SCREENING FOR ANTIBACTERIAL ACTIVITY OF LOCAL PLANTS IN MALAYSIA: *Lobelia chinensis* AND *Ipomoea batatas*

By

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ABSTRACT

SCREENING FOR ANTIBACTERIAL ACTIVITY OF LOCAL PLANTS IN MALAYSIA: *Lobelia chinensis* AND *Ipomoea batatas*

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The increase in the number of microorganisms that are resistant to antimicrobial agents is seriously threatened the control of infectious diseases. Research on new antimicrobial substances by natural sources such as plant extracts must therefore be continued. *Lobelia chinensis* or Chinese lobelia has been used as a diuretic, an antidote, and as carcinostatic agents for stomach cancer in Chinese folk medicine. *Ipomoea batatas* or sweet potato serves as a food source that rich in Vitamin A and carbohydrate as well as acts as an antioxidant and antimutagenicity agent. In this study, the antimicrobial activity of plant extracts from the leaves of *L. chinensis* and *I. batatas* were investigated against four Gram-negative and two Gram-positive bacteria by using colorimetric broth microdilution method. Sequential extraction method was applied to obtain the plant extracts by using solvents of increasing polarity namely hexane, chloroform, ethyl acetate, ethanol, methanol and distilled water accordingly. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of each extract were determined. All extracts except aqueous extract of *L. chinensis* exhibited

inhibitory activity against *K. pneumoniae* and *B. cereus* with the MIC ranged from 0.42-2.50 mg/mL and 0.13-1.67 mg/mL respectively. Four extracts excluded methanol and aqueous extract showed inhibition to *S. aureus* with the MIC ranged from 0.31-2.50 mg/mL. None of the extracts of *L. chinensis* showed antibacterial activity against *A. baumannii*, *E. coli*, and *P. aeruginosa.* For *I. batatas*, only the hexane extract was able to inhibit *K. pneumoniae* and *S. aureus* with the MIC value of 0.31 mg/mL and 0.63 mg/mL respectively. All the extracts except hexane and chloroform extracts inhibited *P. aeruginosa* with MIC values ranged from 1.25-1.67 mg/mL. *Bacillus cereus* was only susceptible to the hexane, ethanol and methanol extracts of *I. batatas* (MIC: 0.16- 2.08 mg/mL). No extracts showed activity against *A. baumannii* and *E. coli*. In term of bactericidal activity, all the extracts of *L. chinensis* except aqueous extract were active against *K. pneumoniae* (MBC= 0.42-2.50 mg/mL). The hexane, chloroform and ethyl acetate extracts were able to kill *B. cereus* and *S. aureus* with MBC ranges of 0.13-0.63 mg/mL and 0.63-2.50 mg/mL respectively. For *I. batatas*, hexane extract was able to kill *K. pneumoniae*, *B. cereus* and *S. aureus* (MBC=0.31 mg/mL, 0.16 mg/mL, 1.25 mg/mL respectively). *Bacillus cereus* (Bacterial Susceptibility Index: 66.67%) was the most susceptible microorganism among six bacterial strains while *A. baumannii*, and *E. coli* were the least susceptible microorganisms which showed 0% of BSI value.

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Lastly, a million thanks to my beloved family members for giving me unconditionally love supports and concerns along the three months period time in completing my final year research project.

DECLARATION

I hereby declare that the project report is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at Universiti Tunku Abdul Rahman or other institutions.

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LEONG WEI MIN

APPROVAL SHEET

This project report entitled **"SCREENING FOR ANTIBACTERIAL ACTIVITY OF LOCAL PLANTS IN MALAYSIA: *Lobelia chinensis* AND *Ipomoea batatas*"** was prepared by LEONG WEI MIN and submitted as partial fulfillment of the requirements for the degree of Bachelor of Science (Hons) in Biomedical Science at Universiti Tunku Abdul Rahman.

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I hereby give permission to my supervisors to write and prepare manuscript of these research findings for publishing in any form, if I did not prepare it within six (6) months time from this date provided that my name is included as one of the author for this article. Arrangement of the name depends on my supervisors.

