on the bacteria which were from LC, LM (*B. malaccensis*) with MIC values of 0.25 mg/mL, 0.5 mg/mL and 1 mg/mL respectively also LM, WH (*K. retusa*) with MIC values 0.25 mg/mL. There is not any significant result shown against *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 10145) for both the Gram-negative bacteria. There was bark extracted with chloroform (BC), flesh extracted with chloroform (FC), flesh extracted with methanol (FM), and seed extracted with hexane (SH) from (*B. malaccensis*) worked specifically against *P. aeruginosa* with MIC value 1 mg/mL. The antibacterial data showed in (Table 8) selective antibacterial activities of all the tested extracts.

The percentage of active extracts was highest on *S. aureus* (ATCC 11632) with 33.33% and lowest in *P. aeruginosa* (ATCC 10145) with 22.22% and *E. coli* (ATCC 8739) with no antibacterial activity. All the results obtained on the bacteria using the plant extracts can be compared with the standard antibiotics

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which acted as the positive control. It is clearly shown that the standard antibiotics work better in inhibiting the bacteria with the concentration of antibiotic needed to inhibit the bacteria was 0.002 mg/mL on *E. coli* using tetracycline and the highest concentration used was 0.125 mg/mL on *P. aeruginosa* using tetracycline.

Determination of the minimum inhibitory concentration (MIC) of extracts from the 2 selected plants by the broth microdilution method (samples with antibacterial activity are only included in Table 8). Broth microdilution test results have verified that Gram-positive bacteria are more resistant than Gram-negative bacteria (Table 8). According to (Rios & Recio, 2015), crude extract with MIC superior to 1000 μg/mL are inactive and proposed interesting activity for MIC of 0.1 mg/mL and below than that defined active crude extracts as having MIC values below 8 mg/mL and according to (Seukep, Sandjo, Ngadjui, & Kuete, 2016) use stricter endpoint criteria, in which crude extracts with MIC values less than 0.1 mg/mL are active. In addition, (Efferth & Kuete, 2010) classifies as weakly active extracts with MIC above 0.625 mg/mL. Following (Seukep, Sandjo, Ngadjui, & Kuete, 2016), no extracts had interesting activities with MIC below 0.1 mg/mL (Table 10). The lowest MIC towards *S. aureus* and *B. subtilis* was demonstrated by the LC (extracted from chloroform) of *B. malaccensis* (0.25 mg/mL).

Antibacterial compounds or extracts are divided into 2 categories: bacteriostatic (MBC/MIC ratio value more than 4) and bactericidal (MBC/MIC ratio value less than or equal to 4) (Brauner, Fridman, Gefen, & Balaban, 2016). Following this classification, LC, LM extracts of *B. malaccensis* with MBC/MIC ratio above 1 which means was bactericidal for *S. aureus* and *B. subtilis*; LM, WH and WC extracts was bactericidal for *K. retusa* against *S. aureus* and *B. subtilis* as well. Here in this experiment LC, LM extracts of *B. malaccensis* and LM, WH and WC extracts of *K. retusa* was strongly bactericidal for *S. aureus* and *B. subtilis* with MIC and MBC of 0.25 mg/mL.

MIC superior to 0.1 mg/mL for phytoconstituent need to be avoided because of mild activity and proposed interesting activity with MIC value of

0.01 mg/mL and below. The antibacterial activity of pure compounds is divided in 3 classes based on the activity rate: MIC < 0.01 mg/mL: high; MIC between 0.01 and 0.1 mg/mL: medium and low for MIC above 0.1 mg/mL'. Following both these classifications, LC from *B. malaccensis* and LM, WH from *K. retusa* with a MIC of 2.5 mg/mL and MIC/MBC ratios of 1.0 and above 1 were strongly bactericidal against *S. aureus* and *B. subtilis* (Brauner, Fridman, Gefen, & Balaban, 2016).

Based on the results obtained, the differences observed were probably due to different chemical compositions in each crude extracts and also the differences in biological actions of the chemical compounds towards the bacteria (Frismantas et al., 2017). The overall activity exerted by the crude extracts on the bacteria is moderate and weak. Three different types of antibiotics were used in this study are chloramphenicol, ampicillin and tetracycline on the selected bacteria and the selection of these antibiotics and their MIC interpretive data were based from the (Kebede & Hibore, 2012). Ampicillin did not bring any activity on *S. aureus* and *B. subtilis* and therefore values are not given in the table (Table 8). However, the reason why most of the MIC value for the antibiotics did not match with data from the CSLI guidelines could be the tested bacteria have already developed resistance towards the antibiotics. However, the MIC values obtained for the positive control based on the results above are according to the CSLI guidelines after a lot of analysis and multiple selections of the antibiotics were done.

Table 8: Minimum inhibitory concentrations (MIC) (mg/mL) and minimum bactericidal concentrations (MBC) (mg/mL) against 4 bacteria cells on *B. malaccensis* and *K. retusa*

Genus- Species Part/Solvent	*S. aureus* (ATCC 11632)	*B. subtilis* ATCC (6633)	*E. coli* ATCC (8739))	*P. aeruginosa* ATCC (10145)
B. malaccensis	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
LH	1.00>1.00	-	-	-
LC	0.25/>1.00	0.25/>1.00	-	-
LM	-0.50/>1.00	-1.00>1.00		-
BC	--	-		1.00>1.00
SH	-	-	-	1.00>1.00
FC	-	-	-	1.00>1.00
FM	-	-	-	1.00>1.00
K. retusa				
LM	0.25/>1.00	0.25/>1.00	-	-
WH	0.25/>1.00	0.25/>1.00	-	-
Standard Antibiotics				
Chloramphenicol	0.03>0.03	0.02>0.03	-	-
Tetracycline	-	-	0.002>0.02	0.012>0.30

Note: samples with no activity are not included here in this table.
The tests were performed in triplicates.
Parts: L: Leave, B: Bark, W: Wood, F: Flesh, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol
S. aureus (ATCC 11632), *B. subtilis* (ATCC6633), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC10145) for K. *retusa* and*B. malaccensis*

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5.3.2 Result of MIC and MBC of *L.* spathacea, *K. maingayi* and *D. hasseltii*

Out of 48 extracts, LC, LM from *L. spathacea*; LC, LM, BM from *K. maingayi*; and LC, BM from *D. hasseltii* were sensitive against Gram-positive bacteria. LC, BC, BM from *L. spathacea*; LM, BC, BM from *K. maingayi*; LH, LM, BC, BM from *D. hasseltii* were active against *E. coli*. LC from *L. spathacea*; BM from *K. maingayi;* and LH, LM, BC, BM from *D. hasseltii* were active against *P. aeruginosa.* LC, LM, BM from *L. spathacea;* BM from *K. maingayi* were active against *A. baumannii.* LC, LM from *L. spathacea* and LC, BC, BM from *D. hasseltii* were active against *K. pneumonia.* From the MIC values (Table 9), six extracts worked on MRSA which are LC, LM from *L. spathacea*, LC, BM from *K. maingayi*, LC and BM from *D. hasseltii*. The lowest MIC obtained was from the LC, LC and LC which was 0.5 mg/mL where other extracts were showing the MIC values of 1 mg/mL. For *S. aureus* (ATCC 29213), there are 4 extracts worked on the bacteria which were from LC (*L. spathacea*), BM (*K. maingayi*) and BM (*D. hasseltii*) with MIC values of 0.5 mg/mL were from and LM with 1 mg/mL.

It can be observed that LC, BH, BC from *L. spathacea*, BC from *K. maingayi*, LH, LM and BC from *D. hasseltii* yield MIC values of 0.5 mg/mL whilst LM, BM yield MIC value of 1 mg/mL. The MIC for BM extract yield 0.25 mg/mL. For *P. aeruginosa*, there were 5 extracts worked on the bacteria which were LC, LC, BM, LC, and BC with MIC values of 0.5 mg/mL, while BM with MIC value of 1 mg/mL. Most of the extracts did not work well against *A. baumanii* except for LC which the MIC value was 0.5 mg/mL, LM, BM and BM with MIC values of 1 mg/mL. There were 5 extracts worked on *K. pneumonia* which were LC, LC, BC, and BM which gave MIC values of 0.5 mg/mL and LM with MIC value of 1 mg/mL. The antibacterial data in (Table 9) exhibited that all the tested extracts demonstrated selective antibacterial activities.

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Leaves of *L. spathacea* (LC) with MIC value of 0.5 mg/mL is active in inhibiting all the types of tested Gram-positive and Gram-negative bacteria strains. In this study bark of *D. hasseltii* which was extracted with methanol (BM) gave the lowest MIC value which was 0.25 mg/mL when being tested on *E. coli* (ATCC 25218). Plants extracted with hexane showed no or little effects on the bacteria except for several extracts of leaves and barks that worked on certain bacteria.

To determine the minimum inhibitory concentration (MIC) out of 15 extracts, the lowest MIC against MRSA and *S. aureus* was demonstrated by LC (extracted from chloroform) of *L. spathacea* (0.5 mg/mL); LC, BM (0.5 mg/mL) from *K. maingayi* and LC of *D. hasseltii* (0.5 mg/mL) against MRSA and BM of *D. hasseltii* (0.5 mg/mL) against *S. aureus.* LC extract of *L. spathacea* against MRSA, *S. aureus, E. coli, P. aeruginosa, A. baumanii*, *K. pneumoniae* with MBC/MIC ratio above 2 and LM extract of *L. spathacea* against MRSA, *A. baumanii*, *K. pneumoniae* above 1 which means is bactericidal; LH of *D. hasseltii.* against *E. coli*, LC against MRSA, *P. aeruginosa* and *K. pneumonia*; LM against *E. coli*, BC *against E. coli, P. aeruginosa* and *K. pneumoniae* and BM in contrast to *S. aureus, P. aeruginosa* and *K. pneumonia* with MBC/MIC ratio above 2 means is bactericidal.

Interestingly BM is only active against *E. coli* of *D. hasseltii* with MBC/MIC ratio above 4 demonstrated bacteriostatic. The antibacterial activity of pure compound is classified in 3 categories: MIC < 0.01 mg/mL: high; MIC between 0.01 and 0.1 mg/mL: medium and low for MIC above 0.1 mg/mL. Following both of these classifications; LC, LM, BH, and BC of *L. spathacea* and LH, LC, LM, BC, BM demonstrated with low activity as bactericidal but only BM against E. coli with MBC/MIC ratio above 4 proved as bacteriostatic (Brauner, Fridman, Gefen, & Balaban, 2016). Extracts were specifically active against Gram-negative bacteria. This is interesting because only a very few natural products from plants are known to be specifically active against Gram-negative bacteria. The specific activity of SH and FC against *P. aeruginosa* are reported for the first time.

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Table 9: Minimum inhibitory concentrations (MIC) (mg/mL) / mimimum bactericidal concentrations (MBC) (mg/mL) against 6 bacteria cells on *L. spathacea*, *K. maingayi* and *D. hasseltii*

Genus- Species Part/Solvent	MRSA	S. aureus (ATCC 29213)	*E. coli* (ATCC 25218)	*P. aeruginosa* (clinical strain)	*A. baumannii* (clinical strain)	*K. pneumonia* (clinical strain)
L. spathacea	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
LC	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00
LM	1.00>1.00	-	-	-	1.00>1.00	1.00>1.00
BH	-	-	0.50/>1.00	-	-	-
BC	--	-	0.50/>1.00	-	-	-
BM	-	-	-	-	1.00>1.00	-
K. maingayi						
LC	0.50/>1.00	-	-	-	-	-
LM	-	1.00>1.00	1.00>1.00	-	-	-
BC	-	-	0.50/>1.00	-	-	-
BM	1.00>1.00	0.50/>1.00	1.00>1.00	1.00>1.00	1.00>1.00	-
D. hasseltii						
LH	-	-	0.50/>1.00	-	-	-
LC	0.50/>1.00	-	-	0.50/>1.00	-	0.50/>1.00
LM	-	-	0.50/>1.00	-	-	-
BH	-	-	-	-	-	-
BC	-	-	0.50/>1.00	0.50/>1.00	-	0.50/>1.00
BM	1.00>1.00	0.50/>1.00	0.25/>1.00	1.00>1.00	-	0.50/>1.00
Standard Antibiotics						
Cefotaxime	0.003/>0.05	-	0.001/>0.003	0.013/>0.05	-	-
Kanamycin	-	0.003/>0.05	-	-	-	0.006/0.01
Vancomycin	-	--	-	-	0.002/>0.05	-

Note: samples with no activity are not included here in this table.
The tests were performed in triplicates.
Parts:
L: Leave, B: Bark
Solvents: H: Hexane, C: Chloroform, M: Methanol
MRSA, *S. aureus* (ATCC 29213), *B. subtilis* (ATCC11778), *E. coli* (ATCC 25218), *P. aeruginosa* (ATCC10145); *Acinetobacter baumannii* (clinical strain), *K. pneumonia (*clinical strain) for *D. hasseltii* and *L. spathacea*
S. aureus (ATCC 11632), *B. subtilis* (ATCC6633), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC10145) for K. *retusa* and *B. malaccensis*

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5.4 Conclusion:

Paper disc diffusion assay experimented as a preliminary qualitative antibacterial assay on different plant species of different families (Chapter IV). Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) was performed to actuate the resistance and susceptibility of bacteria quantitatively. In both antibacterial experiments (paper disc diffusion and MIC, MBC), all the crude extracts of all the selected plant species were analysed to perceive the antibacterial activity against both Gram-positive and Gram-negative bacteria. Extracts were tested against Gram-positive bacteria due to contrast and future furtherance of this research work.

Minimum inhibitory concentrations (MIC) and minimum bactericidal concentrations (MBC) of crude extracts (48 samples) conducted against ATCC and clinical Gram-positive and Gram-negative bacterial strains are depicted in (Table 8 and 9) and (Appendix A3–A4). The lower the MIC values are the most potent the extracts. *E. coli* was the most resistant bacterial species amongst the other tested bacteria for *B. malaccensis* and *K. retusa*. Overall, the Gram-positive bacteria were more sensitive to the extracts than the Gram-negative bacteria.

Out of 48 extracts tested, 12 extracts were (LH, LC, LM from *B. malaccensis*; LM, WH from *K. retusa*; LC, LM from *L. spathacea*; LC, LM, BM from *K. maingayi*; and LC, BM from *D. hasseltii*) sensitive against Gram-positive bacteria. Of note, observed that 4 extracts (BC, SH, FC, and FM from *B. malaccensis*) giving selective activity against *P. aeruginosa*. LC, BC, BM from *L. spathacea*; LM, BC, BM from *K. maingayi*; LH, LM, BC, BM from *D. hasseltii* were active against *E. coli*. LC from *L. spathacea*; BM from *K. maingayi;* and LH, LM, BC, BM from *D. hasseltii* were active against *P. aeruginosa.* LC, LM, BM from *L. spathacea;* BM from *K. maingayi* were active against *A. baumannii.* LC, LM from *L. spathacea* and LC, BC, BM from *D. hasseltii* were active against *K. pneumonia.* None of the extracts from *B. malaccensis, K. retusa, L. spathacea, D. hasseltii* and *K. maingayi* is as efficient as the antibiotics (positive control) against Gram-negative bacteria. The

interesting thing is BC, SH, FC, and FM from *B. malaccensis* are selectively active against *P. aeruginosa.* But the potency exerted are mild compared to reference antibiotic tetracycline.

The MIC/MBC value of 1.00>1.00 mg/ml obtained with extracts BC, SH, FC, and FM against *P. aeruginosa* were greater than antibiotic tetracycline (0.012>0.30) on the corresponding bacteria. The microbial sensitivity to the different extracts against Gram-positive bacteria represented MIC values ranged from 0.25/>1.00 mg/ml to 1.00>1.00 mg/ml with reference to the antibiotic chloramphenicol 0.03>0.03 mg/ml. In correlation with antibiotic extracts played weak bactericidal activity against *P. aeruginosa.*

MIC/MBC values of different extracts from *L. spathacea*, *K. maingayi* and *D. hasseltii* ranged from 0.25/>1.00 mg/ml to 1.00>1.00 against Gram-negative ATCC strains and clinical strains. BM from *D. hasseltii* displayed the lowest and most potent value ranged 0.25/>1.00 mg/ml against *E. coli.* Whereas positive controls demonstrated MIC/MBC values ranged from 0.001/>0.003 mg/ml to 0.013/0.05 mg/ml against Gram-negative ATCC strains and clinical strains. Antibacterial sensitivity to the different extracts against Gram-positive bacteria represented MIC values ranged from 0.50/>1.00 mg/ml to 1.00>1.00 mg/ml. The MIC/MBC values ranged almost similar against both Gram-negative and positive bacteria here for *L. spathacea*, *K. maingayi* and *D. hasseltii.* Compared with antibiotics extracts demonstrated weak to moderate antibacterial activity here. But extracts BC, SH, FC, and FM from *B. malaccensis* were only sensitive towards *P. aeruginosa.* They exerted no activity against Gram-positive bacteria and similarly, positive control tetracycline showed sensitivity only against Gram-negative bacteria. Though the crude extracts showed weak to moderate activity, their synergy might give some potential antibacterial activity.

This was in contrary to the studies of (Tan, Bradshaw, Chu, Khoo & Wiart, 2014, Sowjanya et al. (2013), where displayed MBC values ranged from 0.63 mg/mL to 20 mg/mL against most of the tested ATCC and clinical bacterial strains, which were compared with the positive control, streptomycin sulphate

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(MBC values ranging from 0.3125 mg/mL to 20 mg/mL). According to Krishnan et al. (2012), antibacterial compounds could be categorised into two classes: bacteriostatic (MBC/MIC ratio more than 4) and bactericidal (MBC/MIC ratio less than or equal to 4). Under this classification, extracts from *B. malaccensis* exerted bactericidal activity against *P. aeruginosa* with MBC/MIC ratios ranging from 0. Based on the antibacterial activity attained from previous chapters, isolation and characterisation need to be done afterwards on selected extracts in the next chapter (Chapter VI).

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CHAPTER VI: ISOLATION AND CHARACTERISATION OF ACTIVE CONSTITUENTS

Based on the data attained from qualitative (paper disc assay) and quantitative (MIC and MBC) experiments from previous chapters (Chapter IV and V), decided to identify and characterise the major constituents from 10 samples (5 extracts from *B. malaccensis,* 2 extracts from *L. spathacea,* 1 from *K. maingayi* and 3 from *D. hasseltii*) in contemplation of the goal. Samples got shortlisted eventually for further isolation and characterization due to sufficient yield and antibacterial activity. Samples (LC, FC, SH, BC from *B. malaccensis*; and LC from *D. hasseltii*) with clear separation are only mentioned here with tables (Table 10-14) and figures (Figure 22-25).

Afterwards, PTLC (preparative thin-layer chromatography) was experimented on 2 selected samples: hexane extract of seed (SH) and chloroform extract of flesh (FC) form *B. malaccensis* which were variable with sufficient yield and were specifically active against Gram-negative bacteria (Table 15,16 and Figure 26, 27). GC/MS (gas chromatography/mass spectrometry) has been implemented here in this project right after preparative thin-layer chromatography (PTLC) only on hexane extract of seeds (SH) of *B. malaccensis*. GC/MS was not conducted on chloroform extract of flesh (FC) due to inadequate sample yield. Fractions with activity and with a clear spot were identified through NMR to establish the chemical structures. Fractions obtained from PTLC were examined for their quantitative and qualitative antibacterial activity to observe potential antibacterial compounds amongst all the fractions. This chapter is designed with the overviews, protocols, results, and discussions of all the mentioned techniques sequentially.

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6.1 Overview of all experiments for isolation and characterisation

6.1.1 Overview of TLC

Thin Layer Chromatography (TLC) is an extremely convenient and practical method for isolating compounds. Samples need to be isolated were shortlisted based on the antibacterial activity data against gram-negative bacteria from (Chapter IV and V) and the total yield of the samples for the next experiments. TLC (Thin Layer Chromatography) conducted on 10 selected samples from *B. malaccensis, L. spathacea, K. maingayi*, and *D. hasseltii* (explained in 6.2.1). Samples from *K. retusa* had been excluded on account of no prominent activity against Gram-negative bacteria. Samples only with activity are included and shown in the tables and discussions of this chapter (Table 15 – 19).

The rudimentary principle of thin-layer chromatography (TLC) is the separation of mixtures of compounds in microgram quantities by the movement of a solvent across a flat surface (Biradar, 2013). The compounds migrate at different rates due to differences in their solubility, adsorption capacity or charge. In other words, as a consequence of differences in their partition coefficients between the flat surface (stationary phase) and the solvent (mobile phase) (Biradar & Rachetti, 2013).

It also uses column chromatography to terminate the proper solvent process to isolate the mixtures of substances into their components (Chromatography, Ch, Ch, & Tlc, n.d.). TLC operates by using a stationary phase, commonly prepared with alumina or silica, that is highly polar (standard) or non-polar (reverse) phase, and a mobile phase. There is a need to perform reaction mixture of different solvents and then "run" the plate by allowing a solvent or combination of solvents to move up the plate by capillary action (Biradar & Rachetti, 2013). Samples are used as spots close to one edge of the plate and the mobile process can pass over the samples from that side to the opposite edge. In doing so, the components of the sample move across the

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surface at different rates governed by their physicochemical properties and therefore separate into individual spots or bands Biradar & Rachetti, 2013).

Depending on the polarity of the components of the mixture, various substances travel different distances on the surface. If compounds are more polar then it will "stick" to the polar silica gel and travel short distances on the plate and non-polar substances will disperse into the solvent and travel large distances on the plate (Sherma & Fried, 2013). The separated components may be examined by eye, if they are visible, by chemical staining or by examination under UV light. The measurement of the distance a compound travels is known as Rf. This number (between 0-1) is determined by measuring the distance the compound moved from the baseline divided by the distance.

6.1.2 Overview of PTLC

Preparative thin-layer chromatography (PTLC) is indeed a useful technique for the purification of isolated compounds right after experimenting aluminium sheet TLC with shortlisted samples based on the isolation done from aluminium TLC. 2 samples were selected from *B. malaccensis* with clear isolation for PTLC and in contemplation of the goal of this project selected sampled showed activity against Gram-negative bacteria in the previous antibacterial experiments.

To be specific full-size glass plates with a thick layer of silica are used for preparatory separation by thin-layer chromatography. Preparative TLC separates compounds on a macro scale (0.05–1 g) by utilizing a thick layer of adsorbent up to (0.5–5 mm). Compared to aluminium plates, it is used to isolate pure compounds in enough quantities to allow their properties to be investigated. Preparative TLC has several advantages over column chromatography as to differentiate pure compounds such as rapid element separation, ease of finding suitable irrigants by test runs with layers on microscope slides, clear and easy detectable areas, and ease of isolation from the chromatogram of the compound. It takes practice to determine with methods work best for a particular sample and mostly with the appropriate solvent system (Aljamali, 2015).