Yours truly,

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(LEONG WEI MIN)

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LIST OF ABBREVIATIONS

A/E	Attachment and effacing
AIDS	Acquired immunodeficiency syndrome
ATCC	American Type Culture Collection
BSI	Bacterial susceptibility index
CF	Cystic Fibrosis
CFU	Colony forming unit
CNS	Central nervous system
CQA	Caffeoylquinic acid
CSF	Cerebrospinal fluid
CVD	Cardiovascular disease
CV-I	Crystal violet-iodine
DNA	Deoxyribonucleic acid
DPPH	1, 1-diphenyl-2-picrylhydrazyl
EAEC	Enteroaggregative *Escherichia coli*
EHEC	Enterohemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ESBL	Extended spectrum β-lactamase
ETEC	Enterotoxigenic *E. coli*
HBL	Hemolysin BL
HIV	Human Immunodeficiency Virus

HUS	Hemolytic uremic syndrome
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
INT	Iodonitrotetrazolium Chloride
LDL	Low-density lipoprotein
LPS	Lipopolysaccharides
MAC	*Membrane attack complex*
MBC	Minimum bactericidal concentration
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton Broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
NaCl	Sodium chloride
NAG	N-acetylglucosamine
NAM	N-acetylmuramic acid
NHE	Non-hemolytic enterotoxin
Stx	Shiga toxin
TSS	Toxic shock syndrome
TSST-1	Toxic shock syndrome toxin-1
UPEC	Uropathic *Escherichia coli*
UTI	Urinary tract infections
UV	Ultra-violet

CHAPTER 1

INTRODUCTION

The discovery and development of antibiotics during the 1940s provided potent antimicrobial agents with high specificity for clinical use, which substantially decreased morbidity and mortality from bacterial infections (Onawunmi 2006). Soon after its discovery however, antibiotics became less effective when used, as micro-organisms have the genetic ability to transmit and acquire resistance to therapeutic drugs (Nascimento et al., 2000). Moreover, the widespread use and misuse of antibiotics for human consumption and animal feed has led to the development of resistance in a variety of pathogenic bacteria, which then resulting in the emergence of multiple resistant strains. For example, methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most difficult bacteria to eradicate in hospitals and to treat in patients (Onawunmi 2006). Therefore, proper actions should be taken to solve this problem such as controlling the use of antibiotic or continue studies to develop new drugs, either synthetic or from natural products.

Nature has been a source of medicinal agents for thousands of years and an impressive number of modern drugs have been isolated from natural sources, many based on their use in traditional medicine. Herbal medicine is the oldest form of healthcare known to mankind. In India, among 7500 species of medicinal plants flora, there are 4635 species can be used commercially on a fairly large

scale (Preethi et al., 2010). Natural products play an important role in modern drug discovery and development. It was particularly evident in the areas of cancer and infectious diseases, where over 60% and 75% of these drugs, respectively, were shown to be of natural origin (Newman et al., 2003).

Herbal medicine or phytomedicine refers to the use of any plant's seeds, berries, roots, leaves, bark, or flowers for medicinal purposes (Barrett et al., 1999). Phytochemical compounds are found in plants that are not required for normal functioning of the body, but have a beneficial effect on health or play an active role in amelioration of diseases (Preethi et al., 2010). Some phytochemicals produced by plants have antimicrobial activity allowing these plants to be studied and used for the development of new antimicrobial drugs (Nascimento et al., 2000). Many plants have been used because of their antimicrobial traits, which are due to active compounds synthesized in the secondary metabolism of the plant such as phenolic compounds, tannin, peptides, alkaloids, essential oils, phenols, coumarines and flavonols which confer antimicrobial properties to them. (Nascimento et al., 2000; Ramesh et al., 2008). These compounds have potentially significant therapeutic application against human pathogens, including bacteria, fungi or virus (Ramesh et al., 2008).