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Besides the results got from aluminium TLC, chloroform extract of flesh (FC) and hexane extract of seed (SH) from *B. malaccensis* are the preferred ones for PTLC (explained in 7.3.2) and interestingly these 2 extracts were active only against Gram-negative bacteria according to the objective.

6.1.3 Overview of GC/MS

PTLC helped to get the isolated fractions of the selected 2 samples from *B. malaccensis.* GC/MS (gas chromatography/mass spectrometry) has been implemented here in this project only on hexane extract of seeds (SH) of *B. malaccensis* after PTLC was conducted. GC/MS was not conducted on chloroform extract of flesh (FC) of *B. malaccensis* due to inadequate sample yield.

Gas chromatography/mass spectrometry (GC / MS) is a powerful and useful technique for isolation. Though many compounds are difficult to analyze with GC / MS, it may isolate complex mixtures of air or water chemicals available and used for qualitative and quantitative drug analysis. Different detectors can be used for drug screening in confluence with GC; however, GC-MS and GC-MS / MS are the most sensitive and specific. For broad-spectrum drug screening, GC-MS is still often considered the gold standard. Non-polar, reactive, and low molecular weight compounds are ideal for GC analysis. The most used methods for drug validation testing and broad-spectrum drug screening using GC-MS are electron ionization (EI) with full-scale mass detection and selected ion monitoring (SIM). By comparing their full mass spectrum with a mass spectral library or database, unknown compounds can be identified.

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6.1.4 Overview of NMR

Selected 2 samples from *B. malaccensis* were isolated by TLC, PTLC and GC/MS and then to get the chemical structures of the isolated fractions, NMR was done afterwards. The biggest quantity of data about a compound's structure is provided by a nuclear magnetic resonance spectroscopy (NMR) spectrum. In the NMR Spectroscopic technique, a substance is placed in a strong magnetic field that affects the spin of atomic nuclei. A radio wave passes through the substance and these nuclei are reoriented. When the wave is switched off, the nuclei release an energy pulse that provides data on the substance's molecular structure and can be converted into an image using computer techniques (Sugiki, Furuita, Fujiwara & Kojima, 2018).

The NMR studies were carried out in University Malaysia Pahang (UMP) by Dr Nor Hafizah Zainal Abedin and compound analysis guided by Dr Mark Butler. The synthesized sample was put into an inert solvent {deuterochloroform ($CDCl_3$), deuterium oxide (D_2O), carbon tetrachloride (CCl_4) or deuterated dimethyl sulphoxide (DMSO)} and the solution was placed between the poles of a powerful magnet. The various chemical shifts (δ) of the proton within the molecular environment were evaluated in the NMR device compared to a standard, generally tetramethylsilane (TMS) (Willoughby, Jansma, & Hoye, 2014). Chemical shifts (δ) are measured in ppm units. The signal amplitude can be integrated to show the number of protons resonating at any one frequency. Each chemical shift value in a specific setting corresponds to a set of protons. Each signal's intensity means each type's number of protons (Bingol, 2018).

While MS methods such as gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry (LC-MS) are most frequently used but NMR still offers several significant benefits. In specific, NMR is non-destructive, unbiased, quantitative, requires no separation or derivation, and is suitable for GC-MS and LC-MS compounds that are hard to evaluate. GC-MS, for instance, often needs compound derivation, such as sugars and amines. In contrast, LC-MS usually needs sample preparation, chromatographic separation, and particular circumstances of experimentation

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and ionization, instrumentation and operator skills. These make it difficult standardizing MS analysis.

NMR can provide both qualitative and quantitative data on chemically varied compounds without requirements of extensive sample preparation and fractionation, is extremely reproducible and (Anand, Jeyachandran, & Nandagopalan, 2013). Since NMR is only capable of detecting compounds at a level of 0.1 percent, it is not appropriate for trace component detection. NMR is less sensitive than MS, designed to detect compounds down to components per million concentrations (ppm). NMR and MS are regarded as complementary techniques because of the separate benefits of each technique. NMR is a quantitative spectroscopic method because it is directly proportional to the number of nuclei. The sensitivity and resolving strength of NMR has enhanced with improvements in electronics and the use of greater magnetic field strengths.

The continuing expansion of the use of NMR for metabolomic profiling of natural product extracts is likely to depend on the further growth of analytical methods and the availability of NMR databases for both ^{1}H and ^{13}C nuclei. More compounds are probable to be recognized as enhanced methods. The NMR spectrum is quantitative. The theoretical foundation for its use as a quantitative tool is an understanding of NMR's physical values. Since each signal's frequency is directly proportional to the number of nuclei observed irrespective of setting (Bakiri et al., 2018). There is no pattern for each component in NMR spectroscopy. NMR spectroscopy also provides comprehensive molecular structure data. The use of NMR spectroscopy as a method for profiling natural product extracts therefore provides not only precise and accurate composition, but also structural evidence for each element. Because the reliance of the NMR signal on different variables is already well recognized, resonance positions and intensities are extremely reproducible. These are significant features that offer NMR a distinctive benefit over other methods of analysis (Dayrit & de Dios, 2017).

6.1.4.1 H-NMR spectroscopy

Hydrogen is present in almost every organic molecule and has an abundance of 98.985 % in its significant isotope, ^{1}H. The ^{1}H nucleus reports a frequency in an NMR spectrum that is particular to its immediate vicinity. This frequency is highly sensitive to the electronic environment providing each ^{1}H nucleus in an organic compound a type of identification number, called the NMR chemical shift. Magnetic nuclei, such as ^{1}H, also interact with each other. These interactions are known as couplings and noted as ‘splitting’ lines in an NMR spectrum in solution or liquid-state NMR spectroscopy (Dayrit & de Dios, 2017). In addition to the number and type of bonds separating the interacting pair of ^{1}H nuclei, the magnitude of these couplings relies on the spatial orientation between the nuclei. Both the chemical shifts of NMR and the constants of coupling provide enormous structure and environment data. NMR spectroscopy has thus become a strong instrument for organic structure determination (Dayrit & de Dios, 2017).

Since these NMR interactions (chemical changes and coupling constants) are quite weak and sensitive, only the pulse of excitation, internal magnetic field strength and temperature determines the frequency of an NMR signal. In addition, the chemical shift of the NMR, which represents the resonance frequency variations of the inequivalent ^{1}H nuclei, is very low: the variations are in parts per million (ppm). Therefore, for each ^{1}H in a molecule, the Boltzmann distribution for the two spin states is essentially the same (De Castro, Benedetti, Del Coco & Fanizzi, 2019). Indeed, the region below each peak in a ^{1}HNMR spectrum was shown to be proportional to the number of hydrogen atoms in a compound sharing the same atmosphere as early as 1963.

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6.1.4.2 C-NMR spectroscopy

Since carbon atoms are often connected in organic compounds to hydrogen atoms, the coupling of ^{13}C-^{1}H occurs and leads to the splitting of resonances of ^{13}C. Resembling the impact observed by Overhauser with electron spins, extra improvement of ^{13}C signals is noted when the ^{1}H spin populations are disturbed. Furthermore, because the probability of attaching a ^{13}C nucleus to another ^{13}C nucleus is very low (about 0.0001), couplings of ^{13}C-^{13}C are generally not detected, therefore producing a much simpler ^{13}CNMR spectrum. With the three isomers of a simple hydrocarbon, the use of ^{13}C NMR spectroscopy as a strong analytical instrument can be readily valued. For the above considerations, a non-destructive qualitative and quantitative analysis with ^{13}C NMR spectroscopy is possible (Emsley & Lindon, 2014).

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6.1.4.3 NMR databases in natural products

The convenience of NMR databases is centred on the NMR experiment's quantification starting with sample creation, NMR acquisition and storage through different laboratories. It is essential to prevent circumstances that change the position of chemical shifts, making it hard to identify using database comparisons. Open-access and user-contributed ^{1}H and ^{13}C NMR spectral databases have a strong potential as a beneficial tool for professionals on natural products provided that parameters for sample preparation, instrumentation and acquisition are consistent (Halabalaki, Vougogiannopoulou, Mikros & Skaltsounis, 2014). Only chosen NMR solvents should be used to prepare samples. The magnetic field's energy is more critical than ^{13}C NMR for ^{1}H. The whole process of data acquisition, transfer of vendor-specific raw information documents and data deposition need to be streamlined and standardized to further facilitate the participation of researchers (Williams et al., 2015).

6.1.5 Overview of paper disc assay of the pure fractions

Isolated fractions of selected samples from *B. malaccensis* were then carried for antimicrobial susceptibility testing. As mentioned earlier testing antimicrobial susceptibility of samples, the disc diffusion method is also known as Kirby-Bauer test and one of the classic microbiology techniques and still commonly used in most clinical microbiological laboratories which have already been discussed in (Chapter IV, section 4.1) The traditional technique of assessing antimicrobial susceptibility is focused on methods of broth dilution, which is time-consuming to execute, but still, it is considered as the best standard in current days. This led to the development of a disc diffusion process to determine antimicrobial susceptibility of bacteria. This method was chosen mainly owing to its simplicity, efficiency, and convenience for determining the antimicrobial properties of the studied extract. The purpose of the Kirby-Bauer disc sensitivity assessment is to assess the sensitivity or tolerance of pathogenic bacteria to different antimicrobial compounds.

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The bacterial growth is developed on Mueller-Hinton agar in the presence of specific antibacterial compound impregnated filter paper discs. An indirect measure of the ability of that compound to inhibit that organism is the presence or absence of growth around the discs. In this experiment, the zone of inhibition around the disc which was impregnated with pure fractions of flesh extracted by chloroform and seed extracted by hexane of *B. malaccensis* were measured and that indicated antibacterial susceptibility. The fractions were tested against three Gram-negative bacteria cells: *P. aeruginosa* (ATCC 10145), *E. coli* (ATCC 8739) and *P. putida* (ATCC 49128).

6.1.6 Overview of MIC and MBC of the pure fractions

Lastly MIC and MBC were executed on the pure fractions of selected 2 samples after paper disc assays were done to determine the lowest concentration of an antibacterial agent needed to inhibit the visible growth of bacteria after overnight incubation. By preparing solutions of the chemical *in vitro* at increasing concentrations, MIC is determined which has been discussed already in (Chapter V, section 5.1). The solutions need to be incubated with separate batches of cultured Gram-negative bacteria and then measured the results by using MH agar dilution or MH broth microdilution.

Minimum bactericidal concentration (MBC) is the minimum concentration of an antibacterial agent that results in bacterial death on antibacterial-free media after subculture over specified time limitations such as 18 hours or 24 hours and under a certain set of conditions. MIC shows the lowest antibacterial agent level that greatly inhibits the development of bacteria, the MBC shows the lowest antimicrobial agent level which results in microbial death. The MBC is complementary to the MIC. The closer the MIC is to the MBC, the more bactericidal the compounds are. Different concentrations of the compound are inoculated with cultivated bacteria and the results are calculated using agar dilution or broth dilution to determine the extent at which the MIC endpoint is formed (R. C. Gupta, 2015).

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6.2 Protocols and methods of all experiments for isolation and characterisation

6.2.1 Protocols and methods of TLC

10 samples have been selected for isolation. BC, SH, FC, and FM were chosen from *B. malaccensis.* As LC demonstrated good inhibition towards Gram-positive so LC has been isolated as well for observation basis. LC, LM was chosen from *L. spathacea*, BM from *K. maingayi*, LC and BM from *D. hasseltii.* Samples from *K. retusa* had been excluded on account of no prominent activity against Gram-negative bacteria. Samples which demonstrated good isolation are included and discussed here in this chapter.

There are two sides to the TLC plate (Merck silica gel 60F254), silica side (powdery white substance) and a glass side (smooth reflective) so careful handling is a must in this experiment. Touching the silica side with fingers should be avoided. Generally, silica plates are bought as square glass pieces. Before scoring the glass, use a ruler and a pencil to gently mark baselines (about 1 cm from the bottom) on the silica side of the plate (Sonam, Singh, & Pooja, 2017). It is very important that the surface of the silica gel is not damaged by drawing on it, as if the surface is disrupted, separation will not take place effectively. Once the entire plate is scored by using a sharp glass cutter, you can then break the glass into individual pieces.

By deciding the solvent process, substances can travel different distances on the plate depending on the solvent selected. In non-polar solvents such as pentane and hexane, most polar compounds will not pass, whereas non-polar compounds will migrate some distance on the surface (Wicha-Komsta & Komsta, 2017). Polar solvents, on the other hand, generally move non-polar compounds to the solvent front and drive off the base line's polar compounds (Isa, 2017). A healthy solvent system is one that transfers all the mixture's components of the baseline but places nothing on the solvent side – Rf values between 0.15 and 0.85. It is not possible always, but it should be the goal while

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running TLC. For column chromatography, the actual solvent system should be at Rf value within 0.2 - 0.3.

Choosing a solvent is very important in this technique. Occasionally, a single solvent can be used, or a mixture of solvents is needed. Common solvents and solvent systems which is used usually are methanol ethanol and isopropanol (very polar) where methanol is more polar than isopropanol (Wicha-Komsta & Komsta, 2017). Acetonitrile, ethyl acetate, chloroform dichloromethane, diethyl ether toluene are examples of moderately polar solvents. Before preparing the solvent system for isolation need to check the polarity beforehand as ethyl acetate is more polar than diethyl ether. Some examples of non-polar solvents are cyclohexane petroleum ether, hexane, and pentane.

Some common solvent mixture most popular combination is Ethyl Acetate: Hexane - 0-30% and Ether: Pentane - 0-40% is quite popular as well, easy to remove on the rotary evaporator Ethanol: Hexane/Pentane - 5-30% useful for very polar compounds Dichloromethane: Hexane/Pentane - 5-30% sometimes useful (Wicha-Komsta & Komsta, 2017).

- Dissolve a small amount of each of the samples in a vial with few drops of respective solvent and then filter it through Magnesium sulphate (MgSO4) to ensure that it is no water or vapour inside the sample just prepared.

- For each of the samples, in turn, take up some of the solutions using a capillary tube and place a small spot of the sample on the baseline of the TLC plate. Allow the spot to dry. The spot should be about 1 mm to 2 mm in diameter.

- Fill TLC chamber with 2-3 mL of the desired solvent system. Place a piece of cut filter paper as well in the chamber so that the moisturizer can be soaked in the chamber. Put a lid on the tank and allow it to stand

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in a fume hood until the solvent front has risen to within a few millimetres of the top of the plate.

- Run the TLC and let the solvent go about 80 - 90% of the way up the plate.

- Remove the TLC plate from the tank and mark the solvent front immediately with a pencil.

- Let the solvent dry off.

- The best method to visualize is to detect through UV lamp (250 nm and 365 nm). Place the plate under a UV lamp and circle any UV active spots with a pencil. Sometimes this can be critical for compounds that are not UV-active. Stain it with iodine or spray with the vanillin-sulphuric acid mixture. Then place on a hot plate and observe the development of the spots carefully. Remove the TLC plate once the spots are visible from the heat and before the background colour obscures the spots (Wicha-Komsta & Komsta, 2017).

- Review the solvent process selection based on your initial TLC performance. Consider the solvent system more polar if you want a larger Rf or if you want to grow the Rf. So basically, if large streaks instead of sharp circles are observed, that means sample might be concentrated much so need to dilute the sample and run the TLC once again. If this does not work, they must move to a different solvent system.

- Label TLC and calculate the Rf for each spot accordingly.

$$Rf = \frac{Distance\ spot\ moved}{Distance\ solvent\ moved}$$

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6.2.2 Protocols and methods of PTLC

Preparative thin-layer chromatography (PTLC) was executed with selected 2 samples after observing separation in TLC. Hexane extract from seed (SH) and flesh extract from chloroform (FC) was chosen for PTLC from *B. malaccensis* which is discussed later in this chapter (7.3.2). In contemplation of the goal of this project selected samples showed activity against Gram-negative bacteria with bactericidal activity in the previous antibacterial experiments.

- The relatively concentrated solution was prepared with crude extract with the respective solvent and then was deposited in a horizontal thin line (which was gently marked by a pencil roughly 1-1.5 inches from one side of the plate carefully so that it should scrape the silica gel at the bottom of the glass plate) with help of the capillary tube (Bazzocchi, Ticona, Jiménez & Flores, 2016). It is very important to do this slowly, uniformly and with help of a hairdryer to dry it completely and repeat depositing the sample once again. Make sure to dry in between applications. Not drying fully post-application might cause the solution to diffuse outward, broadening the band and then gently placed it inside the preparative TLC tank or chamber and close the lid by applying paraffin wax to make it air impenetrable.

- The plate was then run with the appropriate solvent system as it was done previously with aluminium TLC plate and with the same concentration of the solvent system. We let it run up to 90% of the plate and then took it off the tank gently. A typical run takes around 40 mins to 1 hour.

- The product is (ideally) located by UV visualization and marked with a pencil.

- A razor blade is used to scrape the silica-containing the product off the plate.

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- The silica is mounted in a fritted funnel and drained with a polar solvent or respective solvent. The pure product can be isolated from the filtrate by evaporating or by using a rotary evaporator.

6.2.3 Protocols and methods of GC/MS

GC/MS (gas chromatography/mass spectrometry) has been implemented here in this project right after preparative thin-layer chromatography (PTLC) only on hexane extract of seeds (SH) of *B. malaccensis*. GC/MS was not conducted on chloroform extract of flesh (FC) of *B. malaccensis* due to inadequate sample yield and shortage of time.

6.2.3.1 Analysis of GC/MS of the phytocomponents of the hexane extracts of seeds of B. malaccensis

6.2.3.1.1 Sample preparation

The hexane fraction of seeds of *B. malaccensis* was taken for GC-MS analysis. 5 mg of the extracts were resuspended in 1 mL of ethyl acetate by ultrasonic extraction. Then, the extracted solution was centrifuged at 10,000 rpm for 10 min and the supernatant was taken for GC-MS analysis.

6.2.3.1.2 Chromatography- mass spectrum analysis

The analysis was performed on Shimadzu QP2010 gas chromatograph mass spectrometer coupled with AOC-20i auto-injector. Chromatographic column was HP-5MS capillary column (30 m × 0.25 mm, 0.25 μm). The temperature programme was as follows: the initial temperature was 50 °C and held for 5 minutes; then rose to 140 °C with the rate of 5 °C /min, held for 5 minutes; increased to 230 °C with the rate of 2°C /min; finally elevated to 260 °C with the rate of 10 °C /min and held for 10 minutes Helium was used as carrier gas here (1 mL/min). The volume of injection was 2μl with a split ratio of 20:1 and 10:1 respectively. Mass spectra were taken at 70 eV; a scan-interval

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of 0.2 s and fragments from 35to 650 m/z. Based on the total ion chromatogram (TIC).

6.2.4 Protocols and methods of NMR

Pure fractions were isolated from samples of *B. malaccensis* by TLC, PTLC and GC/MS and then to get the chemical structures of the isolated samples, NMR was done afterwards. 2 samples (chloroform extract of flesh - FC and hexane extract of seeds - SH) from *B. malaccensis* were selected for PTLC after aluminium The pure fractions were analyzed by using conventional NMR method and the entire procedure was conducted in University Pahang Malaysia.

6.2.5 Protocols and methods of paper disc assays on the isolated pure fractions of *B. malaccensis*

The antibacterial activities of the selected pure fractions of *B. malaccensis* were assessed against 3 ATCC Gram-negative bacteria cells using Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (CLSI 2012), formerly known as National Committee for Clinical Laboratory Standards (NCCLS). Regarding the aim of the project isolated pure fractions were tested against Gram-negative bacteria to check the antibacterial activity against these bacteria cells.

6.2.5.1 Preparation of agar plates

Mueller-Hinton agar (MHA) (Difco Laboratories, USA) was prepared according to the manufacturer's instructions. The whole procedure was carried out in a microbiology lab under a biohazard safety cabinet as prepared at the beginning of this research and explained in (Chapter IV, section 4.2.1).

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6.2.5.2 Preparation of impregnated filter paper discs

Qualitative filter paper (Grade 3) (Whatman International Ltd., England) filter paper was used to prepare paper discs for the antibacterial testing here. The paper was hole-punched, to a width of 6 mm, and autoclaved to ensure sterility. Solutions were prepared for each fraction so that 50 µL contains 500 µg of the compound and 50 µL of this were used to soak each paper disc using a volumetric pipette.

Discs were placed on glass discs to dry; these discs were being dried under the biohazard safety cabinet. Negative control discs were also prepared. The paper discs were then allowed to dry for 24 hours before being placed onto the agar medium. The antibiotic chloramphenicol and ampicillin were used as positive controls and DMSO (R & M Chemicals, UK) used as the negative controls which were pre-prepared discs (50 µL in each disc).

6.2.5.3 Growing the bacteria

MHB was required for the bacterial growth and this 21 g of MHB was weighed accurately and added to an autoclaved Schott bottle with 1 L of distilled water. The mixture then sent for autoclaving to ensure sterility. 10 mL of the broth (autoclaved) was measured into glass vials. Three different Gram-negative bacteria cells were used for *B. malaccensis* and in this experiment and those were *E. coli* (ATCC 8739) and *P. aeruginosa* (ATCC 10145) and *P. putida* (ATCC 49128) were selected to assess the susceptibility patterns against the pure fractions. By using a sterilized inoculating loop, bacterial colonies were taken and mixed into the MHB contained glass vials, then placed into an incubator shaker for 60-90 minutes depending on bacteria which allowed the bacteria to grow in the broth. The UV absorbance was measured at an optical density to ensure the bacteria had colonised enough or not. A sample from each vial placed in a cuvette and its UV absorbance (625 nm) was tested, to ensure bacterial optimum density ranged within 0.08 to 0.1.

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6.2.5.4 Disc assay

Disc assay was done as the same method explained in (Chapter IV, section 4.2.4).

6.2.5.5 Reading plates and interpreting results

The Kirby-Bauer standardized disc diffusion method was applied to test the antibacterial activity of the crude extracts of *B. malaccensis* in opposition to several Gram-negative bacteria cells. Result interpretations were done in the same way as done in (Chapter IV, section 4.2.5).

6.2.6 Protocols and methods of MIC and MBC on the isolated pure fractions of *B. malaccensis*

Lastly MIC and MBC were executed on the pure fractions (9 fractions from chloroform extract of flesh- FC and 2 fractions from hexane extract of seeds- SH) from *B. malaccensis* after paper disc assays were done.