Numerous researchers have been involved in the investigation of antimicrobial properties possessed by plants world widely, especially in Latin America. As evidence, a research was done in Argentina, to test the usage of 122 known plant

species in therapeutic treatments (Anesini and Parez 1993). The result showed that among these plant extracts, 12 inhibited the growth of *Staphylococus aureus*, 10 inhibited *Escherichia coli*, and four inhibited *Aspergillus niger* and also reported that the most potent compound was one extracted from *Tabebuia impetiginosa*.

Consequently, successive finding in many researches had proven that discovery and development of new antimicrobial drug can be obtained from natural products or medicinal plants.

Therefore, the objectives of this project are:

I) To obtain various extracts from stems and leaves of *Lobelia chinensis* by sequential extraction and determine the percentage yield of various extracts.

II) To analyse the extracts of *Lobelia chinensis* and *Ipomoea batatas* for antimicrobial activity against Gram-negative (*Acinetobacter baumanii, Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa,*) and Gram-positive (*Bacillus cereus, Staphylococcus aureus*) bacteria, using the colorimetric broth microdilution method.

III) To determine the minimum inhibitory concentration (MIC), and minimum bactericidal concentration (MBC) values of plant extracts against Gram-negative and Gram-positive bacteria.

IV) To calculate the total activities of plant extracts as well as bacterial susceptibility index (BSI).

CHAPTER 2

LITERATURE REVIEW

2.1 Plant of the study: *Lobelia chinensis*

2.1.1 Description

The plant sample, *Lobelia chinensis* as shown in Figure 2.1 is belongs to family Campanulaceae and commonly known as Chinese Lobelia or Chinese cardinal flower. Other synonyms of *L. chinensis* are *Lobelia radicans* Thunb and *Pratia radicans* G. Don (Wiart 2006). *Lobelia chinensis* is a glabrous and perennial herb which grows to about 10 cm high and 15 cm wide (Leung and Ho 2001). It can be found mainly in wet places, especially around paddy fields and in lowland in China, Japan, Korea, India, North Thailand, Vietnam and Taiwan (Wiart 2006; Leung and Ho 2001). It is a hermaphrodite herbaceous plant which means it has both male and female organs (Leung and Ho 2001). The stems are slender, decumbent, creeping and contain bearing upright simple branches with 5 cm-20 cm long. The leaves are simple and alternate while the blade is narrowly elliptical or lanceolate with an acute apex (Wiart 2006; Hong and Lammers 2011). The leaves margin is entire or very finely serrate at the upper part (Hong and Lammers 2011). The flowers with diameter less than 1 cm are solitary in the upper axillary branches and are attached to 1.5 cm–3 cm long pedicels (Wiart 2006; Ren 2008; Hong and Lammers 2011). The calyx lobes are 3 mm–4 mm long, narrowly deltoid with very finely lobed margins. The small corolla with 10–15 mm long is

vary from white to rose-purple or bluish, consisting of five lanceolate–oblong lobes which are grouped at one side of the corolla (Wiart 2006; Hong and Lammers 2011). The anther consists of five stamens are fused tightly into a tube in which the stigma and style are packed. The filaments are also fused at the upper parts, forming a column-like structure surrounding the pistil (Wiart 2006; Ren 2008). The style is approximately 8 mm long while the stigma consists of three lobes. The fruits are conical and 5 mm–7 mm long capsules containing several ovate, smooth and reddish seeds **(**Wiart 2006**).** It can be harvested in summer by pulling the whole herb out, remove impurities and use it when fresh or dry it in the sun.