6.2.6.1 Preparation of Mueller-Hinton broth

Mueller-Hinton broth (MHB) (Difco Laboratories, USA) was prepared in accordance with the manufacturer's orders as explained in (Chapter V, section 5.2.2).

6.2.6.2 Preparation of microdilution plates

2.5 mg of isolated fractions were diluted in 1 mL of a mixture prepared with purified water, DMSO and respective solvent. 20 µL of MHB was added to 80 µL of each isolated compound (final concentrations varying from 7.8125 µg / mL to 1000 µg / mL) before inoculation in a 96-well microtiter plate (Jet Biofil, China). Tetracycline was used as a positive control with respect to the resistance to Gram-negative bacteria. A mixture consisted of with DMSO,

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purified water and respective solvent to the tested sample were served as a negative control.

6.2.6.3 Preparation of inoculums

Using the growth technique as stated earlier above, a standardized inoculum was prepared. The adjusted inoculum suspension was optimally diluted in broth and place that in the shaker machine at 180 rpm for about 40-90 minutes at 37° C to obtain a final test concentration of approximately 5×10^5 CFU / mL in each well.

Minimum inhibitory concentration (MIC) was defined as the lowest concentration of the compound to inhibit the growth of microorganism (Zeedan, Abdalhamed, Abdeen, Ottai, & Abdel-shafy, 2014). So as mentioned above that the sterile 96-well plates were prepared by distributing 20 µL of MHB as diluents and media for bacterial growth followed by 20 µL of diluted plant extracts from the stock solution which contain the concentration of 2.5 mg/mL into the first row of well that make up a total of 100 µL of suspension into the first row. Another 50 µL of fresh MHB was added from the second row to the eighth row of the well. Serial dilutions were performed by transferring 50 µL of the mixture of extracts and MHB from the first well to the subsequent wells and the last 50 µL was discarded into a beaker. The MIC test was done in triplicates and under the biohazard safety cabinet to ensure the sterility. After the serial dilutions, 50 µL of bacterial suspension was added into each well afterwards. The concentrations of plant extracts from the first well to the eighth well were 1000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL, 15.625 µg/mL, and 7.8125 µg/mL respectively. Negative controls contained the bacterial suspension and the solvent used to dilute the extracts which were purified water, DMSO and respective solvent but without the plant extract.

Tetracycline was used as a positive control. As for the positive control, the MIC values were taken following MIC interpretive standards from the CSLI, M02-A10 and M07-A8 (Advisory Committee on Immunization Practices, 2000). The quantity of growth in the wells containing isolated compounds was

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contrasted with the quantity of the positive growth control wells (without isolated compounds) used in each test set when determining growth endpoints. The MIC value was eventually recorded as the smallest concentration of each isolated compound that inhibited the development of bacteria in the reservoirs of microdilution as identified by the unaided eye.

6.2.6.4 Inoculation of microdilution plates

Inoculation was done in the same way explained in (Chapter V, section 5.2.6).

6.2.6.5 Incubation of microdilution plates

The inoculated microdilution plates were incubated as discussed in (Chapter V, section 5.2.6).

6.3 Result and discussion of all experiments for isolation and characterisation

6.3.1 Result and discussion of TLC

As stated before 10 samples have been selected for isolation. BC, SH, FC, and FM were chosen from *B. malaccensis.* As LC demonstrated good inhibition towards Gram-positive so LC has been isolated as well for observation basis. LC, LM was chosen from *L. spathacea*, BM from *K. maingayi*, LC and BM from *D. hasseltii. K. retusa* has been excluded here due to not having potential antibacterial activity against Gram-negative bacteria. Samples with clear and readable separations are explained here below.

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6.3.1.1 TLC of chloroform extract of leaves from B. malaccensis

Chloroform extracts from leaves of *B. malaccensis* exhibited the presence of 9 spots having Rf values of 0.213, 0.234, 0.277, 0.383, 0.468, 0.543, 0.681, 0.723 and 0.787 respectively and the solvent phase used was chloroform: ethyl acetate (40:60) (Table 10). The stains observed after vanillin-sulphuric acid spray were pink for fractions 1-7 and green for fractions 8 and 9 (Figure 22, 23 and 24). Pink stain indicates the presence of phenols in the vanillin-sulphuric acid reagent. Ferric chloride did not show any significant change in colour. Fractions 2, 8 and 9 developed greenish-brown stain after dragendorff spray which does not clearly indicate any compound according to MSDS and other references.

Table 10: TLC of Leaves Chloroform *(B. malaccensis)* at the concentration of Chloroform: Ethyl Acetate

Leaves Chloroform *(B. malaccensis)* Sample	Rf value	254 nm	365 nm	Vanillin spray	Dragendorff spray
Chloroform: Ethyl Acetate (40:60)				(15g van+2.5 mL con. H_2SO_4 +250 mL Ethanol)	
1	0.213	Black	-	Pink	Greenish brown
2	0.234	Black	-	Pink	-
3	0.277	Black	-	Pink	-
4	0.383	Black	Dark red	Pink	-
5	0.468	Black	Dark red	Pink	-
6	0.543	Black	Dark red	Pink	-
7	0.681	Black	Dark red	Pink	-
8	0.723	Black	-	Green	Greenish brown
9	0.787	Black	-	Green	Greenish brown

(40:60)

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6.3.1.2 TLC of chloroform extract of flesh from B. malaccensis

Chloroform extracts from flesh of *B. malaccensis* exhibited the presence of 9 spots having Rf value*s* of 0.187, 0.242, 0.286, 0.341, 0.396, 0.462, 0.659, 0.769 and 0.846 respectively when a solvent phase of chloroform: ethyl acetate: diethyl ether (40:40:20) was used (Table 11). The stains observed hereafter vanillin-sulphuric acid spray were pink and dark pink for most of them which indicate the compounds could be phenols or steroids (Figure 24). Ferric chloride did not show any change in colour. Fractions F2, F3 developed orange stain and F6 and F7 gave light orange stains after dragendorff spray which indicates that the compounds could be any nitrogenous compounds or phenols (Figure 24) (Sonam, 2017).

Table 11: TLC of Flesh Chloroform *(B. malaccensis)* at the concentration of Chloroform: Ethyl Acetate: Diethyl Ether (40:40:20)

Flesh Chloroform *(B. malaccensis)* Sample	Rf value	254 nm	365 nm	Vanillin spray	Dragendorff spray
Chloroform: Ethyl Acetate: Diethyl Ether (40:40:20)				(15g van+2.5 mL con. H_2SO_4 +250 mL Ethanol)	
1	0.187	Black	-	Pink	-
2	0.242	Black	-	Dark Pink	orange
3	0.286	Black	-	Dark Pink	orange
4	0.341	N/A	-	Pink	-
5	0.396	N/A	-	Pink	-
6	0.462	N/A	-	Pink	Light orange
7	0.659	Black	-	Pink	Light orange
8	0.769	Black	-	Pink	-
9	0.846	Black	-	-	-

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6.3.1.3 TLC of hexane extract of seeds from B. malaccensis

Hexane extracts from seeds of *B. malaccensis* revealed the presence of 2 spots having Rf values of 0.25, 0.75 respectively when a solvent phase of hexane: ethyl acetate: dichloromethane (40:30:30) was used (Table 12). After applying several combinations of extracts, two clear spots were able to isolate at 40% of hexane, 30% of ethyl acetate and 30% of dichloromethane concentration (Figure 22, 23 and 24). After vanillin-sulphuric acid spray, the stains were pink in colour for both which indicate the compounds could be phenols or steroids. Ferric chloride and dragendorff reagent did not show any significant change in colour here.

Table 12: TLC of seed hexane *(B. malaccensis)* at the concentration of hexane: ethyl acetate: dichloromethane (40:30:30)

Seed Hexane *(B. malaccensis)* Sample	Rf value	254 nm	365 nm	Vanillin spray	Dragendorff spray
Hexane: Ethyl Acetate: Dichloromethane (40:30:30)				(15g van+2.5 mL con. H_2SO_4 +250 mL Ethanol)	
1	0.25	Black	Fluorescent blue	Pink	-
2	0.75	Black	-	Pink	-

6.3.1.4 TLC of chloroform extract of bark from B. malaccensis

Chloroform extracts from barks of *B. malaccensis* exhibited 5 spots having Rf values of 3.63, 1.60, 1.509, 1.429 and 1.194 respectively when a solvent phase of chloroform: ethyl acetate: dichloromethane (40:30:30) was used (Table 13). Ferric chloride and vanillin-sulphuric acid reagent did not show any significant change in colour here but after applying the dragendorff reagent fractions 2 and 4 developed light orange stain indicates that the compounds could be any nitrogenous compounds or phenols (Figure 24).

Table 13: TLC of bark chloroform *(B. malaccensis)* at the concentration of chloroform: ethyl acetate: dichloromethane (40:30:30)

Bark Chloroform *(B. malaccensis)* Sample	Rf value	254 nm	365 nm	Vanillin spray	Dragendorff spray
Chloroform: Ethyl Acetate: Dichloromethane (40:30:30)				(15g van+2.5 mL con. H_2SO_4 +250 mL Ethanol)	
1	3.63	Black	-	-	-
2	1.60	Black	-	-	Light orange
3	1.509	Black	-	-	Light orange
4	1.429	Black	Fluorescent blue	-	-
5	1.194	Black	Fluorescent red	-	

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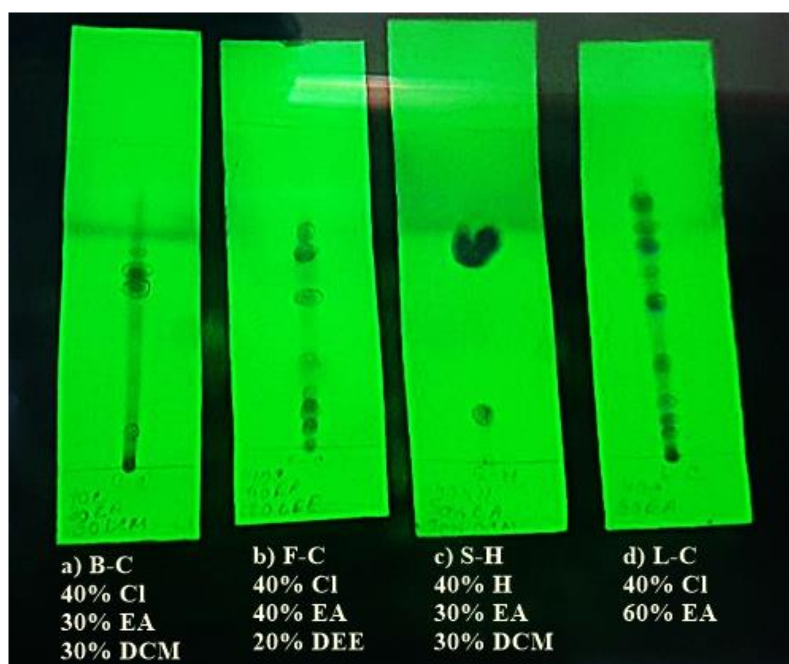

Figure 22: Appearance of TLC plate at 254nm a) bark-chloroform with concentration (chloroform: ethyl acetate: dichloromethane-40:30:30%), b) flesh-chloroform with concentration (chloroform: ethyl acetate: diethyl ether-40:40:20%), c) seed-hexane with concentration (hexane: ethyl acetate: dichloromethane-40:30:30%), leaves-chloroform with concentration (chloroform: ethyl acetate-40:60%)

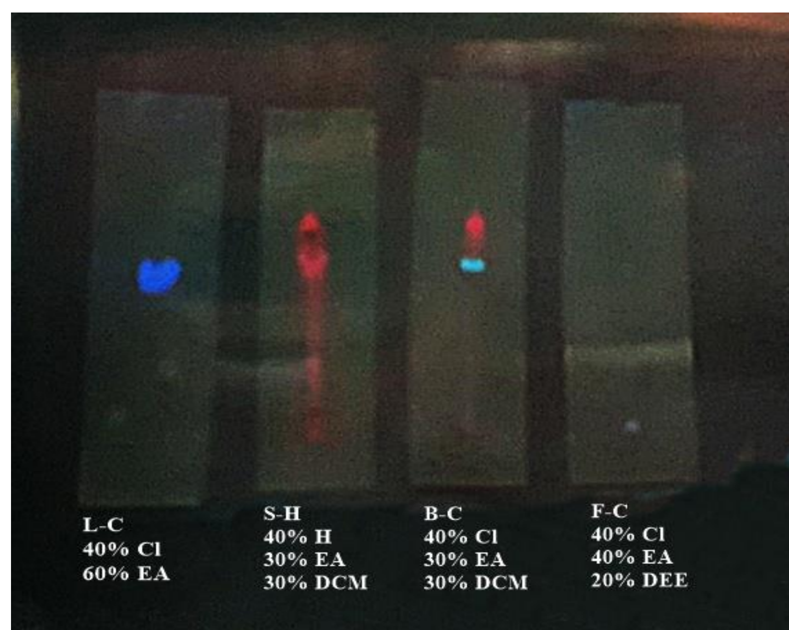

Figure 23: Appearance of TLC plate at 365nm a) bark-chloroform with concentration (chloroform: ethyl acetate: dichloromethane-40:30:30%), b) flesh-chloroform with concentration (chloroform: ethyl acetate: diethyl ether-40:40:20%), c) seed-hexane with concentration (hexane: ethyl acetate: dichloromethane-40:30:30%), leaves-chloroform with concentration (chloroform: ethyl acetate-40:60%)

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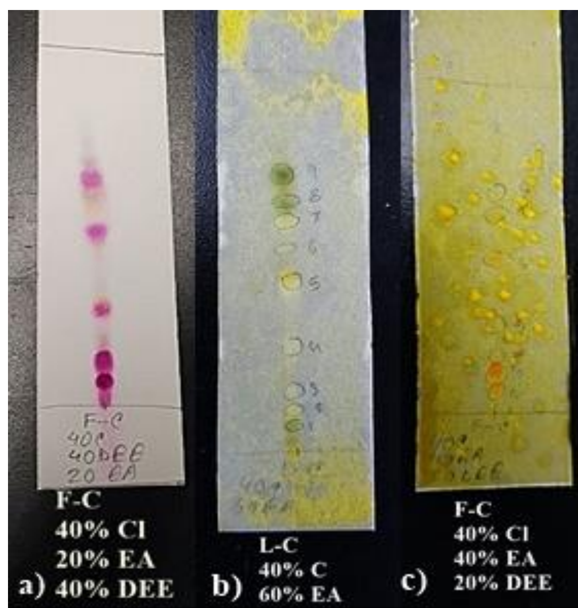

Figure 24: Appearance of TLC plate at visible light a) flesh-chloroform with concentration (chloroform: ethyl acetate: diethyl ether-40:40:20%) sprayed with vanillin spray, b) leaves-chloroform with concentration (chloroform: ethyl acetate-40:60%) after spraying dragendorff reagent and c) flesh-chloroform with concentration (chloroform: ethyl acetate: diethyl ether-40:40:20%) after spraying dragendorff reagent.

6.3.1.5 TLC of chloroform extract of leaves from D. hasseltii

There were a total of six spots observed on the TLC plate. However, only four spots labelled 1, 2, 3 and 4 as showed in the (Figure 25) were focused on suggesting compounds at these spots involved in the antimicrobial activities. On the other hand, two spots from the top of the plate (appeared as green) were considered as chlorophyll by comparison with reference TLC plate of spinach with a different solvent system, showed green appearance on the TLC plate is a chlorophyll B pigments. So, TLC of LC exhibited the presence of 4 compounds having Rf values of 0.26, 0.28, 0.45 and 0.57 respectively when a solvent phase of Diethyl ether: Methanol (9:1) was used (Table 14). Notably, it was found a blue colour appeared at the third spot suggesting the presence of steroids compound, one of the main components isolated from *D. hasseltii.*

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Table 14: TLC of leaves chloroform *(D. hasseltii)* at the concentration of diethyl ether: methanol (9:1)

Leaves Chloroform (Diospyros sp) Sample	Rf value	254 nm	365 nm	Vanillin spray	Dragendorff spray
Diethyl ether: Methanol (9:1)				(15g van+2.5 mL con. H_2SO_4 +250 mL Ethanol)	
1	0.26	Black	-	Green	-
2	0.28	Black	-	Greenish blue	-
3	0.45	Black	-	Blue	-
4	0.57	Black	-	Green	-

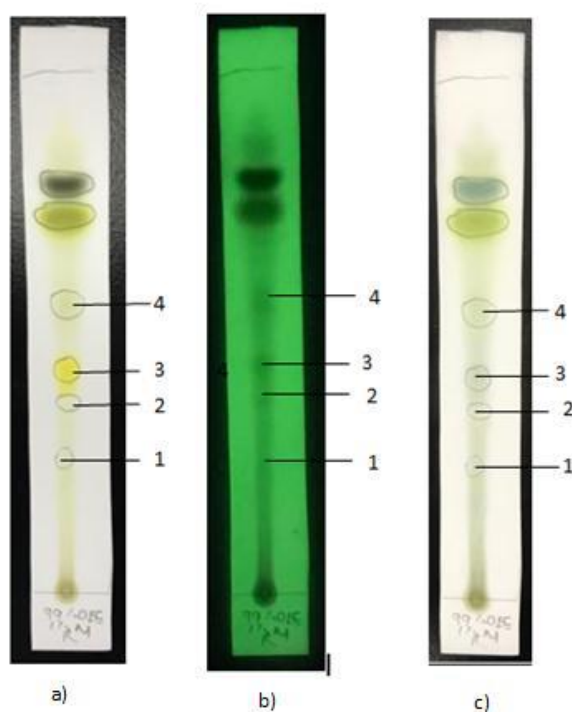

Figure 25: Appearance of TLC plate of leaves chloroform of *D. hasseltii* at visible light (a), under UV light at 254nm (b) and at visible light after sprayed with vanillin-sulphuric acid reagent (c). with a concentration of diethyl ether: methanol-9:1

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6.3.2 Result and discussion of PTLC

PTLC terminated right after TLC was done. 2 samples (chloroform fractions of flesh) and hexane fractions of seed from *B. malaccensis* were selected for PTLC based on clear sharp separations of the compounds got from TLC.

6.3.2.1 PTLC for chloroform fractions of the flesh of B. malaccensis

PTLC of chloroform extracts from the flesh of *B. malaccensis* exhibited the presence of 9 compounds through 9 layers (Table 15) with the solvent phase of chloroform: ethyl acetate: diethyl ether (40:40:20) (Figure 26). Once they are well dried, all the separated layered of compounds were then scrapped by a razor blade inside the fume hood with proper caution. The silica was placed in an autoclaved glass vials and flushed with respective solvent. Then the pure product was isolated from the filtrate by evaporating the solvent. The yield of the 9 compounds were 13, 3, 11, 5, 10, 10, 30, 92 and 140 mg respectively for fractions Fx, F1, F2, F3, F4, F5, F6, F7 and F8.

Table 15: Flesh Chloroform of *B. malaccensis* pure compounds yield:

Flesh Choloroform	Before (g)	After (g)	Yield (g)	Yield (mg)
x	13.70	13.713	0.013	13
1	13.77	13.773	0.003	3
2	13.81	13.821	0.011	11
3	13.79	13.795	0.005	5
4	13.65	13.66	0.01	10
5	13.72	13.73	0.01	10
6	13.74	13.77	0.03	30
7	13.75	13.842	0.092	92
8	13.79	13.93	0.14	140

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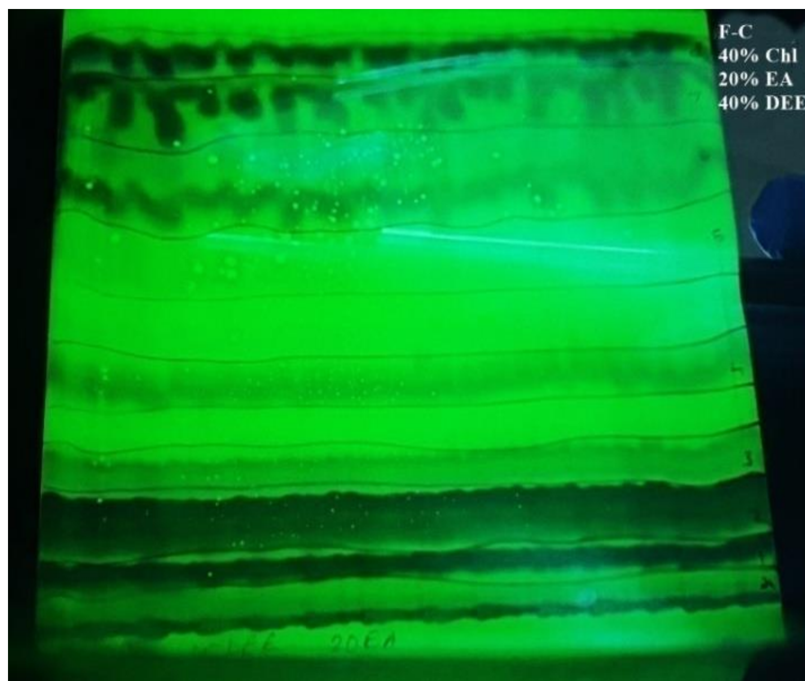

Figure 26: PTLC on flesh-chloroform with concentration (chloroform: ethyl acetate: diethyl ether-40:40:20%) at 254 nm

6.3.2.2 PTLC for hexane extract of seed of B. malaccensis

PTLC of hexane extracts from seeds of *B. malaccensis* revealed the presence of 2 compounds with a solvent phase of Hexane: Ethyl Acetate: Dichloromethane (40:30:30) was used (Table 16 and Figure 27). The scrapped silica was placed in an autoclaved glass vials and flushed with the respective solvent. Then the pure product was isolated from the filtrate by evaporating the solvent. The yields of 2 compounds were 7 and 119 mg for fractions S1 and S2.

Table 16: Seed Hexane of *B. malaccensis* pure compounds yield:

Seed Hexane	Before (g)	After (g)	Yield (g)	Yield (mg)
1	13.743	13.75	0.007	7
2	13.705	13.824	0.119	119

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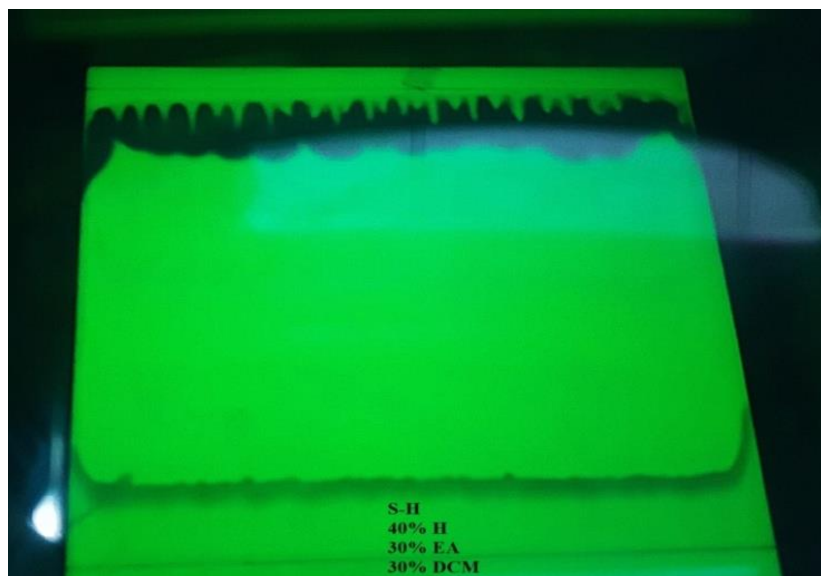

Figure 27: PTLC on seed-hexane with concentration (hexane: ethyl acetate: dichloromethane-40:30:30%) at 254 nm.

6.3.3 Result and discussion of GC/MS

6.3.3.1 Result of GC-MS of the hexane extracts of seeds of B. malaccensis

Based on the above chromatographic pattern analysis of hexane extracted seeds of *B. malaccensis* two major peaks were observed. Similarly, TLC showed 2 major spots of hexane extracted seeds of *B. malaccensis.* At retention time 51.685, one peak was detected with the mass of 409 and at retention time 54.161 another peak with mass spectrometry 392 was detected.

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Sample Information

Analyzed by	: Admin
Analyzed	: 14/12/2018 9:59:17 PM
Sample Type	: Unknown
Sample Name	: S-Hex-141218a
Sample ID	: S-Hex-141218a
Vial#	: 3
Injection Volume	: 1.00
Data File Method	: C:\GCMSsolution\Data\2018\Prof Khozirah\DrWiart\S-Hex-141218a.qgd
File Report File	: C:\GCMSsolution\Data\Essential Oil Analysis\Essential oil_MEOH50-700 Oct2018.qgm
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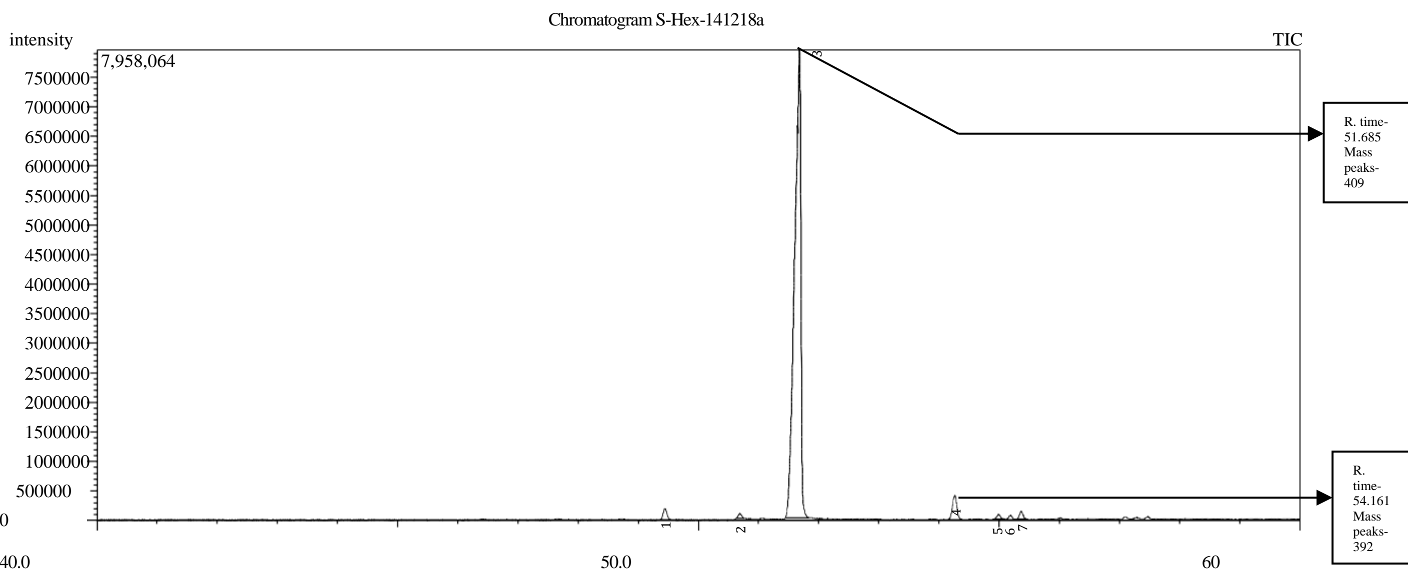

Peak Report TIC

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2	50.6897	50.6317	50.7483	286636.00	0.47	81123.00	0.94	3.5300
3	**51.6850**	**51.4517**	**51.8417**	**57138794.00**	**93.14**	**7719507.00**	**89.17**	**7.4000**
4	**54.2697**	**54.1617**	**54.3683**	**2025729.00**	**3.30**	**398302.00**	**4.60**	**5.0900**
5	54.9905	54.9350	55.0950	297749.00	0.49	76999.00	0.89	3.8700
6	55.1885	55.1233	55.2567	278056.00	0.45	69871.00	0.81	3.9800
7	55.3733	55.2950	55.4550	543983.00	0.89	127749.00	1.48	4.2600
				61348019.00	100.00	8656675.00	100.00	

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Method

[Comment]

===== Analytical Line 1 =====

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Injection Temp.	:250.00 °C	
Injection Mode	:Split	
Flow Control Mode	:Pressure	
Pressure	:37.1 kPa	
Total Flow	:11.8 mL/min	
Column Flow	:0.80 mL/min	
Linear Velocity	:32.4 cm/sec	
Purge Flow	:3.0 mL/min	
Split Ratio	:10.0	
High Pressure Injection	:OFF	
Carrier Gas Saver	:OFF	
Splitter Hold	:OFF	
Oven Temp. Program		
Rate	Temperature(°C)	Hold Time(min)
-	50.0	0.00
3.00	300.0	10.00

Column: Rxi-5ms (30.0m Length x 0.25mm ID x 0.25um Thickness)

[Comment]

===== Analytical Line 1 =====

[GCMS-QP2010 ultra]

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Interface Temp.	:250.00 °C
Solvent Cut Time	:2.00 min
Detector Gain Mode	:Relative
Detector Gain	:0.91 kV +0.00 kV
Threshold	:0

[MS Table]

--Group 1 - Event 1--

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Event Time	:0.10sec
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Start m/z	:40.00
End m/z	:700.00

Sample Inlet Unit : GC

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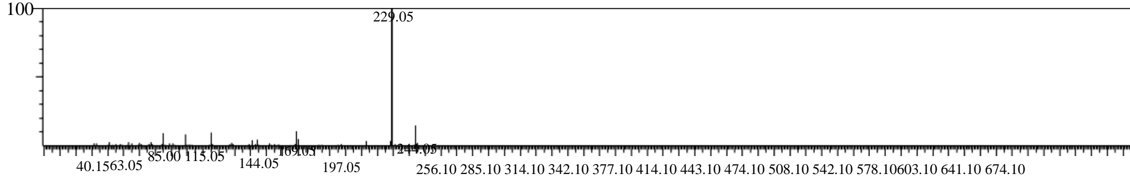

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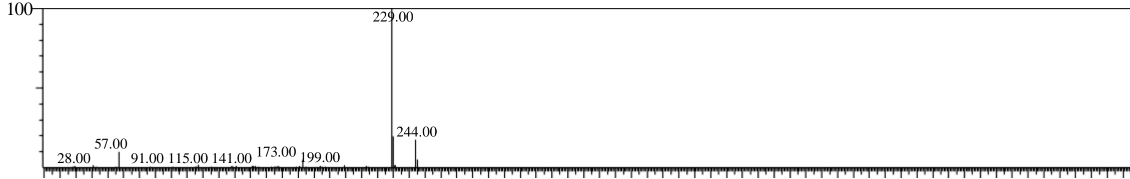

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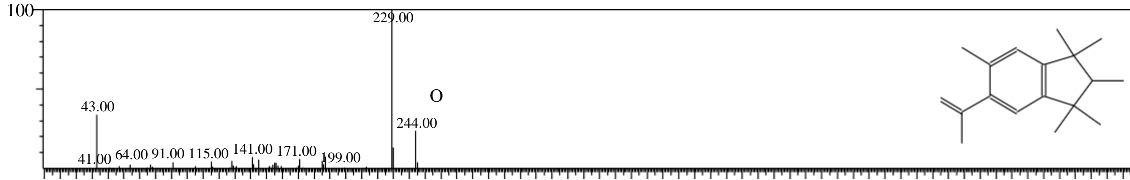

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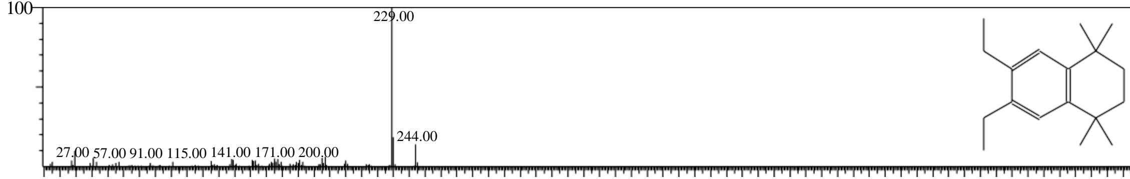

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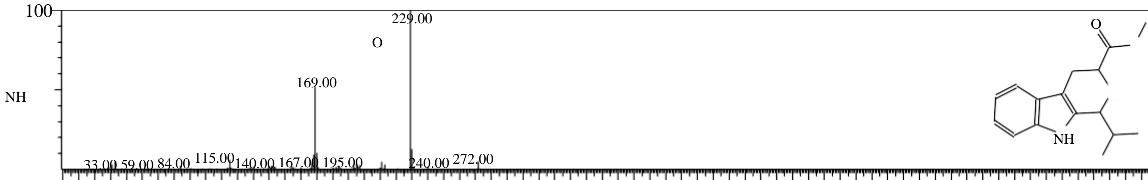

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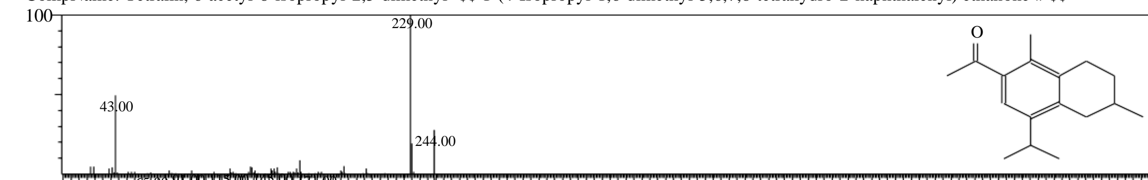

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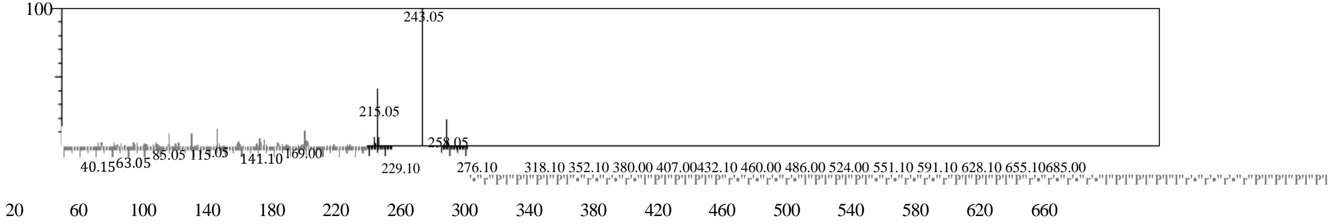

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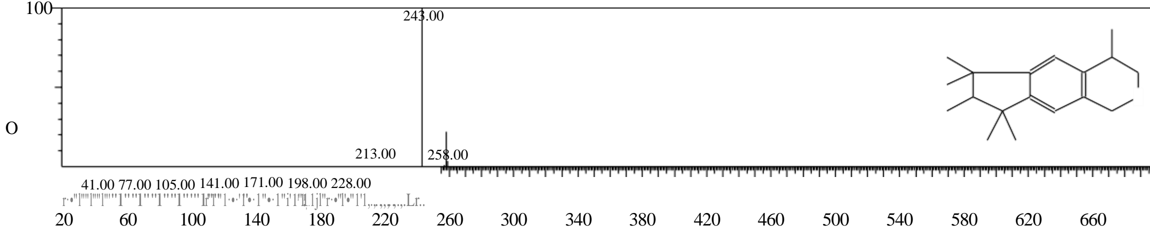

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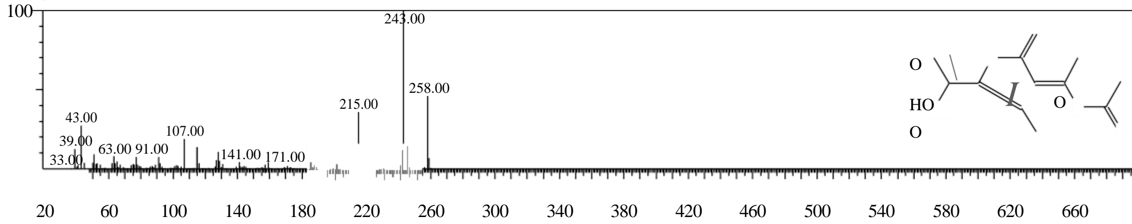

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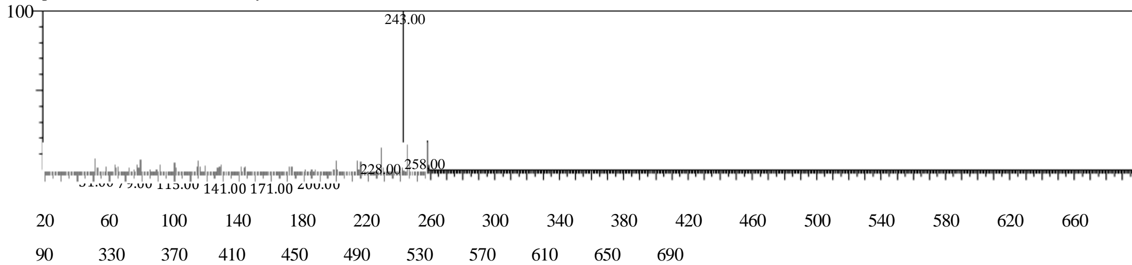

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6.3.4 Result and discussion of NMR

The entire NMR process was conducted in University Pahang Malaysia by Dr Nor Hafizah Zainal Abidin and compound analysis was guided by Dr Mark Butler. NMR was conducted on 9 fractions from chloroform extract of the flesh of *B. malaccensis* and 1 fraction of hexane extract of seed from *B. malaccensis*. Compounds isolated are rare and still no current findings are available yet. So expanded structural analysis is not included here due to insufficient current data of these compounds.

Out of 9 fractions of FC of *B. malaccensis,* able to detect sample 1 (F1) by observing the NMR spectrum. It has been detected that the sample has derivatives of dihydroxy acidissiminol $C_{25}H_{34}NO_5$, (molecular mass 427.2359). Dihydroxy acidissiminol, is an alkaloid tyramine derivative, was isolated from the chloroform extract of the flesh of *B. malaccensis*. The HNMR and CNMR data of dihydroxy acidissiminol are shown in (Table 17). The structure of the compound was determined as benzamide-N- {p [(3,7-dimethyl-4,6,7-trihydroxy-2-octenyl) oxy] phenethyl} based on spectroscopic evidence (P, R, PS & RM, 2020). The ^{13}C chemical shift value δ 72.6 for the hydroxy-bearing C-7 of dihydroxy acidissiminol was consistent with that of the hydroxy-bearing tertiary carbon of 2.3~dimethyl-2-butanol (C-l, δ 26.3; C-2, δ 72.2), thus supporting the proposed structure for dihydroxy acidissiminol. 2DNMR data were consistent with the proposed structure of dihydroxy acidissiminol and the presence of benzamide (Figure 28). Benzamide has been found to play a crucial role in conferring antibacterial activity (Pitchai P, 2013). Dihydroxy acidissiminol is a tyramine alkaloid which has been isolated before from the plant *Limonia acidissima* in the family Rutaceae (Ghosh et al., 1994). To date, there is no report on the occurrence of this alkaloid from *B. malaccensis*. Further, no reports on the biological activity of this compound have been found. It is a mid-polar substance with a long 7 carbon side chain and theoretically, it might also possibly penetrate the membrane of bacteria and exert antibacterial effect (Ghosh et al., 1994).

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Fraction 1 from the hexane extracts of seeds (S1) of *B. malaccensis* demonstrated that it has derivatives of werneria chromene ($C_{15}H_{16}O_3$, molecular mass 244.28 g/mol) by observing the NMR spectrum (Bohlmann, Beltran & Leiva, 2018). The NMR spectrum gave a molecular ion at 244 consistent with a formula of $C_{15}H_{16}O_3$. The HNMR stipulated a 1,2,4-trisubstituted benzene ring, a methoxyl group, two identical C-methyl groups and one cis- and one trans-disubstituted double bond. The CNMR and APT data supported these structural features and showed five quaternary carbons: one sp3 and one sp2 carbon each attached to oxygen, two additional sp2 carbons and one carbonyl carbon in the ester region. The position of the methoxyl carbon resonance indicated an ester. The NMR data clearly matched those of Werneria chromene which was first isolated from *Werneria stuebelii* and confers the presence of chromanone (Hong, Minter, Franzblau & Reinecke, 2008), (Table 18 and Figure 29). The biological evaluation revealed that the chromanone group also proved to be a determining factor for antibacterial activities (Feng et al., 2014). The isolation of Werneria chromene from the *B. malaccensis* and the family Rutaceae, in general, has never been reported before. In addition to this werneria chromene has never been tested for any biological activity either.

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6.3.4.1 Result of sample 1 (F1) from chloroform extract of the flesh of B. malaccensis

Dihydroxy acidissiminol $C_{25}H_{34}NO_5$, molecular mass 427.2359, Amorphous semi-solid, HNMR (400 MHz, $CDCl_3$), CNMR (100 MHz, $CDCl_3$): δ 7.70 (1H, d, H-2') and 7.70 (1H, d, H-6'), 7.40 (1H, t, H-3') and 7.40 (1H, t, H-5'), 7.49 (1H, t, H-4'), 6.15 (1H, t, CO-NH), 3.70 (2H, m, N-CH_2), 2.80 (2H, t, Ar-CH_2), 6.87 (1H, d, H-2") and 6.87 (1H, d, H-6"), 7.16 (1H, d, H-3") and 7.16 (1H, d, H-5"), 4.60 (2H, d, H-1), 5.78 (1H, d, H-2), 1.75 (3H, s, 3-Me), 4.35 (1H, dd, H-4), 3.65 (1H, m, H-6), 1.17 (3H, s, H-7 Me), 1.75 (s, OH-4), 1.65 (s, OH-6) and 1.65 (s, OH-7), δ 135.00 (C-1'), 126.50 (C-2', C-6'), 128.90 (C-3', C-5'), 130.00 (C-4'), 167.50 (CO-NH), 41.50 (N-CH2), 35.00 (Ar-CH2), 157.00 (C-1"), 115.00 (C-2", C-6"), 129.90 (C-3", C-5"), 131.50 (4"), 64.50 (C-1), 121.50 (C-2), 140.50 (C-3), 12.90 (C-3 Me), 79.00 (C-4), 77.50 (C-6), 24.00 (7-Me).