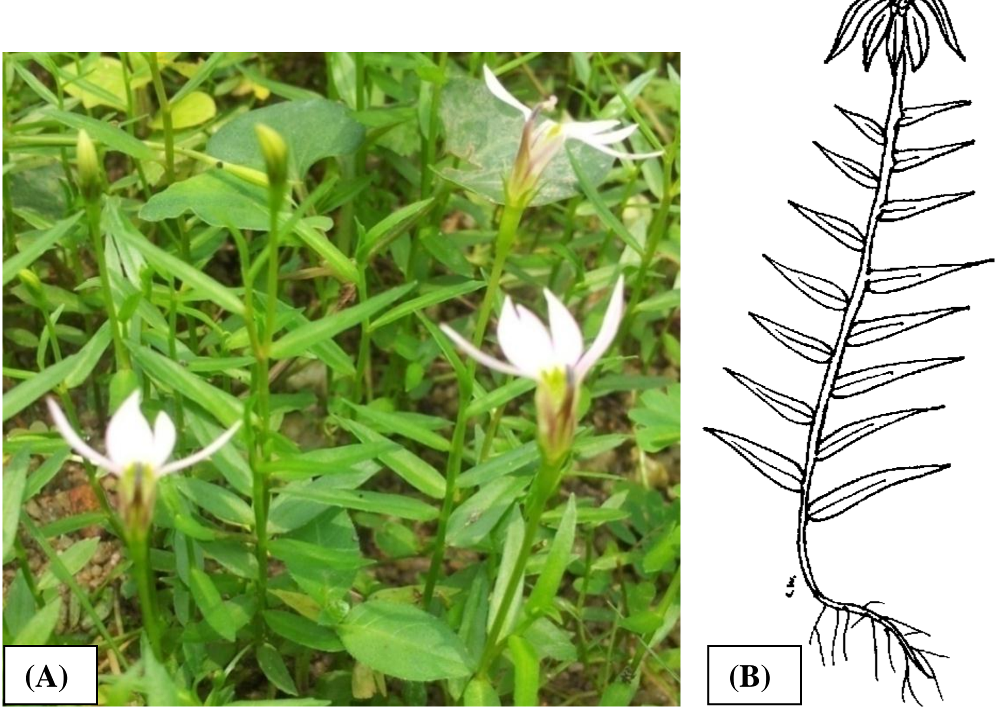


Figure 2.1: ***Lobelia chinensis***

(A) Leaves, stems and flowers

(B) Leaves, stems, flowers and roots (Wiart 2006)

2.1.2 Chemical Constituent

The major constituent of *L. chinensis* consists of several alkaloids, including lobeline, lobelanine, and lobelanidine which have been isolated from *L. chinensis* (Shibano et al., 2001). Lobeline diminishes the behavioral and neurochemical effects of nicotine and amphetamines, and is considered a potential pharmacotherapy for drug abuse and addiction. Lobeline has high affinity for nicotinic acetylcholine receptors and inhibits the function of vesicular monoamine and dopamine transporters (Miller et al., 2007).

Besides, radicamine A and B are important groups of naturally occurring polyhydroxylated pyrrolidine alkaloids isolated from *L. chinensis* which have shown potent inhibitory activity against α-glucosidase (Shibano et al., 2001; Mallesham et al., 2011).

2.1.3 Medicinal Uses

Lobelia chinensis is commonly used in Chinese herbalism, where it is considered to be one of the 50 fundamental herbs (Leung and Ho 2001). It has been used as an antidote, a hemostat, and as carcinostatic agents for stomach cancer in Chinese folk medicine (Shibano et al., 2001). During 19th century, *L. chinensis* was one of the most medicinally important plants and was used as a valuable remedy for asthma especially the rhizomes of *L. chinensis* (Felpin and Lebreton 2004; Joshi et al., 2011). The whole plant can be used for anti-inflammatory, depurative and febrifuge purposes. The fresh plant can also be crushed and used as a poultice, a soft substance to be applied to sores, inflamed parts of the body (Leung and Ho 2001). The roots are considered depurative and antirheumatic in Indo-China (Joshi et al., 2011). Besides, the root is used for antisyphilitic, cathartic, and diuretic in Vietnam (Leung and Ho 2001; Wiart, 2006). Moreover, this plant is one of the constituents of a tincture formulation used for the treatment of scars (Joshi et al., 2011). A paste is used to counteract putrefaction and poisonous snake bites, soothe the swollen parts, treat ascite, and heal insect bites (Wiart, 2006; Leung and Ho 2001).