Table 17: HNMR spectroscopic data (400MHz, $CDCl_3$) and CNMR (100MHz, $CDCl_3$) data of dihydroxy acidissiminol

	1HNMR _Parthasarathi	Sample 1 (δH)	Integration	CNMR_ Parthasarathi	Sample 1 (δC)
1'	-	-	-	134.60	135.00
2', 6'	7.69 d	7.70 d	1, 1	126.80	126.50
3',5'	7.41 t	7.40 t	1, 1	128.60	128.90
4'	7.49 t	7.49 t	1	131.40	130.00
CO-NH	6.10 m	6.15 m	1	167.60	167.50
N-CH2	3.70 q	3.70 m	2	41.30	41.50
Ar- CH2	2.88 t	2.80 t	2	34.80	35.00
1"	-	-	-	157.30	157.00
2",6"	6.87 d	6.87 d	1, 1	114.90	115.00
3",5"	7.18 d	7.16 d	1, 1	129.80	129.90
4"	-	-	-	131.00	131.50
1	4.58 d	4.60 d	2	64.50	64.50
2	5.80 d	5.78 d	1	121.10	121.50
3	-	-	-	142.0	140.50
3- Me	1.76 s	1.75 s	3	12.40	12.90
4	4.35 dd	4.35 dd	1	77.40	79.00
5	-	-	-	-	-
6	3.64 m	3.65 m	1	78.70	77.50
7	-	-	-	72.60	74.00
7- Me	1.18 s	1.17 s	3	23.70	24.00
4- OH	1.55 s	1.75 s	-	-	-
6,7 OH	1.55 s	1.65 s	-	-	-

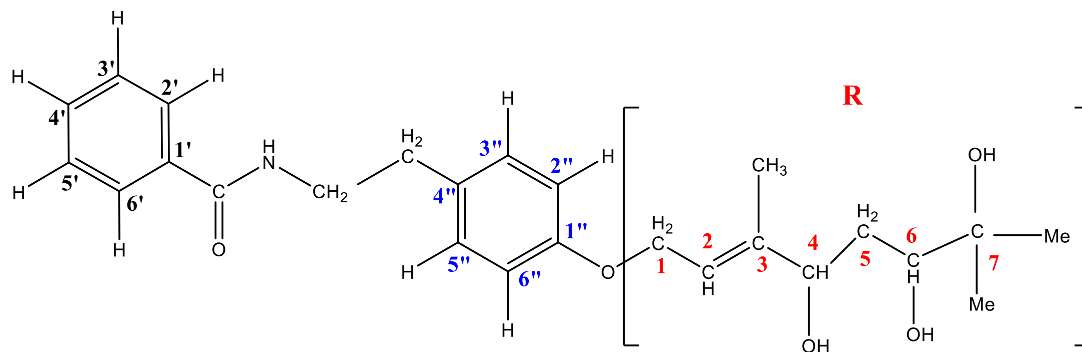

Figure 28: Dihydroxy acidissiminol chemical structure

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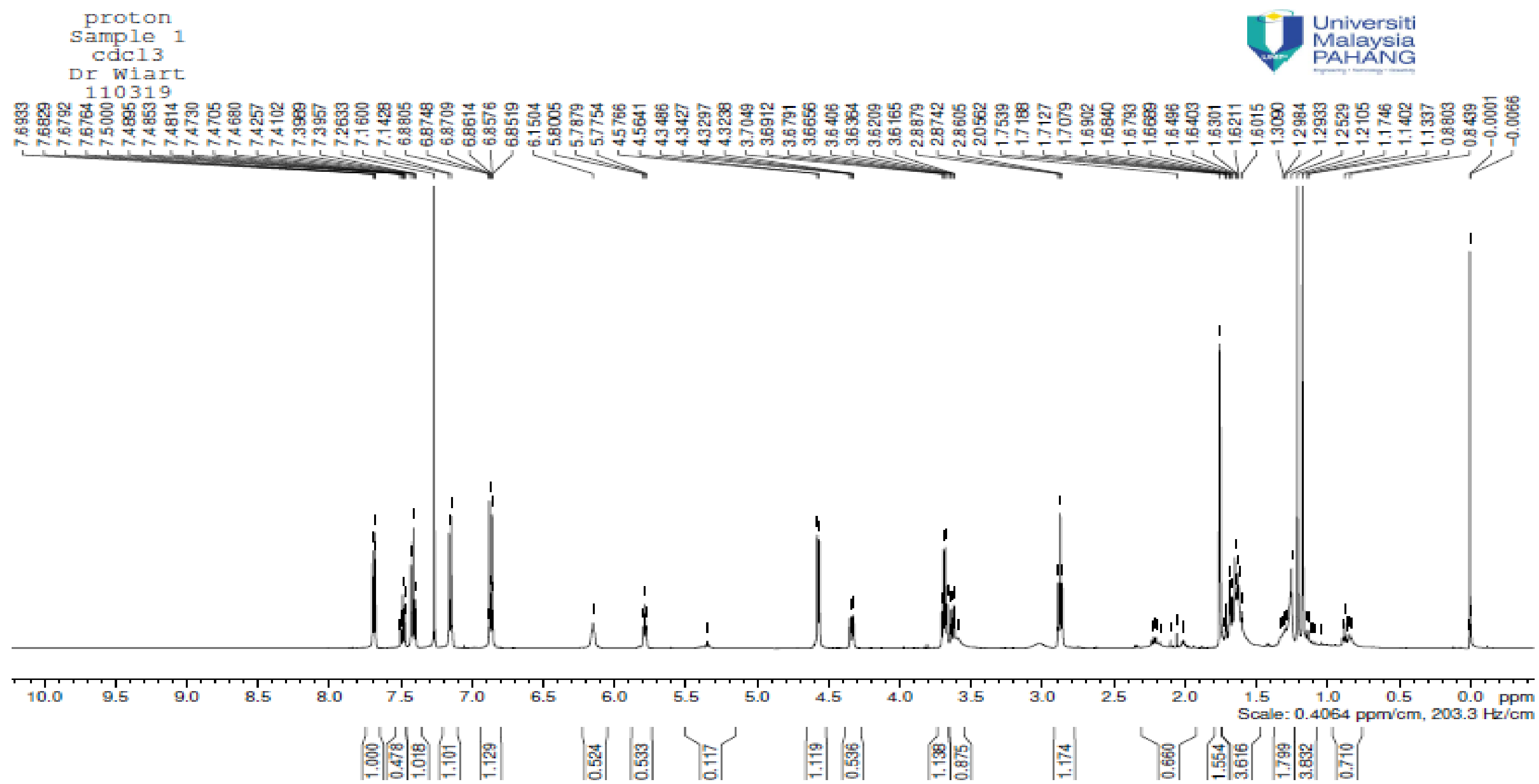

Figure 29: HNMR of compound 1 from chloroform extract of the flesh of *B. malaccensis*.

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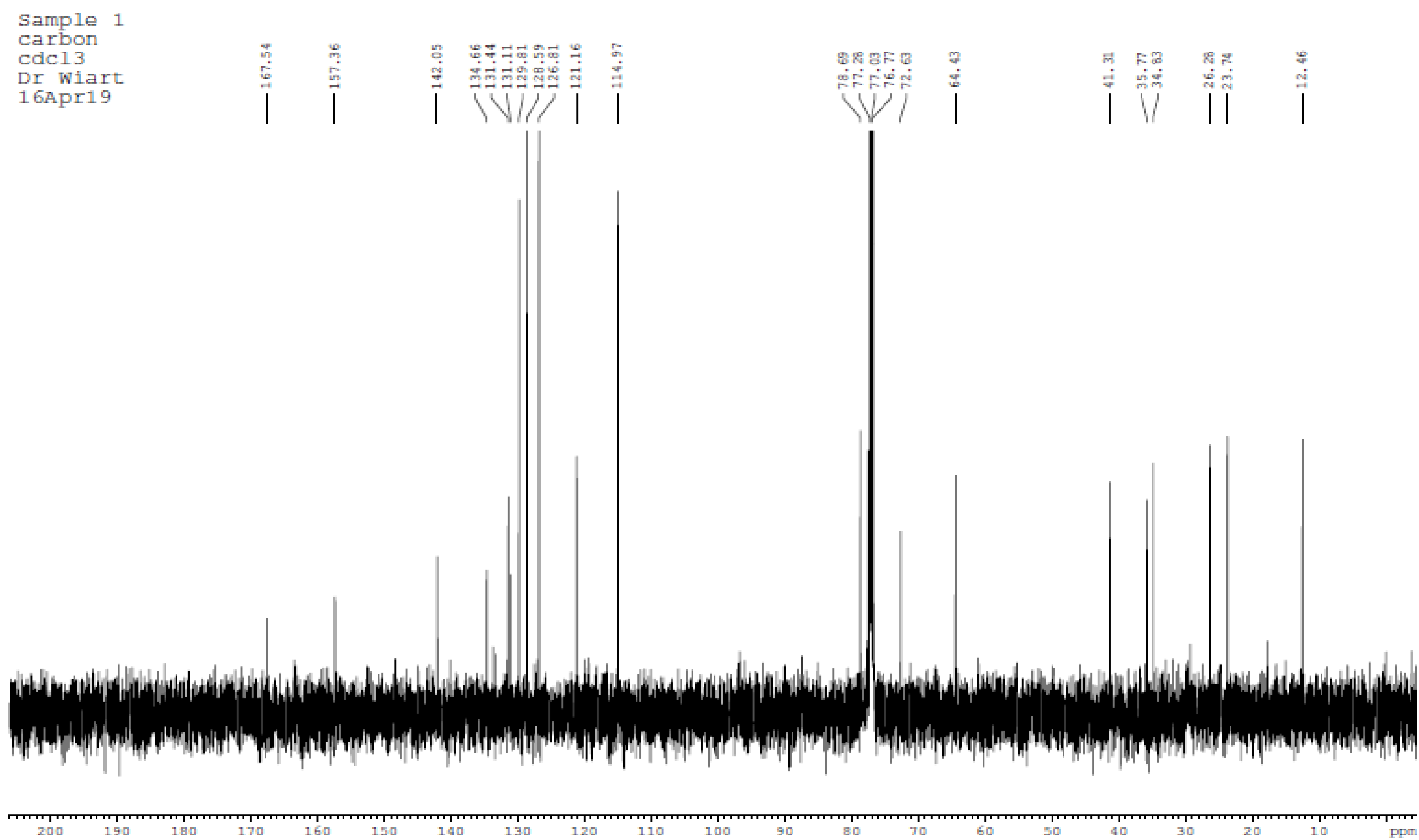

Figure 30: CNMR of compound 1 from chloroform extract of the flesh of *B. malaccensis*

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6.3.4.2 Result of sample 1 (S1) from hexane extract of seeds of B. malaccensis

Werneria chromene $C_{15}H_{16}O_3$, pale yellow crystal, molecular mass 244.28 g/mol, HNMR (400 MHz, $CDCl_3$), CNMR (100 MHz, $CDCl_3$): δ 5.65 (1H, d, H-3), 6.30 (1H, d, H-4), 7.18 (1H, d, H-5), 7.25 (1H, d, H-7), 6.78 (1H, d, H-8), 7.6 (1H, d, H-9), 6.2 (1H, d, H-10), 7.57 (2H, d, H-12) and 7.57 (2H, d, H-13), 3.8 (3H, s, O-Me), δ 78 (C-2), 132 (C-3), 122 (C-4), 134 (C-5), 128 (C-6), 129 (C-7), 116 (C-8), 122 (C-10), 115 (C-11), 145 (C-12).

Table 18: HNMR spectroscopic data (400MHz, $CDCl_3$) and CNMR (100MHz, $CDCl_3$) data of Werneria Chromene

	1HNMR _Bohlman	Sample 1 (δ H)	Integration		CNMR_Hong	Sample 1 (δC)
H3	5.62 d	5.65 d	1	C2	77.1 e	78
H4	6.28 d	6.30 d	1	C3	131.3 o	132
H5	7.12 d	7.18 d	1	C4	121.7 o	122
H7	7.26 dd	7.25 dd	1	C5	134.3 o	134
H8	6.74 d	6.78 d	1	C6	127.1 e	128
H9	7.58 d	7.60 d	1	C7	129.4 o	129
H10	6.26 d	6.20 d	1	C8	116.7 o	116
H12,13	7.58 d	7.57 d	2, 2	C10	121.3 e	122
OMe	3.76 s	3.80 s	3	C11	115 o	115
				C12	144.6 o	145

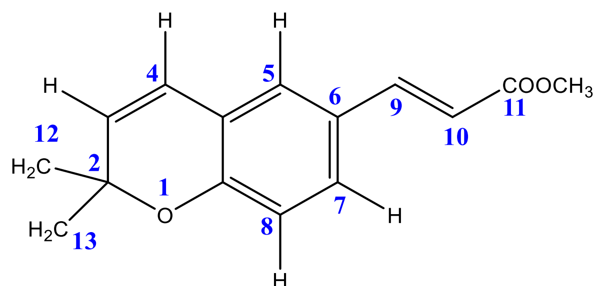

Figure 31: Werneria chromene chemical structure

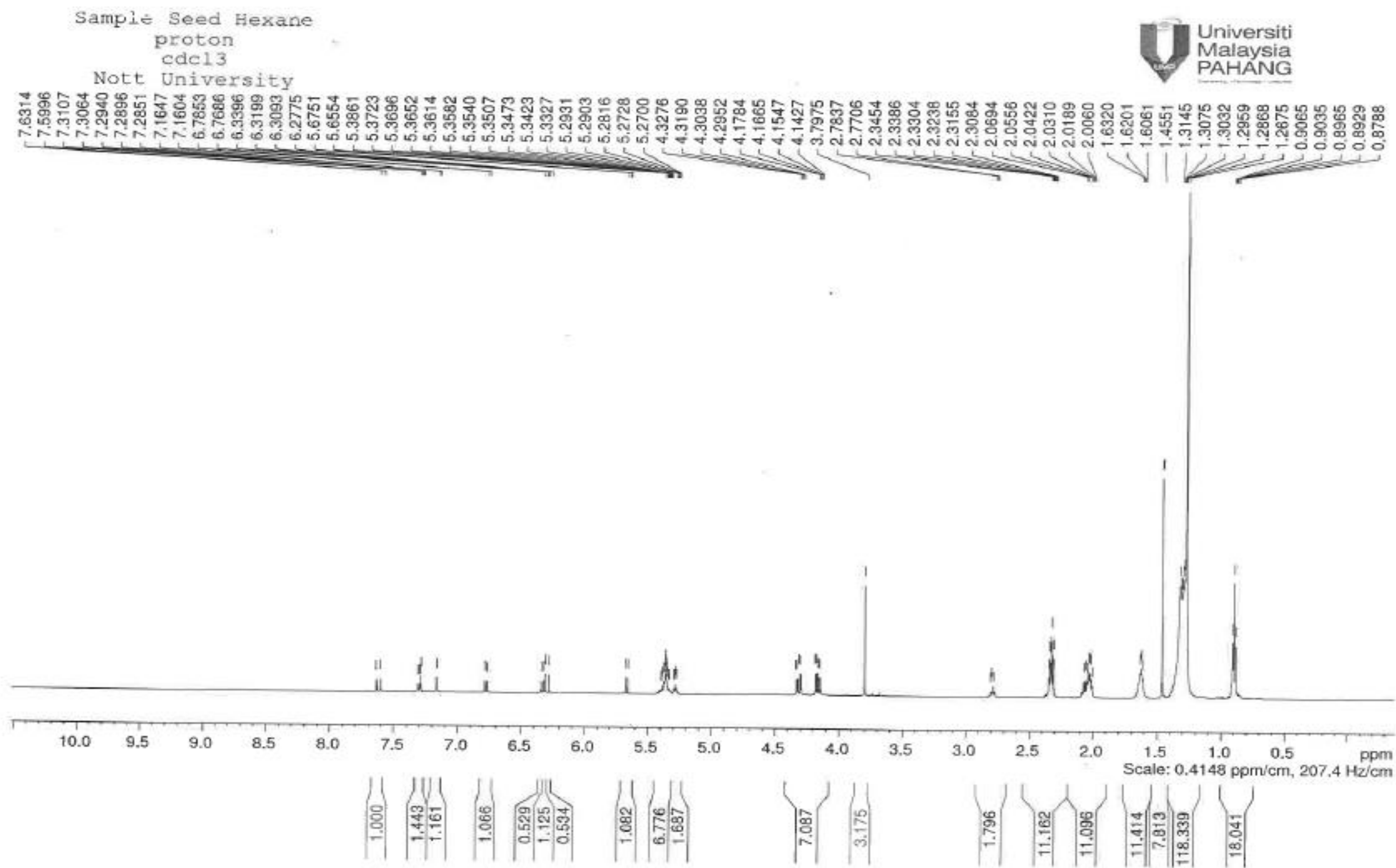

Figure 32: HNMR of compound 1 from Hexane extract of seed of *B. malaccensis*

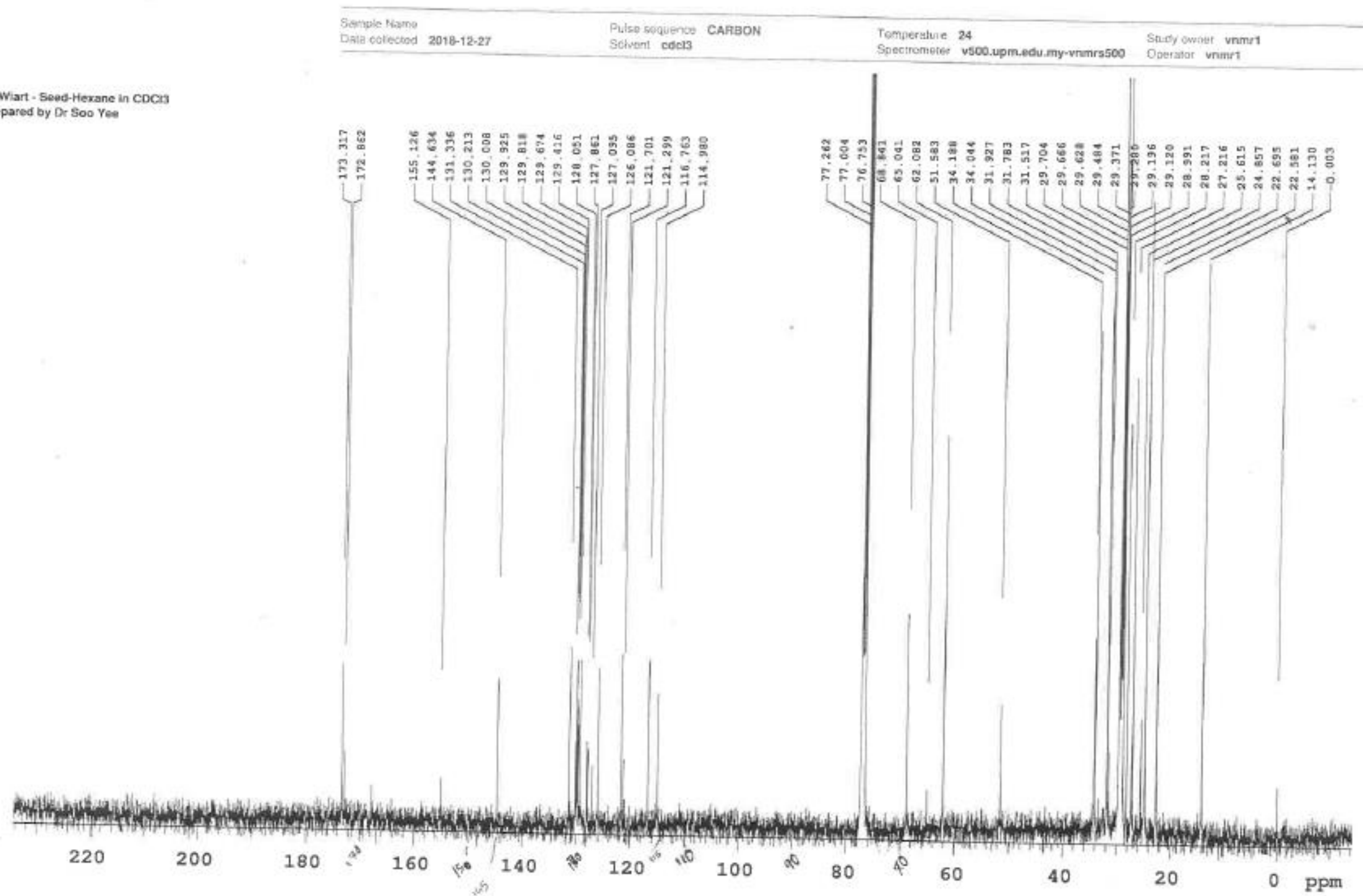

Figure 33: CNMR of compound 1 from hexane extract of seed of *B. malaccensis*

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6.3.5 Result and discussion of paper disc assay on pure fractions against Gram-negative bacteria:

A total of 8 pure fractions from the plant *B. malaccensis* were assayed through the paper disc. In Chapter IV and V, chloroform extracted flesh samples and hexane extracted seeds sample showed activity against *P. aeruginosa.* So before experimenting on MIC and MBC samples had been tested on agar plates to see the zone of inhibitions of these fractions. Here, *P. putida* and *E. coli* along with *P. aeruginosa* have been used. Bacteria cells selected here were due to the availability at that time. Fractions F4, F5, F6, F7, F8, Fx exhibited 8.00 mm of zones of inhibitions whereas, tetracycline exhibited 30.00 mm of inhibition. Surprisingly, fractions showed quite nice zones of inhibitions against *E. coli* which they did not show any during previously conducted paper disc assay with crude extracts.

Fractions F2, F6 and S1 displayed around (7.00-8.00) mm of the zone of inhibitions against *P. aeruginosa*. Samples were examined with *P. putida* as well because although this organism causes health-related infections, due to the prevalence, relatively lower virulence, and higher antimicrobial resistance of *P. putida* compared to other *Pseudomonas* organisms, there is a lack of clinical data on *P. putida* infections, especially *P. aeruginosa*. Recently, the emergence of multidrug-resistant (MDR) and carbapenem-resistant *P. putida* has become a major concern (Kim, 2012). Though it did not show any significant inhibition with *P. putida,* but slight inhibition observed with fractions F2 and F6 with 10^8 the concentration of *P. putida* and 200.00 µg per disc (Figure 36). So assuming that, if *P. putida* can be diluted a little more and decrease the concentration to 10^6 and increase the concentration to 500.00 µg per disc-like with other fractions and bacteria then it might will work better and show better inhibition (Kim, 2012).

A total of 11 pure compounds from the plant *B. malaccensis.* were screened by the disc diffusion method from 100.00 to 500.00 µg/disc. Amongst the 11 compounds from *B. malaccensis,* only 3 fractions (F2, F6, and F8) from flesh-chloroform and 1 fraction (S1) from seed-hexane showed activity against

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P. aeruginosa (Figure 34). On the other hand, 6 fractions from flesh-chloroform displayed activity against *E. coli* at the highest amount (500.00 µg) tested (Figure 35). None of the fractions showed any activity against Gram-positive bacteria. As the positive standards tetracycline and gentamycine have been used here in this experiment (Llamas, Ramos, & Rodríguez-Herva, 2015). Based on some antibacterial activity categories using the disc procedure the most active fractions were F2, F6 and F8. They exhibited moderate activity (between 7.00 mm-8.00 mm). agar disc diffusion and the agar dilution method were used here for the determination of inhibition zones and the Minimum Inhibitory Concentration (MICs) respectively. However, at 200.00 µg per disc, it did not exhibit any activity against any bacterial strains.

The results of the *in vitro* antibacterial activity of the chloroform extracted pure fractions are determined by the diameters of inhibition zones are presented in (Table 19). The results showed that inhibition zones varied in diameters from 6.50 mm to 8.00 mm. Tetracycline and Gentamycin used as a standard antibiotic at the concentration of 30.00 µg and 10.00 µg/disc exhibited higher diameters of inhibition than the fractions alone. The solvent dilution of the extract or the negative standard did not show any antibacterial activity. Results also revealed that the diameters of inhibition zones increased with the concentration of extract. In the range of 100.00 – 200.00 µg/disc, the fractions did not exhibit any antibacterial activity against all the bacteria strains used.

Among the 11 isolates, F2, F4, F5, F6, F7, F8 and FX sensitive against *E. coli* at 500.00 µg/disc and the zone of inhibition varied from 6.50 mm to 8.00 mm (Figure 35). According to the inhibition, diameters can be classified into four categories. No activity considered when (diameter of inhibition < 7.00 mm); weak activity (diameter of inhibition between 7.00 mm and 10.00 mm), moderate activity (diameter of inhibition between 11.00 mm and 16.00 mm) and good or higher activity (diameter of inhibition between > 16.00 mm). Based on this information, these fractions are moderately sensitive towards *E. coli.*

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F2, F6, F8 and S1 were sensitive against *P. aeruginosa* at 500.00 µg/disc and above and the zone of inhibition varied from 7.00 to 8.00 mm (Table 19). So, based on the previously stated information these fractions are moderately sensitive towards *P. aeruginosa.* Based on the MIC/ MBC value criteria our fractions did not manifest any activity lower than 500.00 µg/mL and it did show activity at 500.00 µg/ mL and above that which is clearly emphasizing the moderate activity.

Amongst these 11 fractions, only 3 of them (F2, F6 and F8) were active against both *P. aeruginosa* and *E. coli.* F4 and Fx were sensitive only against *E. coli* and S1 only against *P. aeruginosa.* So, based on the above data that, werneria chromene was isolated and detected by NMR from sample 1 of seed hexane so it has weak activity against *P. aeruginosa* and P. *putida* was susceptible to F1, F2, F5 and F6 at 200 µg/disc concentration. Dihroxy acidissiminol was isolated and detected through NMR and it is reasonable to infer that dihydroxy acidissiminol is weakly active against *P. putida.*

Table 19: Paper disc asssay (500 µg/disc) against 3 Gram-negative bacteria cells with the pure fractions of flesh-chloroform and seed-hexane from *B. malaccensis*

Genus- Species Part/Solvent	*E. coli* ATCC (8739))	*P. aeruginosa* ATCC (10145)	*P. putida* (ATCC 49128)
B. malaccensis Flesh- Chloroform	Zone of inhibition (mm)	Zone of inhibition (mm)	Zone of inhibition (mm)
F1	-	-	+/-
F2	6.5	7	6.5
F4	8	-	-
F5	8	-	6.5
F6	8	7/8	6.5
F7	8	-	-
F8	8	7	-
Fx	8	-	-
B. malaccensis Seed- Hexane			
S1	-	7	-
Standard Antibiotics			
Tetracycline	30		
Gentamycin		35	35

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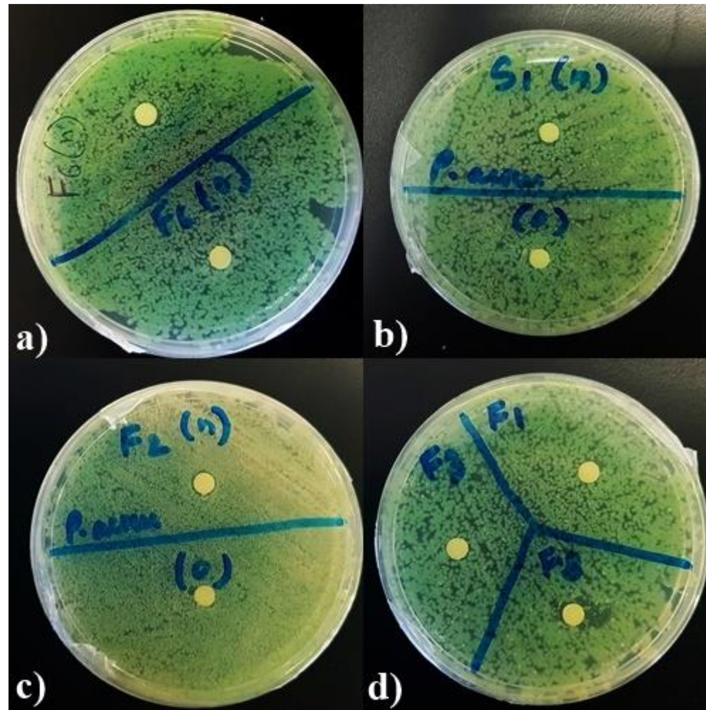

Figure 34: Paper disc assay (500 µg/disc) on fractions of *B. malaccensis* against *P. aeruginosa* a) F6 from flesh-chloroform b) S1 from seed-hexane, c) F2 from flesh-chloroform, d) F8 from flesh-chloroform

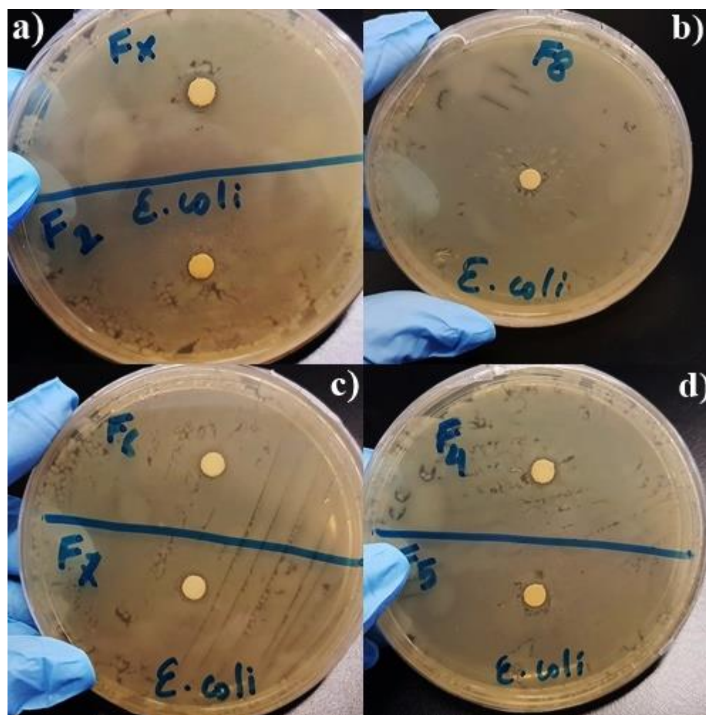

Figure 35: Paper disc assay (500 µg/disc) on fractions of *E. coli* against *P. aeruginosa* a) Fx from flesh-chloroform b) F8 from flesh-chloroform, c) F6, F7 from flesh-chloroform, d) F4, F5 from flesh-chloroform

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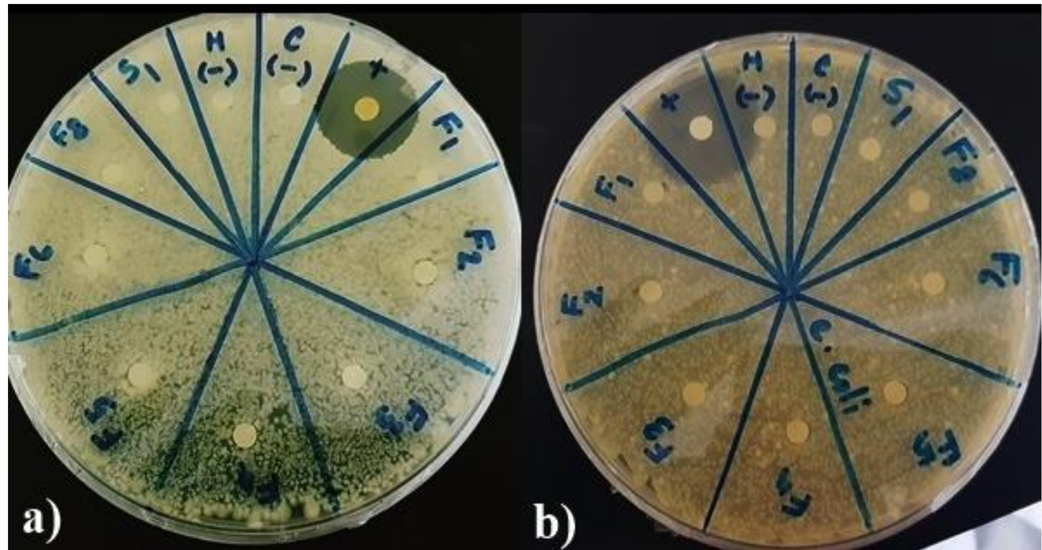

Figure 36: Paper disc assay (200 µg/disc) on pure fractions of *B. malaccensis*, a) against *P. putida,* b) against *E. coli*

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6.3.6 Result and discussion of MIC and MBC on pure fractions

MIC and MBC were after conducting the disc diffusion assay with the pure fractions and observed that all these fractions showed moderate activity against specifically with Gram-negative bacteria. F2, F4, F5 and S2 got MIC value 1.0>1.0 against *E. coli*. Fractions F2, F6, F8 and S1 showed MIC value 1.0>1.0 against *P. aeruginosa.* Fractions F1, F2, F3, F4, F6, F8, Fx, S1 and S2 delivered MIC value of 1.0>1.0. Although these delivered weak activity towards Gram-negative bacteria but in contemplation to aim, an antibacterial compound with bactericidal activity and active particularly against Gram-negative bacteria was the objective of this entire project.

In consonance with the goal, flesh, and seed from *B. malaccensis* provide antibacterial activity only against *P. aeruginosa*. Here *E. coli* and *P. putida* have been used as well. Antimicrobial susceptibility of *P. putida* is higher if compared with other *Pseudomonas* species, especially *P. aeruginosa*. But in this study, both *Pseudomonas* species showed quite similar activity along with *E. coli.*

Crude compounds with MIC superior to 1000.00 µg/mL are inactive and proposed interesting activity for MIC of 0.10 mg/mL and below than that active as having MIC values below 8 mg/mL. Kuete (2013) and according to (García-Arribas, de Cos, & Barandiarán, 2015) use stricter endpoint criteria, in which compounds with MIC values less than 0.10 mg/mL are active. In addition, Kuete (2013) classifies as weakly active extracts with MIC above 0.63 mg/mL. Following them, no extracts had interesting activities with MIC below 0.10 mg/mL (Table 20). So, based on that our fractions did not manifest any activity below than 500.00 µg/mL and which in both ways clearly emphasizes the weak to moderate antibacterial activity. Compounds or extracts are categorized into 2 classes: bacteriostatic (MBC/MIC ratio value more than 4) and bactericidal (MBC/MIC ratio value less than or equal to 4). So according to that, these 7 fractions emphasize the bactericidal value.

To summarise according to (Table 20), sample F1 from flesh chloroform of *B. malaccensis* exhibited weak bactericidal activity against *P. putida* and sample S1 from seed hexane of *B. malaccensis* exhibited weak bactericidal activity against *P. aeruginosa* and *P. putida*. Our study provides evidence for the first time that werneria chromene and dihydroxy acidissinminol have selective mild bacticidal activities against P. aeruginosa. In addition, we are the first to report the activity of F1 against *P. putida.* This types of selective natural products against *Pseudomonus* are so far not known in the existing available literature.

Table 20: Minimum inhibitory concentrations (MIC) (mg/mL)/mimimum bactericidal concentrations (MBC) (mg/mL) against 3-Gram-negative bacteria cells on pure fractions of flesh-chloroform and seed-hexane from *B. malaccensis*

Genus- Species Part/Solvent	*E. coli* ATCC (8739)	*P. aeruginosa* ATCC (10145)	P. putida ATCC 49128)
B. malaccensis Flesh- Chloroform	MIC/MBC	MIC/MBC	MIC/MBC
F1	-	-	1.0>1.0
F2	1.0>1.0	1.0>1.0	1.0>1.0
F3			1.0>1.0
F4	1.0>1.0		1.0>1.0
F5	1.0>1.0	-	
F6	-	1.0>1.0	1.0>1.0
F8		1.0>1.0	1.0>1.0
Fx			1.0>1.0
B. malaccensis Seed- Hexane			
S1		1.0>1.0	1.0>1.0
S2	1.0>1.0		1.0>1.0
Standard Antibiotics			
Tetracycline	0.002>0.01	0.012>0.3	0.012>0.2

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6.4 Conclusions of all the experiments for isolation and characterisation

6.4.1 Conclusion of TLC

10 antibacterial active samples have been selected for isolation (TLC and PTLC). After running TLC on selected samples of *B. malaccensis*, chloroform extracted flesh sample (FC) and hexane extracted seed (SH) sample were narrowed down for PTLC as the spots observed were quite defined and clear. 9 spots from FC and 2 spots from SH have been isolated.

6.4.2 Conclusion of PTLC

PTLC was conducted on FC and SH of *B. malaccensis* as the spots observed were clearly defined in TLC. 9 spots were isolated from FC and 2 spots from SH of *B. malaccensis.* The yields of these isolated fractions were preserved in the autoclaved glass vials and the mass was measured. These isolated fractions were then tested against Gram-negative bacteria to see the efficacy as an antibacterial compound.

6.4.3 Conclusion of GC/MS

GC/MS (gas chromatography/mass spectrometry) has been implemented here in this project only on hexane extract of seeds (SH) of *B. malaccensis*. GC/MS was not conducted on chloroform extract of flesh (FC) of *B. malaccensis* due to inadequate sample yield. Based on the above chromatographic pattern analysis (6.3.3) of seed hexane of *B. malaccensis* two major peaks were observed. Similarly, 2 spots were detected at TLC as well. At retention time 51.685, one peak was detected with the mass of 409 and at retention time 54.161 another peak with mass spectrometry 392 was detected.

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6.4.4 Conclusion of NMR

NMR was conducted to get the chemical structure of the fractions of the selected samples from *B. malaccensis* which were active against Gram-negative bacteria. From the previous antibacterial assays, FC and SH have antibacterial activity against Gram-negative bacteria and has bactericidal activity according to the goal of this project. The compounds were isolated through TLC, PTLC, GC-MS and performed NMR afterwards to get the chemical structures of the isolated fractions.

The NMR spectrum was able to detect 1 compound (dihydroxy acidissiminol) from F1 of FC and 1 (werneria chromene) from S1 of SH from *B. malaccensis*. Dihydroxy acidissiminol is a new tyramine alkaloid derivative and indicated the presence of benzamide and alkaloid. Benzamide has been found to play a crucial role in conferring antibacterial activity. The position of the methoxyl carbon resonance in werneria chromene indicated an ester. The NMR data clearly matched those of werneria chromene which was first isolated from *Werneria stuebelii* and confers the presence of chromanone (Hong, Minter, Franzblau & Reinecke, 2008), (Table 17, 18 and Figure 29, 30). The biological evaluation revealed that the chromanone group also proved to be a determining factor for antibacterial activities (Feng et al., 2014).

6.4.5 Conclusion of paper disc assay on pure fractions

Paper disc assay on pure isolated fractions exhibited zones of inhibitions here ranged from 6.5-8 mm. Though it has low to moderate activity, but an interesting fact is that it is only active against Gram-negative bacteria cells. *P. putida* was susceptible to F1, F2, F5 and F6 at 200 µg/disc concentration. So, dihroxy acidissiminol from sample 1 of FC is weakly active against *P. putida* and werneria chromene from sample 1 of SH has weak activity against *P. aeruginosa.*

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6.4.6 Conclusion of MIC and MBC on pure compounds

According to the MIC and MBC result, fractions have mild bactericidal activity with the MIC/MBC value of 1.0>1.0. and displayed bactericidal activity. According to the value in (Table 20), sample F1 from chloroform extracts from the flesh (FC) of *B. malaccensis* exhibited antibacterial activity against *P. putida* and infer bactericidal activity. Sample S1 from hexane extracts from seed (SH) of *B. malaccensis* exhibited weak bactericidal activity against *P. aeruginosa* and *P. putida*.

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CHAPTER VII: CONCLUSION AND FUTURE CONSIDERATIONS

7.1 Conclusion

As one of the world's most developed and diverse habitats, Malaysia's tropical rainforest represents a vast untapped natural resource biodiversity. This has culminated in the current research on the *in vitro* antibacterial activities on 5 different rare medicinal plants. We have successively extracted seed with hexane (lipophilic-nonpolar) and flesh with chloroform (amphiphilic-mid polar) obtain lipophilic (nonpolar) substance which might be able to penetrate the outer membrane of Gram-negative bacteria. Research started with antibacterial studies on 5 different rare medicinal plant parts where the selected species were never studied before. Dried plant parts were extracted with hexane, chloroform, and methanol to obtain lipophilic (non-polar), amphiphilic (mid-polar), and hydrophilic (polar) extracts respectively (Harbone 2013). The average yield values ranged from (0.27 – 109.23) % indicating good extraction process (Parthasarathy et al 2012). Calculated averages yield for hexane, chloroform**,** and methanol extracts were 3.89 %, 7.20 %, and 13.44 %, respectively. Methanol extract had the highest average extraction yields. Considering the yield of extracts left with, continued further quantitative experiments such as minimum inhibitory concentrations (MIC), minimum bactericidal concentrations (MBC) on selected plant samples right after conducting the qualitative antibacterial assays.

Antibacterial assays started with qualitative Kirby-Bauer paper disc diffusion assay against Gram-positive and negative bacterial strains. Their zone of inhibition of the crude extracts was also observed and out of 48 extracts, 4 extracts demonstrated selective activity against *P. aeruginosa*. the hexane extract of seed (SH) and chloroform extract of flesh (FC) specifically inhibited the growth of *P. aeruginosa* with the inhibition zone diameters of 11.30±1.50 mm and 23.30±2.00 mm, respectively. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) were performed to actuate the resistance and susceptibility of bacteria quantitatively right after paper disc

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diffusion and in the microdilution broth assay, both these extracts selectively inhibited the growth of *P. aeruginosa* with the MIC value of 1000 ug/ml.

To focus on the objective of this project chloroform extracts from the flesh (FC) and hexane extracts from seed (SH) from *B. malaccensis* exhibited moderate bactericidal activity explicitly against Gram-negative bacteria. Based on the antibacterial activity attained and adequate yield for further experiments, isolation and characterisation were perceived afterwards on selected samples in the next chapter (Chapter VI). TLC executed on 10 samples from 2 different plants (*B. malaccensis* and *D. hasseltii*). Thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC) was conducted to isolate the compounds inside the active samples. 9 clear spots from FC and 2 clear spots from SH were isolated by chromatographic isolation. PTLC terminated right after TLC was done.

Gas chromatography mass spectrometry was conducted afterwards to get an idea about the molecular mass and compounds names. Based on the GC-MS chromatographic pattern analysis of SH of *B. malaccensis* two major peaks were observed. Similarly, 2 spots were detected at TLC as well which ensures the presence of 2 major compounds. At retention time 51.685, one peak was detected with the mass of 409 and at retention time 54.161 another peak with mass spectrometry 392 was detected.

Isolated pure fractions of SH and FC were sent to University Malaysia Pahang for nuclear magnetic resonance spectroscopy (NMR). NMR was conducted to get the chemical structure of the fractions SH and FC. The NMR spectrum was able to detect 1 compound (dihydroxy acidissiminol) from FC (Fraction 1) and 1 (werneria chromene) from SH (Fraction 1) of *B. malaccensis*. Dihydroxy acidissiminol is a new tyramine alkaloid which has been isolated before from the plant *Limonia acidissima* in the family Rutaceae once in 1994 (Ghosh et al., 1994) and indicated the presence of benzamide. Benzamide has been found to play a crucial role in conferring antibacterial activity. Werneria chromene which was first isolated from *Werneria stuebelii* and confirms the presence of chromanone and ester. The biological evaluation revealed that the

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chromanone group also proved to be a determining factor for antibacterial activities

Paper disc assay was done on isolated pure fractions again which exhibited zones of inhibitions ranging from (6.5-8) mm. Disc diffusion assay was done with the isolated pure fractions (F1, F2, F4, F5, F6, F7, F8, Fx and S2) of *B. malaccensis* at (500 µg/disc). *P. putida* was susceptible to F1, F2, F5 and F6 at 200 µg/disc concentration. It is reasonable to infer that dihydroxy acidissiminol is weakly active against *P. putida* and werneria chromene from sample 1 of seed hexane has weak activity against *P. aeruginosa.*

Lastly MIC and MBC result on isolated pure fractions, they have provided mild to moderate bactericidal activity with the MIC/MBC value of 1.0>1.0. According to the value in (Table 20), sample F1 from FC exhibited positive activity against *P. putida* and infer bactericidal activity. Sample S1 from SH exhibited weak bactericidal activity against *P. aeruginosa* and *P. putida*.

To conclude, this research provides that FC and SH exhibited weak to moderate antibacterial activity specifically against *Pseudomonus* genus. And managed to get 2 active antibacterial compounds through isolation nuclear magnetic resonance spectroscopy (NMR). Dihydroxy acidissiminol from F1 from FC has susceptibility against *P. putida* and infer weak bactericidal activity and werneria chromene from fraction S1 of SH has also weak bactericidal activity against *P. aeruginosa* and *P. putida.* Though the compounds exert weak bactericidal activity but it is assumed that synergy might display much more potentiality against Gram-negative bacteria.

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7.2 Future considerations

An aim was triggered before starting this project and that was to isolate and characterise pure antibacterial compounds from 5 rare medicinal plants with specifically against Gram-negative bacteria. We successively have isolated 2 compounds (dihydroxy acidissiminol of F1 from FC and werneria chromene of S1 from SH) from the fractions of flesh and seeds of *B. malaccensis* which exhibited bactericidal properties.

For future research, this plant could become the antibiotic potentiators which can help in enhancing the current antibiotics. The novelty of this project is, no scientific data or research has ever been published on these species so far by known. Virtually no records of any studies, more specifically antibacterial studies performed by researchers and hence not much information have been revealed on their phytochemicals content. In the near future, further isolation is going to take place to get all the chemical compounds which are responsible for antibacterial activity. These isolated pure compounds might have potential synergistic activity and in future synergism assays. Even though the compounds exert weak to moderate activity but with natural products which exert mild activity can be very potential with synergism. Here only flesh and seed were selected and tested for isolation because of their activity against Gram-negative bacteria but further isolation can be done with other plant parts.

Eventually, all chemical compounds are needed to be isolated and synergistic assays are needful on *B. malaccensis* and other selected plants to detect the active molecules with antibacterial activity. As a conclusion, exploration of the *in vitro* pharmacological properties of *B. malaccensis* revealed that acidisssiminol and werneria chromene may represent a new generation of potential drug candidates for the treatment of bacterial infections. Therefore, further *in vivo* studies and clinical trials are needed to ascertain the efficacy, safety, and mechanisms of action of acidisssiminol and werneria chromene before application in the pharmaceutical industry as natural therapeutic agents.

`

References:

Abdallah, E. (2016). Preliminary phytochemical and antibacterial screening of methanolic leaf extract of Citrus aurantifolia. *Pharm Biotechnol Curr Res, 1*(2), 1-5.

Adzitey, F., Ekli, R., & Abu, A. (2019). Prevalence and antibiotic susceptibility of Staphylococcus aureus isolated from raw and grilled beef in Nyankpala community in the Northern Region of Ghana. *Cogent Food & Agriculture*(just-accepted), 1671115.

Afouda, P., Durand, G. A., Lagier, J. C., Labas, N., Cadoret, F., Armstrong, N., Dubourg, G. (2019). Noncontiguous finished genome sequence and description of Intestinimonas massiliensis sp. nov strain GD 2T, the second Intestinimonas species cultured from the human gut. *MicrobiologyOpen, 8*(1), e00621.

Aljamali, N. M. (2015). Choosing a Solvent System In Tests of (TLC).

Aminov, R. I. (2010). A brief history of the antibiotic era: lessons learned and challenges for the future. *Frontiers in microbiology, 1*, 134.

Nuzillard, J.-M. (2018). Reconstruction of HMBC Correlation Networks: A Novel NMR-Based Contribution to Metabolite Mixture Analysis. *Journal of chemical information and modeling, 58*(2), 262-270.

Balouiri, M., Sadiki, M., & Ibnsouda, S. K. (2016). Methods for *in vitro* evaluating antimicrobial activity: A review. *Journal of pharmaceutical analysis, 6*(2), 71-79.

Beaucourt, S., & Vignuzzi, M. (2014). Ribavirin: a drug active against many viruses with multiple effects on virus replication and propagation.