2.2 Plant of the Study: *Ipomoea batatas*

2.2.1 Description

Ipomoea batatas as shown in Figure 2.2 or commonly known as sweet potato is an important food crop belongs to the family Convolvulaceae. Other synonyms of *I. batatas* are *Ipomoea fastigiata*, *Ipomoea tiliacea* and *Ipomoea triloba* (USDA 2013). It is widely grown in tropical, subtropical and warm temperate regions (Srisuwan 2006). *Ipomoea batatas* is a perennial plants in which the upper part of the plants grows as hairy herbaceous vine. The stems can grow several meters long while the root in contact with soil. The leaves are ovate to oblong-ovate, approximately 6 to 14 cm long (Stuart 2012). The leaves are alternating heart-shaped and pointed at the tip. The flowers look alike to morning glory, from white or pale violet, which is a self sterile and rarely producing seed (Duke 1983). Sweet potato varieties exist in many colors of skin and flesh, ranging from white to deep purple, although white and yellow orange flesh are the most common.

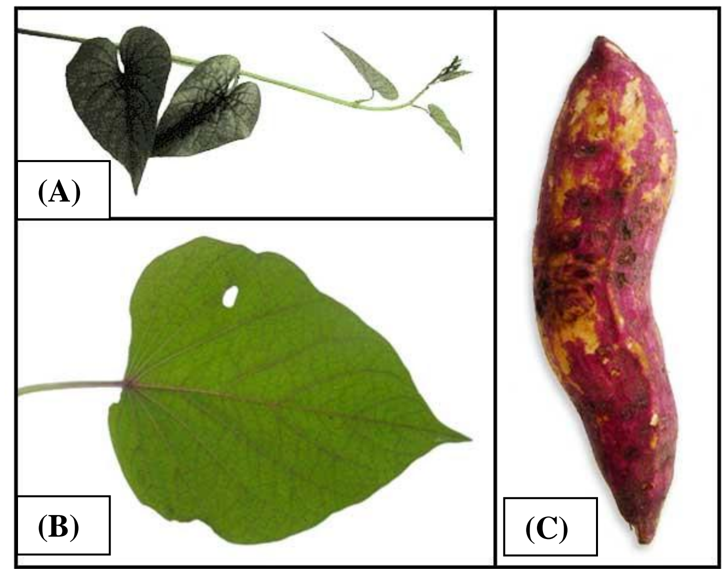


Figure 2.2: ***Ipomoea batatas***

(A) Leaves and flower

(B) Leaf

(C) Skin color (Stuart 2012)

2.2.2 Chemical Constituent

Ipomoea batatas's leaves contained abundant polyphenol compounds, mainly caffeoylquinic acid (CQA) derivatives, indicating that sweet potato leaves could be a strongly antioxidative leafy vegetable due to high amount of polyphenol (Nagai et al., 2010). *Ipomoea batatas*'s leaves had the highest phenolic acid content followed by the peel, whole root, and flesh tissues (Truong et al., 2007). Other than polyphenol, *I. batatas*'s leaves also contain various antioxidants such as vitamin E, β-carotene and lutein which might be contribute to their radical scavenging effects (Chandrika et al., 2009; Dini et al., 2009; Zhou et al., 2012).

Besides, flavonoids and chitinase are present in *I. batatas*' s leaves as well (Hue et al., 2012). Saponin which acts as anti-inflammatory agent can be isolated from tuber of *I. batatas* (Banno et al., 2004; Dini et al., 2009).

2.2.3 Medicinal Uses

Presence of polyphenol in abundant amount and various antioxidants such as vitamin E, β-carotene and lutein in *I. batatas* allow it to carry out high DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging activity and prevent free radical-induced lipid peroxidation in low-density lipoprotein (LDL) (Oki et al., 2006; Nagai 2010). The oxidative modification of LDL is related to foam cell formation, and can result in the initiation and progression of atherosclerosis leading to cardiovascular disease (CVD). It can reduce the risk of CVD since it is highly inhibiting the LDL oxidation (Nagai 2010). Furthermore, *I. batatas*'s leaves are aid in inhibition of HIV replication, mutagenicity, diabetes, and the proliferation of cancer cells (Yoshimoto et al., 2002). The presence of chitinase in *I. batatas* are able to catalyze the hydrolysis of chitin, the main structural component of fungal walls and arthropod integuments, proposed that it is important in defencing against pathogens (Stuart 2012). Furthermore, the *I. batatas*'s leaves help to reduce stomach distress, nausea and diarrhea (DeVries 2010). Nevertheless, the saponin isolated from tuber of *I. batatas* provides some pharmacological activities such as anti-diabetic and anti-inflammatory effects (Banno et al., 2004; Dini et al., 2009).

2.3 Bacteria

2.3.1 Introduction of Bacteria

Bacteria are relatively simple, single-celled or unicellular microscopic organisms. The bacteria cells are called as "Prokaryote", from the Greek words meaning prenucleus due to the absence of a special nuclear membrane which enclosed the genetic material (Tortora et al., 2010). Bacteria are found in every habitat on Earth such as soil, rock, oceans and even arctic snow. Some live in or on other organisms including plants and animals as well as humans body (Noel 1998; Costello et al., 2009)

2.3.2 Size, Shape and Structure of Bacteria

In general, bacteria come in many sizes and several shape. Most of the bacteria range from 0.2-2.0 μm in diameter and from 2-8 μm in length (Ryan and Ray 2010; Tortora et al., 2010). The major morphology forms of bacteria including spherical or oval coccus, rod-shaped bacillus, bent or curved rods and spirals (Carter 1997; Kaiser 2006). Cocci are typically arranged in a chainlike pattern called streptococci or in a form of grapelike cluster or broad sheets are known as staphylococci. Bacilli that are small and pleomorphic to the point of resembling cocci are often called as coccobacilli and streptobacilli occur in chains. Spiral bacteria have one or more twists, they may be rigid or flexible and undulating (Carter 1997; Kaiser 2006; Ryan and Ray 2010; Tortora et al., 2010).

Structurally, bacteria cells consist of three architectural regions which are appendages, cell envelope and cytoplasmic region. Appendages to the cell surface can appear in the form of flagella or pili as well as fimbriae which important for bacterial movement and mobilization (Todar 2012a). Moreover, bacteria cell envelope consisting of a capsule or slime layer which is made up of polysaccharides that able to protect bacteria from immune system mechanism such as phagocytic engulfment, killing or digestion (Bailey 2013). Internal to capsule but still outside the cell proper, there is a rigid bacteria cell wall which composed of a macromolecular network called peptidoglycan. Peptidoglycan is absent in eukaryotic cells and it is essential to prevents osmotic lysis of cell protoplast and confers rigidity and shape of bacteria cells (Royet and Dziarski 2007). Besides, bacteria cell membrane consists of phospholipid-protein bilayer and lack of sterols. It acts as permeability barrier and aids in electron and solutes transportation and energy generation. The cytoplasmic region includes nucleoid which consists of the naked, circular bacterial chromosome (DNA), ribosomes that act as the site for protein synthesis and lastly, various sorts of inclusions which often reserves nutrients for bacteria cells (Ryan and Ray 2010; Tortora et al., 2010; Todar 2012a).

Some bacteria can form endospores which are extremely resistant to hostile physical and chemical conditions such as heat, ultra-violet (UV) radiation and disinfectants. This allows them to survive and remain viable even after 50 years of dormancy. Many endospore-producing bacteria are nasty pathogens, for

example *Bacillus anthracis* is the cause of anthrax and food poisoning (Heyndrickx 2011).