Molecular basis of ribavirin resistance. *Current opinion in virology, 8*, 1015.

`

Biradar, S. R., & Rachetti, B. D. (2013). Extraction of some secondary metabolites & thin layer chromatography from different parts of Centella asiatica L.(URB). *American Journal of Life Sciences, 1*(6), 243-247. Bishop, R. E. (2008). Structural biology of membrane-intrinsic β-barrel enzymes: Sentinels of the bacterial outer membrane. *Biochimica et*

Brauner, A., Fridman, O., Gefen, O., & Balaban, N. Q. (2016). Distinguishing between resistance, tolerance and persistence to antibiotic treatment. *Nature Reviews Microbiology, 14*(5), 320.

Brown, A. E., & Catteruccia, F. (2006). Toward silencing the burden of malaria: progress and prospects for RNAi-based approaches. *Biotechniques, 40*(S4), S38-S44.

Brown, E. D., & Wright, G. D. (2016). Antibacterial drug discovery in the resistance era. *Nature, 529*(7586), 336.

Chandra, H., Bishnoi, P., Yadav, A., Patni, B., Mishra, A. P., & Nautiyal, A. R. (2017). Antimicrobial resistance and the alternative resources with special emphasis on plant-based antimicrobials—a review. *Plants, 6*(2), 16.

Choi, J., Yoo, J., Lee, M., Kim, E.-G., Lee, J. S., Lee, S., Lee, J. C. (2014). A rapid antimicrobial susceptibility test based on single-cell morphological analysis. *Science translational medicine, 6*(267), 267ra174-267ra174.

Choudhary, N., & Sekhon, B. S. (2011). An overview of advances in the standardization of herbal drugs. *Journal of Pharmaceutical Education and Research, 2*(2), 55.

Compean, K., & Ynalvez, R. (2014). Antimicrobial activity of plant secondary metabolites: A review. *Research Journal of Medicinal Plants,*

Clinical and Laboratory Standards Institute (CLSI) and European Committee on Antimicrobial Susceptibility Testing (EUCAST) broth microdilution reference

`

methods and with the Sensititre YeastOne and Etest techniques for *in vitro* detection of antifungal resistance in yeast isolates. *Journal of clinical microbiology, 48*(5), 1782-1786.

Dayrit, F. M., & de Dios, A. C. (2017). 1H and 13C NMR for the Profiling of Natural Product Extracts: Theory and Applications. *Spectroscopic Analyses-Developments and Applications*.

Delfianti, M., Yuniastuti, E., & Cahyani, V. (2019). *Propagation and growth of persimmon (Diospyros kaki L.) in Indonesia.* Paper presented at the IOP Conference Series: Earth and Environmental Science.

Delgado-Valverde, M., Sojo-Dorado, J., Pascual, Á., & Rodríguez-Baño, J. (2013). Clinical management of infections caused by multidrug-resistant Enterobacteriaceae.

Arakawa, H., Takasaki, M., Tajima, N., Fukamachi, H., & Igarashi, T. (2014). Antibacterial Activities of Persimmon Extracts Relate with Their Hydrogen Peroxide Concentration. *Biological And Pharmaceutical Bulletin*, *37*(7), 1119-1123. doi: 10.1248/bpb.b13-00952

Diep, T. T., Van Kiem, P., Dong, N. T., Tung, N. H., Bang, B. T., Van Minh, C., & Braca, A. (2007). Pinoresinol and 3, 4', 5, 7-tetrahydroxy-3'-methoxyflavanone from the fruits of Silybum marianum (L.) Gaertn. *Vietnam Journal of Chemistry, 45*(2), 219.

Duedu, K. O., Offei, G., Codjoe, F. S., & Donkor, E. S. (2017). Multidrug resistant enteric bacterial pathogens in a psychiatric hospital in Ghana: implications for control of nosocomial infections. *International journal of microbiology, 2017*.

El-Arabi, T. F., Griffiths, M. W., She, Y.-M., Villegas, A., Lingohr, E. J., & Kropinski, A. M. (2013). Genome sequence and analysis of a broad-host range

`

lytic bacteriophage that infects the Bacillus cereus group. *Virology journal, 10*(1), 48.

ELANGOMATHAVAN, S. T. R. (2013). Bio-prospecting of Cleistanthus collinus and its antibacterial activity. *Asian J Pharm Clin Res, 6*(4), 106-209.

Fabricant, D. S., & Farnsworth, N. R. (2001). The value of plants used in traditional medicine for drug discovery. *Environmental health perspectives, 109*(suppl 1), 69-75.

African medicinal plants. *Journal of ethnopharmacology, 60*(1), 79-84. Fischetti, V. A. (2019). Surface proteins on Gram-positive bacteria. *Microbiology spectrum, 7*(4).

Jenni, S. (2017). Ex vivo drug response profiling detects recurrent sensitivity patterns in drug-resistant acute lymphoblastic leukemia. *Blood, 129*(11), e26-e37.

Gavamukulya, Y., Abou-Elella, F., Wamunyokoli, F., & AEl-Shemy, H. (2014). Phytochemical screening, anti-oxidant activity and *in vitro* anticancer potential of ethanolic and water leaves extracts of Annona muricata (Graviola). *Asian Pacific journal of tropical medicine, 7*, S355-S363.

Gonzalez-Bello, C. (2017). Antibiotic adjuvants–A strategy to unlock bacterial resistance to antibiotics. *Bioorganic & medicinal chemistry letters, 27*(18), 4221-4228.

Govindachari, T., Sathe, S., Viswanathan, N., Pai, B., & Srinivasan, M. (1969). Chemical constituents of Cleistanthus collinus (Roxb.). *Tetrahedron, 25*(14), 2815-2821.

Gupta, P. D., & Birdi, T. J. (2017). Development of botanicals to combat antibiotic resistance. *Journal of Ayurveda and integrative medicine, 8*(4), 266-275.

`

Gupta, R. C. (2015). *Handbook of toxicology of chemical warfare agents*: Academic Press.

Hossain, M. A., Al-Hdhrami, S. S., Weli, A. M., Al-Riyami, Q., & Al-Sabahi, J. N. (2014). Isolation, fractionation and identification of chemical constituents from the leaves crude extracts of Mentha piperita L grown in Sultanate of Oman. *Asian Pacific journal of tropical biomedicine, 4*, S368-S372.

Analysis of antibiotic resistance genes in multidrug-resistant Acinetobacter sp. isolates from military and civilian patients treated at the Walter Reed Army Medical Center. *Antimicrobial agents and chemotherapy, 50*(12), 4114-4123.

Joppa, L., Visconti, P., Jenkins, C., & Pimm, S. (2013). Achieving the convention on biological diversity's goals for plant conservation. *science, 341*(6150), 1100-1103.

Kalwij, J. M. (2012). Review of 'The Plant List, a working list of all plant species'. *Journal of Vegetation Science, 23*(5), 998-1002.

Karunamoorthi, K., Jegajeevanram, K., Vijayalakshmi, J., & Mengistie, E. (2013). Traditional medicinal plants: a source of phytotherapeutic modality in resource-constrained health care settings. *Journal of Evidence-Based Complementary & Alternative Medicine, 18*(1), 67- 74.

Kebede, A., & Hibore, M. E. (2012). *Prevalence and Antibiotic Susceptibility Patterns of Selected Bacterial Uropathogens Among Patients With Urinary Tract Infection Cases in Wonji Hospital, Ethiopia'.* Haramaya University,

Khazaei, S. (2016). Determination of chemical composition and potential anticancer activities of alliatroviolaceum extract on selected cancer cell lines

Kim, S. E., Park, S.-H., Park, H. B., Park, K.-H., Kim, S.-H., Jung, S.-I., Kang,

`

S. J. (2012). Nosocomial Pseudomonas putida bacteremia: high rates of carbapenem resistance and mortality. *Chonnam medical journal, 48*(2), 91-95.

Lewis, K. (2013). Platforms for antibiotic discovery. *Nature reviews Drug discovery, 12*(5), 371.

Llor, C., & Bjerrum, L. (2014). Antimicrobial resistance: risk associated with antibiotic overuse and initiatives to reduce the problem. *Therapeutic advances in drug safety, 5*(6), 229-241.

Lone, A., Anany, H., Hakeem, M., Aguis, L., Avdjian, A.-C., Bouget, M., . . . G riffiths, M. W. (2016). Development of prototypes of bioactive packaging materials based on immobilized bacteriophages for control of growth of bacterial pathogens in foods. *International journal of food microbiology, 217*, 49-58.

Antimicrobial Agents and Chemotherapy, 15(3), 368-371. Milow, P., Malek, S. B., Edo, J., & Ong, H.-C. (2014). Malaysian species of plants with edible fruits or seeds and their valuation. *International journal of fruit science, 14*(1), 1-27.

Mohr, K. I. (2016). History of antibiotics research. In *How to Overcome the Antibiotic Crisis* (pp. 237-272): Springer.

Munita, J. M., & Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiology spectrum, 4*(2).

Galdiero, S., Falanga, A., Cantisani, M., Tarallo, R., Elena Della Pepa, M., D'Oriano, V., & Galdiero, M. (2012). Microbe-Host Interactions: Structure and Role of Gram-negative Bacterial Porins. *Current Protein And Peptide Science*, *13*(8), 843-854. doi: 10.2174/138920312804871120

Antimicrobial activity of crude leaf extracts from medicinal plants against Enterococcus faecalis. (2016). *International Journal Of Traditional And Complementary Medicine*. doi: 10.28933/rachuonyo-ijtcm-2016

`

Nordin, M. S., & Zakaria, N. H. (2016). Plants used for medicines by the indigenous Malay of Pahang, Malaysia. *Medicinal Plants- International Journal of Phytomedicines and Related Industries, 8*(2), 137-145.

Norhayati, I., Getha, K., Haffiz, J. M., Ilham, A. M., Sahira, H. L., Syarifah, M. S., & Syamil, A. M. (2013). *In vitro* antitrypanosomal activity of Malaysian plants. *Journal of Tropical Forest Science*, 52-59.

G. P., & Spring, D. R. (2013). Combating multidrug-resistant bacteria: current strategies for the discovery of novel antibacterials. *Angewandte Chemie International Edition, 52*(41), 10706-10733. Organization, W. H. (2014a). *Antimicrobial resistance global report on surveillance: 2014 summary*. Retrieved from Organization, W. H. (2014b). *Antimicrobial resistance: global report on surveillance*:

World Health Organization. Organization, W. H. (2018). Global antimicrobial resistance surveillance system (GLASS) report: early implementation 2017-2018.

Pan, S.-Y., Zhou, S.-F., Gao, S.-H., Yu, Z.-L., Zhang, S.-F., Tang, M.-K., .Fong, W.-F. (2013). New perspectives on how to discover drugs from herbal medicines: CAM's outstanding contribution to modern therapeutics. *Evidence-Based Complementary and Alternative Medicine, 2013*.

Renwick, M. J., Brogan, D. M., & Mossialos, E. (2016). A systematic review and critical assessment of incentive strategies for discovery and development of novel antibiotics. *The Journal of antibiotics, 69*(2), 73.

Renwick, M. J., Simpkin, V., & Mossialos, E. (2016). Targeting innovation in antibiotic drug discovery and development. *Health Policy Series of the European Observatory on Health Systems and Policies*.

`

Roemer, T., & Krysan, D. (2014). Antifungal drug development: challenges, unmet clinical needs, and new approaches. Cold Spring Harb Perspect Med 4: a019703. In.

Roemer, T., & Krysan, D. J. (2014). Antifungal drug development: challenges, unmet clinical needs, and new approaches. *Cold Spring Harbor perspectives in medicine, 4*(5), a019703.

Rolain, J.-M., Abat, C., Jimeno, M.-T., Fournier, P.-E., & Raoult, D. (2016). Do we need new antibiotics? *Clinical Microbiology and Infection, 22*(5), 408-415.

Kamin, I., & Kong, R. C. (2017). Plant Checklist of the Bukit Nanas Forest Reserve, Kuala Lumpur, Malaysia. *One Ecosystem, 2*, e13708.

Salleh, W. M. N. H. W., & Ahmad, F. (2017). Phytochemistry and biological activities of the genus Knema (Myristicaceae). *Pharm Sci, 23*(4), 249-255.

Savoia, D. (2012). Plant-derived antimicrobial compounds: alternatives to antibiotics. *Future microbiology, 7*(8), 979-990.

Seukep, J., Sandjo, L., Ngadjui, B., & Kuete, V. (2016). Antibacterial activities of the methanol extracts and compounds from Uapaca togoensis against Gram-negativemulti-drug resistant phenotypes.

South African Journal of Botany, 103, 1-5. Shakoor, A., Hussain, A., Abbas, T., Muhammad, S., & Kashif, M. (2014).

Singariya, P., Mourya, K. K., & Kumar, P. (2012). Antimicrobial Activity of the Crude Extracts of Withania somnifera and Cenchrus setigerus In-vitro. *Pharmacognosy journal, 4*(27), 60-65.

Sonam, M., Singh, R. P., & Pooja, S. (2017). Phytochemical screening and TLC profiling of various extracts of Reinwardtia indica. *Inter J of Pharma and Phytochem Res, 9*(4), 523-527.

`

Spellberg, B. (2012). New antibiotic development: barriers and opportunities in 2012. *APUA Clinical Newsletter, 30*(1), 8-10. Subramani, R., Narayanasamy, M., & Feussner, K.-D. (2017). Plant-derived antimicrobials to fight against multi-drug-resistant human pathogens. *3 Biotech, 7*(3), 172.

Suratman, M. N. (2012). Tree species diversity and forest stand structure of Pahang National Park, Malaysia. *Biodiversity enrichment in a diverse world*, 473-492.

Surawicz, C. M., Brandt, L. J., Binion, D. G., Ananthakrishnan, A. N., Curry, S. R., Gilligan, P. H., . . . Zuckerbraun, B. S. (2013).

Tan, K. K. (2015). *In vitro pharmacological properties of an indigenous medicinal plant, Artabotrys crassifolius Hook. f. & Thomson (Family: Annonaceae Juss.).* University of Nottingham,

Teka, A., Rondevaldova, J., Asfaw, Z., Demissew, S., Van Damme, P., Kokoska, L., & Vanhove, W. (2015). *In vitro* antimicrobial activity of plants used in traditional medicine in Gurage and Silti Zones, south central Ethiopia. *BMC complementary and alternative medicine, 15*(1), 286.

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L., & Fowler, V. G.(2015). Staphylococcus aureus infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clinicalmicrobiology reviews, 28*(3), 603-661.

Typas, A., Banzhaf, M., Gross, C. A., & Vollmer, W. (2012). From the regulation of peptidoglycan synthesis to bacterial growth and morphology. *Nature Reviews Microbiology, 10*(2), 123.

Vashist, H., & Jindal, A. (2012). Antimicrobial activities of medicinal plants– Review. *Int J Res Pharm Biomed Sci, 3*(1), 222-230. Vázquez, L. H., Palazon,

J., & Navarro-Ocaña, A. (2012). The Pentacyclic Triterpenes□,□-amyrins: A Review of Sources and Biological Activities. In *Phytochemicals-A global perspective of their role in nutrition and health*: IntechOpen.

Ventola, C. L. (2015a). The antibiotic resistance crisis: part 1: causes and threats. *Pharmacy and therapeutics, 40*(4), 277.

Ventola, C. L. (2015b). The antibiotic resistance crisis: part 2: management strategies and new agents. *Pharmacy and Therapeutics, 40*(5), 344.

Wang, H., Wilksch, J. J., Lithgow, T., Strugnell, R. A., & Gee, M. L. (2013). Nanomechanics measurements of live bacteria reveal a mechanism for bacterial cell protection: the polysaccharide capsule in Klebsiella is a responsive polymer hydrogel that adapts to osmotic stress. *Soft Matter, 9*(31), 7560-7567.

Wijesekera, R. (2017). Plant-derived medicines and their role in global health. In *The Medicinal Plant Industry* (pp. 1-18): Routledge.

Willoughby, P. H., Jansma, M. J., & Hoye, T. R. (2014). A guide to small-molecule structure assignment through computation of (1 H and 13 C) NMR chemical shifts. *Nature protocols, 9*(3), 643.

Wohlleben, W., Mast, Y., Stegmann, E., & Ziemert, N. (2016). Antibiotic drug discovery. *Microbial biotechnology, 9*(5), 541-548.

Manandhar, S., Luitel, S., & Dahal, R. (2019). *In Vitro* Antimicrobial Activity of Some Medicinal Plants against Human Pathogenic Bacteria. *Journal Of Tropical Medicine*, *2019*, 1-5. doi: 10.1155/2019/1895340

Wongsrichanalai, C. (2013). Artemisinin resistance or artemisinin-based combination therapy resistance? *The Lancet Infectious Diseases, 13*(2), 114-115.

`

Wongsrichanalai, C., & Sibley, C. (2013). Fighting drug-resistant Plasmodium falciparum: the challenge of artemisinin resistance. *Clinical microbiology and infection, 19*(10), 908-916.

Yao, L., Fiona, S., Ten-Jin, K., & Christophe, W. (2014). Antileishmanial assay and antimicrobial activity on crude extracts of Melodinus eugeniifolus barks and leaves from Malaysia. *Pharmacol Pharm, 5*, 747- 754.

Yuan, H., Ma, Q., Ye, L., & Piao, G. (2016). The traditional medicine and modern medicine from natural products. *Molecules, 21*(5), 559. Zeedan, G. S., Abdalhamed, A. M., Abdeen, E., Ottai, M. E., & Abdel-Shafy, S. (2014). Evaluation of antibacterial effect of some Sinai medicinal plant extracts on bacteria isolated from bovine mastitis. *Veterinary World, 7*(11).

Zhang, G., Meredith, T. C., & Kahne, D. (2013). On the essentiality of lipopolysaccharide to Gram-negative bacteria. *Current opinion in microbiology, 16*(6), 779-785.

Medhi, B., Goyal, P., Semwal, A., & Prakash, A. (2019). Emerging antimicrobial resistance and newer tools to address the resistance. *Indian Journal Of Pharmacology*, *51*(5), 291. doi: 10.4103/ijp.ijp_607_19

Loreto Sanhueza, Ricardo Melo, Ruth Montero, Kevin Maisey, Leonora Mendoza, Marcela Wilkens. Synergistic interactions between phenolic compounds identified in grape pomace extract with antibiotics of different classes against *S. aureus* and *E. coli,* (2017)

World Conservation Monitoring Centre. 1998. *Burkillanthus sp.* In: IUCN 2013. IUCN Red List of Threatened Species. Version 2013.1. Downloaded on 05 August 2013.

Wan Mohd Nuzul Hakimi Wan Salleh & Farediah Ahmad. Phytochemistry and biological activities of the genus *Knema (Myristicaceae*). (2017). Pharmaceutical Sciences. 23: 249-255

`

Mohammad Amzad Hossain, Seham Salim Al-Hdhrami, Afaf Mohammed Weli,Qasim Al-Riyami, andJamal Nasser Al-Sabahi. Isolation, fractionation and identification of chemical constituents from the leaves crude extracts of *Mentha piperita* grown in Sultanate of Oman. (2014) Asian Pac J Trop Biomed. 4: 368-372.

M.P. Francino. Antibiotics and the human gut microbiome: dysbioses and accumulation of resistances. (2015) 6: 1543Ma, H.Q., (2006). The formulation, manufacture and evaluation of capsules containing freeze-dried aqueous extracts of Leonotis leonorus or Mentha longifolia. MPharm thesis, University of the Western Cape.

A.G. Patil, S.P. Koli, D.A. Patil & A.V. Phatak. Evaluation of extraction techniques with various solvents to determine extraction efficiency of selected medicinal plants. (2012) International journal of pharmaceutical sciences and research 33: 2607-2612

Wilkins, T. D. *et al.* (2018) 'Review of Methods for Antibiotic Susceptibility Testing of Anaerobic Bacteria', 7(4).

Ishnava, K. B. *et al.* (2012) 'Antibacterial and phytochemical studies on Calotropis gigantia (L.) R. Br. latex against selected cariogenic bacteria', *Saudi Journal of Biological Sciences*. King Saud University, 19(1), pp. 87–91. doi: 10.1016/j.sjbs.2011.10.002.

Zeedan, G. S. G. *et al.* (2014) 'Evaluation of antibacterial effect of some Sinai medicinal plant extracts on bacteria isolated from bovine mastitis', 7, pp. 991–998. doi: 10.14202/vetworld.2014.991-998.

Tchinda, C. F. *et al.* (2017) 'Antibacterial activities of the methanol extracts of Albizia adianthifolia, Alchornea laxiflora, Laportea ovalifolia and three other Cameroonian plants against multi-drug resistant Gram-negative bacteria', *Saudi*

`

Journal of Biological Sciences. King Saud University, 24(4), pp. 950–955. doi: 10.1016/j.sjbs.2016.01.033.

Teka, A. *et al.* (2015) ‘*In vitro* antimicrobial activity of plants used in traditional medicine in Gurage and Silti Zones, south central Ethiopia’, *BMC Complementary and Alternative Medicine*. BMC Complementary and Alternative Medicine, 15(1), pp. 1–7. doi: 10.1186/s12906-015-0822-1.

Valle, D. L. *et al.* (2015) ‘Antibacterial activities of ethanol extracts of Philippine medicinal plants against multidrug-resistant bacteria’, *Asian Pacific Journal of Tropical Biomedicine*. Elsevier (Singapore) Pte Ltd, 5(7), pp. 532–540. doi: 10.1016/j.apjtb.2015.04.005.

González-Bello, C. (2017) ‘Antibiotic adjuvants – A strategy to unlock bacterial resistance to antibiotics’, *Bioorganic and Medicinal Chemistry Letters*, 27(18), pp. 4221–4228. doi: 10.1016/j.bucL.2017.08.027.

de Kok, R. P. J. (2017) ‘Two new records of Litsea (Lauraceae) from Singapore and the lectotypification of twenty-two names from several Lauraceae genera’, *Gardens’ Bulletin Singapore*, 69(2), pp. 167–177. doi: 10.26492/gbs69(2).2017-02.

Bereksi, M., Hassaïne, H., Bekhechi, C., & Abdelouahid, D. (2018). Evaluation of Antibacterial Activity of some Medicinal Plants Extracts Commonly Used in Algerian Traditional Medicine against some Pathogenic Bacteria. *Pharmacognosy Journal*, *10*(3), 507-512. doi: 10.5530/pj.2018.3.83

Elisha, I., Botha, F., McGaw, L., & Eloff, J. (2017). The antibacterial activity of extracts of nine plant species with good activity against Escherichia coli against five other bacteria and cytotoxicity of extracts. *BMC Complementary And Alternative Medicine*, *17*(1). doi: 10.1186/s12906-017-1645-z

`

Aslam, B., Wang, W., Arshad, M., Khurshid, M., Muzammil, S., & Rasool, M. et al. (2018). Antibiotic resistance: a rundown of a global crisis. *Infection And Drug Resistance*, *Volume 11*, 1645-1658. doi: 10.2147/idr.s173867

Podolsky, S. (2018).

The evolving response to antibiotic resistance (1945–2018). *Palgrave Communications*, *4*(1). doi: 10.1057/s41599-018-0181-xGuerriero, G., Berni, R., Muñoz-Sanchez, J., Apone, F., Abdel-Salam, E., & Qahtan, A. et al. (2018).

Production of Plant Secondary Metabolites: Examples, Tips and Suggestions for Biotechnologists. *Genes*, *9*(6), 309. doi: 10.3390/genes9060309

List of plants in the family Rutaceae. (2020). Retrieved 16 May 2020, from https://www.britannica.com/topic/list-of-plants-in-the-family-Rutaceae-2038278

Apocynaceae: Characters, Distribution and Types. (2020). Retrieved 16 May 2020, from https://www.biologydiscussion.com/angiosperm/dicotyledons/Apocynaceae-characters-distribution-and-types/48288

Antimicrobial resistance remains a threat. (2017). *C&EN Global Enterprise*, *95*(9), 17-17. doi: 10.1021/cen-09509-govcon003

Brown, E., & Wright, G. (2016). Antibacterial drug discovery in the resistance era. *Nature*, *529*(7586), 336-343. doi: 10.1038/nature17042

Lewis, K. (2015). Challenges of Antibiotic Discovery. *Microbe Magazine*, *10*(9), 363-369. doi: 10.1128/microbe.10.363.1

Bulgarelli, D., Schlaeppi, K., Spaepen, S., van Themaat, E., & Schulze-Lefert, P. (2013). Structure and Functions of the Bacterial Microbiota of Plants. *Annual Review of Plant Biology*, *64*(1), 807-838. doi: 10.1146/annurev-arplant-050312-120106

`

Clifton, L., Skoda, M., Le Brun, A., Ciesielski, F., Kuzmenko, I., Holt, S., & Lakey, J. (2014). Effect of Divalent Cation Removal on the Structure of Gram-negative Bacterial Outer Membrane Models. *Langmuir*, *31*(1), 404-412. doi: 10.1021/la504407v

Ilangovan, A., Connery, S., & Waksman, G. (2015). Structural biology of the Gram-negative bacterial conjugation systems. *Trends In Microbiology*, *23*(5), 301-310. doi: 10.1016/j.tim.2015.02.012

Opal, S. (2016). Non-antibiotic treatments for bacterial diseases in an era of progressive antibiotic resistance. *Critical Care*, *20*(1). doi: 10.1186/s13054-016-1549-1

Edwin MD, D. (2018). Antibacterial activity of Psidium guajava L. against certain multidrug resistant Gram-negative and Gram-positive bacteria. *Journal of Medical Science and Clinical Research*, *6*(9). doi: 10.18535/jmscr/v6i9.29

Kayış, Ş., Er, A., & İpek, Z. (2017). Effects of albinism on Pseudomonas putida infection on convict cichlid (Cichlasoma nigrofasciatum). *Journal of Anatolian Environmental and Animal Sciences*, *2*(3), 51-52. doi: 10.35229/jaes.351597

Thomas, B., Okamoto, K., Bankowski, M., & Seto, T. (2013). Lethal Case of Pseudomonas putida Bacteremia Due to Soft Tissue Infection. *Infectious Diseases In Clinical Practice*, *21*(3), e13-e15. doi: 10.1097/ipc.0b013e318276956b

Aminu, M., Usman-Sani, H., & Usman, M. (2014). Characterization and determination of antibiotic susceptibility pattern of bacteria isolated from some fomites in a teaching hospital in northern Nigeria. *African Journal Of Microbiology Research*, *8*(8), 814-818. doi: 10.5897/ajmr2013.6512

Brem, J., van Berkel, S., Aik, W., Rydzik, A., Avison, M., & Pettinati, I. et al. (2014). Rhodanine hydrolysis leads to potent thioenolate mediated metallo-β-

lactamase inhibition. *Nature Chemistry*, *6*(12), 1084-1090. doi: 10.1038/nchem.2110

Stanton, T. (2013). A call for antibiotic alternatives research. *Trends In Microbiology*, *21*(3), 111-113. doi: 10.1016/j.tim.2012.11.002

Xu, R., Peng, Y., Wang, M., Fan, L., & Li, X. (2014). Effects of broad-spectrum antibiotics on the metabolism and pharmacokinetics of ginsenoside Rb1: A study on rats′ gut microflora influenced by lincomycin. *Journal of Ethnopharmacology*, *158*, 338-344. doi: 10.1016/j.jep.2014.10.054

Prevel, R., Berdaï, D., & Boyer, A. (2019). Antibiotics for Ceftriaxone-Resistant Gram-negative Bacterial Bloodstream Infections. *JAMA*, *321*(6), 613. doi: 10.1001/jama.2018.19349

Rahman, T., Yarnall, B., & Doyle, D. (2017). Efflux drug transporters at the forefront of antimicrobial resistance. *European Biophysics Journal*, *46*(7), 647-653. doi: 10.1007/s00249-017-1238-2

Bilal, M., Rasheed, T., Iqbal, H., Hu, H., Wang, W., & Zhang, X. (2017). Macromolecular agents with antimicrobial potentialities: A drive to combat antimicrobial resistance. *International Journal of Biological Macromolecules*, *103*, 554-574. doi: 10.1016/j.ijbiomac.2017.05.071

Weng, X., & Huang, Y. (2014). Relationship structure-antioxidant activity of hindered phenolic compounds. *Grasas Y Aceites*, *65*(4), e051. doi: 10.3989/gya.0225141

Piasecka, A., Jedrzejczak-Rey, N., & Bednarek, P. (2015). Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. *New Phytologist*, *206*(3), 948-964. doi: 10.1111/nph.13325

Vinale, F., Sivasithamparam, K., Ghisalberti, E., Woo, S., Nigro, M., & Marra, R. et al. (2014). Trichoderma Secondary Metabolites Active on Plants and

`

Fungal Pathogens. *The Open Mycology Journal*, *8*(1), 127-139. doi: 10.2174/1874437001408010127

Jonville, M., Dive, G., Angenot, L., Bero, J., Tits, M., Ollivier, E., & Frédérich, M. (2013). Dimeric bisindole alkaloids from the stem bark of Strychnos nux-vomica L. *Phytochemistry*, *87*, 157-163. doi: 10.1016/j.phytochem.2012.11.002

Schwartz, T., Nylinder, S., Ramadugu, C., Antonelli, A., & Pfeil, B. (2016). The Origin of Oranges: A Multi-locus Phylogeny of Rutaceae Subfamily Aurantioideae. *Systematic Botany*, *40*(4), 1053-1062. doi: 10.1600/036364415x690067

ASTIANI, D. (2016). Tropical peatland tree-species diversity altered by forest degradation. *Biodiversitas, Journal Of Biological Diversity*, *17*(1), 102-109. doi: 10.13057/biodiv/d170115

Turner, I., Low, Y., Rodda, M., Wong, K., & Middleton, D. (2018). The plant taxa of H.N. Ridley, 5. The Gentianales. *Gardens' Bulletin Singapore*, *70*(2), 307-395. doi: 10.26492/gbs70(2).2018-08

Gerbig, D., Aubihl, H., & Engohang-Ndong, J. (2013). A New Twist to the Kirby-Bauer Antibiotic Susceptibility Test Activity—Increasing Antibiotic Sensitivity of Pseudomonas fluorescens through Thermal Stress. *Journal Of Microbiology & Biology Education*, *14*(2), 269-270. doi: 10.1128/jmbe.v14i2.617

The MIC and MBC of Silver Nanoparticles against Enterococcus faecalis - A Facultative Anaerobe. (2015). *Journal Of Nanomedicine & Nanotechnology*, *06*(03). doi: 10.4172/2157-7439.1000285

Tan, K., Bradshaw, T., Chu, J., Khoo, T., & Wiart, C. (2014). *In vitro* anticancer effect of Artabotrys crassifolius Hook F & Thompson against human carcinoma

cell lines *Journal Of Drug Delivery And Therapeutics*, *4*(1). doi: 10.22270/jddt.v4i1.728

Kłodzińska, S., Priemel, P., Rades, T., & Nielsen, H. (2018). Combining diagnostic methods for antimicrobial susceptibility testing – A comparative approach. *Journal Of Microbiological Methods*, *144*, 177-185. doi: 10.1016/j.mimet.2017.11.010

Jahanshahi-Anbuhi, S., Kannan, B., Pennings, K., Monsur Ali, M., Leung, V., & Giang, K. et al. (2017). Automating multi-step paper-based assays using integrated layering of reagents. *Lab On A Chip*, *17*(5), 943-950. doi: 10.1039/c6lc01485b

Nahi, A., Othman, R., & Omar, D. (2016). Effects of Sb16 bacterial strain and herbicides on endophytic bacterial populations and growth of aerobic rice. *Plant, Soil And Environment*, *62*(No. 10), 453-459. doi: 10.17221/289/2016-pse

Nagy, E., Boyanova, L., & Justesen, U. (2018). How to isolate, identify and determine antimicrobial susceptibility of anaerobic bacteria in routine laboratories. *Clinical Microbiology and Infection*, *24*(11), 1139-1148. doi: 10.1016/j.cmi.2018.02.008

Feng, L., Maddox, M., Alam, M., Tsutsumi, L., Narula, G., & Bruhn, D. et al. (2014). Synthesis, Structure–Activity Relationship Studies, and Antibacterial Evaluation of 4-Chromanones and Chalcones, as Well as Olympicin A and Derivatives. *Journal Of Medicinal Chemistry*, *57*(20), 8398-8420. doi: 10.1021/jm500853v
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4207537/

Hong, Q., Minter, D., Franzblau, S., & Reinecke, M. (2008). Anti-tuberculosis Compounds from two Bolivian Medicinal Plants, Senecio Mathewsii and Usnea Florida. *Natural Product Communications*, *3*(9), 1934578X0800300. doi: 10.1177/1934578x0800300901

`

Sugiki, T., Furuita, K., Fujiwara, T., & Kojima, C. (2018). Current NMR Techniques for Structure-Based Drug Discovery. *Molecules*, *23*(1), 148. doi: 10.3390/molecules23010148

Bingol, K. (2018). Recent Advances in Targeted and Untargeted Metabolomics by NMR and MS/NMR Methods. *High-Throughput*, *7*(2), 9. doi: 10.3390/ht7020009

De Castro, F., Benedetti, M., Del Coco, L., & Fanizzi, F. (2019). NMR-Based Metabolomics in Metal-Based Drug Research. *Molecules*, *24*(12), 2240. doi: 10.3390/molecules24122240

Emsley, J., & Lindon, J. (2014). *NMR Spectroscopy Using Liquid Crystal Solvents*. Kent: Elsevier Science.

Ghosh, P., Ghosh, M., Thakur, S., (Datta), J., Akihisa, T., Tamura, T., & Kimura, Y. (1994). Dihydroxy acidissiminol and acidissiminol epoxide, two tyramine derivatives from Limonia acidissima. *Phytochemistry*, *37*(3), 757-760. doi: 10.1016/s0031-9422(00)90353-4

Halabalaki, M., Vougogiannopoulou, K., Mikros, E., & Skaltsounis, A. (2014). Recent advances and new strategies in the NMR-based identification of natural products. *Current Opinion In Biotechnology*, *25*, 1-7. doi: 10.1016/j.copbio.2013.08.005

Wicha-Komsta, K., & Komsta, Ł. (2017). Unconventional TLC systems in lipophilicity determination: A review. *Journal Of Liquid Chromatography & Related Technologies*, *40*(5-6), 219-225. doi: 10.1080/10826076.2017.1298023

P, P., R, U., PS, M., & RM, G. (2020). Derivatives of Coumarins, Cinnamic acid and Tyramine from Leaves of Limonia crenulata. Retrieved 18 May 2020, from https://scienztech.org/ijrpp/article/view/835

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Appendix

A.a Antibacterial activity assessment with disc diffusion assay by *B. malaccensis, K. retusa, L. spathacea, D. hasseltii and K. maingayi*

Table A.1: Zones of inhibitions against 4 bacteria cells by *K. retusa* (c100.00 µg/disc), *B. malaccensis* (c100.00 µg/disc)

	Zone of Inhibition (mm)			
Genus species Part/Solvent	*S. aureus*	*B. subtilis*	*E. coli*	*P. aeruginosa*
K. retusa (c100.00 µg/disc)				
LH	-	-	-	-
LC	6.66 ± 0.30	-	-	-
LM	8.50 ± 0.50	-	-	-
BH	8.00 ± 0.90	7.20 ± 0.30	-	-
BC	7.80 ± 0.30	7.00 ± 0.50	-	-
BM	-	-	-	-
WH	7.80 ± 0.30	7.80 ± 1.00	-	-
WC	8.20 ± 0.30	8.00 ± 1.70	7.30 ± 0.60	-
WM	-	-	-	-
B. malaccensis (c100.00 µg/disc)				
LH	6.50 ± 0.50	6.50	-	6.60 ± 0.30
LC	7.00 ± 0.50	6.50	-	
LM	7.60 ± 0.30	7.20 ± 0.80	6.60 ± 0.30	-
BH	10.60 ± 2.80	8.30 ± 1.50	-	-
BC	7.00	6.50	-	-
BM	6.80 ± 0.30	6.60 ± 0.30	-	-
WH	8.00	9.50 ± 1.80	-	6.80 ± 0.30
WC	8.50 ± 0.90	8.30 ± 2.30	-	-
WM	-	-	-	7.00
FH	-	-	-	-
FC	-	-	-	11.30±1.50
FM	-	-	-	20.30±1.20
PH	-	-	-	-
PC	-	-	-	-
PM	-	-	-	-
SH	-	-	-	23.30±2.00
SC	-	-	-	-
SM	-	-	-	-
Standard Antibiotics				
Chloramphenicol	27.00 ± 3.00	27.00 ± 3.00	28.00 ± 5.00	30.00 ± 2.00
Ampicillin (10 ug)	10.00 ± 2.00	20.00 ± 4.00	16.50 ± 3.00	-
Cephalexin (30 ug)	29.29 ± 0.20	30.00 ± 0.50	22.40 ± 0.60	10.00 ± 0.50

The testes were performed in triplicates.

Plant Parts:
L: Leave, B: Bark, W: Wood, F: Flesh, S: Seed, P: Peel
Solvents: H: Hexane, C: Chloroform, M: Methanol
S. aureus: *Staphylococcus aureus* (ATCC 11632), *B. subtilis: Bacillus subtilis* (ATCC 6633), *E. coli*: *Escherichia coli* (ATCC 8739), *P. aeruginosa*: *Pseudomonas aeruginosa* (ATCC 10145) for *K. retusa* and *B. malaccensis*

6 mm diameter paper disc and Values are given as mean of triplicate, c: concentration (100 µg/disc) or (1 mg/disc)

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Table A.2: Zones of inhibitions (c 200.00 µg/disc) against 3 bacteria cells by *D. hasseltii, L. spathacea* and (c1.00mg/disc) against 3 bacteria cells by *D. hasseltii*

	Zone of Inhibition (mm)			
Genus species Part/Solvent	*S. aureus*	*B. subtilis*	*P. aeruginosa*	*E. coli*
D. hasseltii (c200.00ug/disc)				
LH	-	-	-	-
LC	7.00 ± 1.00	7.50 ± 1.50	7.50	-
LM	6.80 ± 0.10	-	10.10	-
BH	-	-	-	-
BC	7.80 ± 0.20	-	-	-
BM	-	-	-	-
F[1]H	7.00 ± 0.80	-	-	-
F[1]C	8.10 ± 0.10	-	-	-
F[1]M	9.10	-	-	-
Standard Antibiotics				
Chloramphenicol	22.00 ± 2.00	26.00 ± 2.20	32.00 ± 2.00	-
Ampicillin	9.00 ± 3.00	20.00 ± 3.50	-	-
D. hasseltii (c1.00mg/disc)				
LH	7.00	-	-	-
LC	6.80 ± 0.10	-	7.50	-
LM	-	-	-	-
BH	7.00	-	-	-
BC	8.10 ± 0.10	-	-	-
BM	-	-	-	-
Standard Antibiotics				
Amoxicillin	11.33 ± 2.52	27.00 ± 2.30	28.00 ± 2.50	-
Ceftazidine	19.00 ± 3.00	18.00 ± 3.00	-	-
L.spathacea (c200.00ug/disc)				
LH	-	-	-	-
LC	7.10 ± 0.20	-	-	-
LM	-	-	-	-
BH	7.00 ± 0.07	-	-	-
BC	6.90 ± 0.07	-	-	-
BM	-	-	-	-
Standard Antibiotics				
Chloramphenicol	22.00 ± 2.00	27.00 ± 2.30	28.00 ± 2.50	-
Ampicillin	9.00 ± 3.00	18.00 ± 3.00	-	-
K. maingayi (c200.00ug/disc)				
LH	-	-	-	-
LC	-	-	-	-
LM	-	-	-	-
BH	-	-	-	-
BC	-	-	-	-
BM	-	-	-	-
Standard Antibiotics				
Chloramphenicol	18.00 ± 2.00	17.00 ± 2.30	18.00 ± 2.50	-
Ampicillin	10.00 ± 3.50	15.00 ± 3.00	-	-

The testes were performed in triplicates.

Plant Parts:
L: Leave, B: Bark, F[1]: Fruit, F: Flesh, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol

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S. aureus: *Staphylococcus aureus* (ATCC 29213), *B. subtilis: Bacillus subtilis* (ATCC11778), *E. coli*: Escherichia coli (ATCC 25218), *P. aeruginosa*: *Pseudomonas aeruginosa* (ATCC10145); for *D. hasseltii, L. spathacea* and *K. maingayi*

6 mm diameter paper disc and Values are given as mean of triplicate, c: concentration (100,200 µg/disc) or (1 mg/disc)

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A.b Result of MIC and MBC of *B. malaccensis, K. retusa, L. spathacea, D. hasseltii* and *K. mangayi*

Table A.3: Minimum inhibitory concentrations (MIC) (mg/mL) and minimum bactericidal concentrations (MBC) (mg/mL) against 4 bacteria cells on *B. malaccensis* and *K. retusa*

Genus- Species Part/Solvent	*S. aureus* (ATCC 11632)	*B. subtilis* ATCC (6633)	*E. coli* ATCC (8739))	*P. aeruginosa* ATCC (10145)
B. malaccensis	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
LH	1.00>1.00	-	-	-
LC	0.25/>1.00	0.25/>1.00	-	-
LM	0.50/>1.00	1.00>1.00	-	-
BH	-	-	-	-
BC	-	-	-	1.00>1.00
BM	-	-	-	-
WH	-	-	-	-
WC	-	-	-	-
WM	-	-	-	-
SH	-	-	-	1.00>1.00
SC	-	-	-	-
SM	-	-	-	-
PH	-	-	-	-
PC	-	-	-	-
PM	-	-	-	-
FH	-	-	-	-
FC	-	-	-	1.00>1.00
FM	-	-	-	1.00>1.00
K. retusa				
LH	-	-	-	-
LC	-	-	-	-
LM	0.25/>1.00	0.25/>1.00	-	-
BH	-	-	-	-
BC	-	-	-	-
BM	-	-	-	-
WH	0.25/>1.00	0.25/>1.00	-	-
WC	-	-	-	-

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WM	-	-	-	-
Standard Antibiotics				
Chloramphenicol	0.03>0.03	0.02>0.03	-	-
Tetracycline	-	-	0.002>0.02	0.012>0.30

The testes were performed in triplicates.
Parts:
L: Leave, B: Bark, W: Wood, F: Flesh, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol
S. aureus (ATCC 11632), *B. subtilis* (ATCC6633), *E. coli* (ATCC 8739), *P. aeruginosa* (ATCC10145) for K. *retusa* and *B. malaccensis*

Table A.4: Minimum inhibitory concentrations (MIC) (mg/mL) and minimum bactericidal concentrations (MBC) (mg/mL) against 6 bacteria cells on *L. spathacea*, *K. maingayi* and *D. hasselti*

Genus- Species Part/Solvent	MRSA	S. aureus (ATCC 29213)	*E. coli* (ATCC 25218)	*P. aeruginosa* (clinical strain)	*A. baumanii* (clinical strain)	*K. pneumonia* (clinical strain)
L. spathacea	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC	MIC/MBC
LH	-	-	-	-	-	-
LC	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00	0.50/>1.00
LM	1.00>1.00	-	-	-	1.00>1.00	1.00>1.00
BH	-	-	0.50/>1.00	-	-	-
BC	-	-	0.50/>1.00	-	-	-
BM	-	-	-	-	1.00>1.00	-
$F^{1}H$	-	-	-	-	-	-
$F^{1}C$	-	-	-	-	-	-
$F^{1}M$	-	-	-	-	-	-
K. maingayi						
LH	-	-	-	-	-	-
LC	0.50/>1.00	-	-	-	-	-
LM	-	1.00>1.00	1.00>1.00	-	-	-
BH	-	-	-	-	-	-
BC	-	-	0.50/>1.00	-	-	-
BM	1.00>1.00	0.50/>1.00	1.00>1.00	1.00>1.00	1.00>1.00	-
D. hasselti						
LH	-	-	0.50/>1.00	-	-	-
LC	0.50/>1.00	-	-	0.50/>1.00	-	0.50/>1.00
LM	-	-	0.50/>1.00	-	-	-
BH	-	-	-	-	-	-
BC	-	-	0.50/>1.00	0.50/>1.00	-	0.50/>1.00
BM	1.00>1.00	0.50/>1.00	0.25/>1.00	1.00>1.00	-	0.50/>1.00
Standard Antibiotics						
Cefotaxime	3.10/>50.00	-	1.50/3.10	12.50/>50.00	-	-
Kanamycin	-	3.10/>50.00	-	-	-	6.20/6.20
Vancomycin	-	-	-	-	1.50/>50.00	-

The testes were performed in triplicates.
Parts:
L: Leave, B: Bark, W: Wood, S: Seed
Solvents: H: Hexane, C: Chloroform, M: Methanol

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MRSA, *S. aureus* (ATCC 29213), *B. subtilis* (ATCC11778), *E. coli* (ATCC 25218), *P. aeruginosa* (ATCC10145); *Acinetobacter baumannii* (clinical strain), *K. pneumonia* (clinical strain) for *D. hasseltii* and *L. spatha*