2.3.3 Gram-positive and Gram-negative Bacterial Cell Walls

The major component of bacterial cell wall is a complex structure called peptidoglycan or also known as murein which consists of repeating units of dissacharide molecules which made up of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) (Royet and Dziarski 2007). Both molecules are linked to form a carbohydrate "backbone" by polypeptides bonds. Overall, the structure and function of cell wall is a hallmark of prokaryotes and protects the cell from mechanical as well as chemical disruption (Ryan and Ray 2010; Tortora et al., 2010; Todar 2012a).

Gram-positive bacterial cell wall composed of multiple layers of peptidoglycan, forming a thick, rigid structure that made up 90% of the cell wall. Besides peptidoglycan layer, the teichoic acids which are the polymers of either glycerol phosphate or ribitol phosphate also present in Gram-positive bacterial cell wall. Teichoic acids have strong negative charge which form phosphate groups and therefore allow the movement of cations into and out of the cell and contribute to the antigenic specificity of cell wall. Examples of Gram-positive bacteria are *Bacillus cereus* and *Staphylococcus aureus* (Ryan and Ray 2010; Tortora et al., 2010; Todar 2012a).

For Gram-negative bacteria, their cell wall consists of one or fewer layers of peptidoglycan than Gram-positive bacteria and covered by an outer membrane containing lipopolysaccharides (LPS), lipoprotein, and phospholipids. The outer membrane provides a barrier to certain antibiotics and digestive enzymes such as lysozyme and detergent, and therefore prevents the breakdown of cell wall. The lipopolysaccharides component, called lipid A acts as endotoxin which responsible for some symptoms associated with the infection by Gram-negative bacteria (Hambleton et al., 1996; Raetz and Whitfield 2002). Another component, the O polysaccharides acts as antigen and useful in laboratory tests to differentiate different species of Gram-negative bacteria (Hambleton et al., 1996; Raetz and Whitfield 2002). For example, the food-borne pathogen, *Escherichia coli* O157: H7 is distinguished from other bacteria due to these specific antigens. Examples of Gram-negative bacteria are *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Ryan and Ray 2010; Tortora et al., 2010).

2.3.4 Cell Wall and Gram Stain

Gram stain is a common differential method that used to classify bacteria cells. Different groups of bacteria react differently to Gram stain due to the structural differences of their cell wall. In the procedure, bacteria cells are firstly stained with purple dye called crystal violet and then, treated with iodine, a mordant. Next, the cells are decolorized with alcohol and finally stained with safranin, a counterstain (Tortora et al., 2010; Bruckner 2012).

Since Gram-positive bacteria have thicker peptidoglycan, it can retain a large molecule called crystal violet-iodine (CV-I) complex in the cell. The complex is cannot be washed out of the intact and thick layer of peptidoglycan by alcohol. Consequently, Gram-positive bacteria cells retain the color of crystal violet dye (Tortora et al., 2010; Bruckner 2012).

However, the thin layer of peptidoglycan in Gram-negative bacteria allows alcohol to wash out the CV-I complex by disrupting the lipopolysaccharide layer. As a result, the Gram-negative bacteria cells are colorless until they are counterstained with safranin, after which appeared as pink color (Tortora et al., 2010; Bruckner 2012).

2.4 *Acinetobacter baumannii*

2.4.1 Morphology and Structure

Acinetobacter baumannii as shown in Figure 2.3 is a short, pleomorphic and nonmotile Gram-negative rod or coccobacillus that belonging to family Moraxellaceae. It appears as a coccobacillus at stationary state, however it becomes rod form at growth stage (Dent 2010). This bacterium is commonly isolated from the hospitalized patients' sputum or respiratory secretions, wounds, and urine. *Acinetobacter baumannii* is a water organism and preferentially colonizes aquatic environments (Cunha 2013). Sometimes, it may be misidentified as either Gram-negative or Gram-positive cocci due to the difficulty

of destain process. It is a nonfastidious aerobic organism that produces smooth, sometimes mucoid, pale yellow to grayish white colonies on medium. It is catalase-positive, oxidase-negative and non-fermenter of glucose (Peleg 2008; Dent 2010).

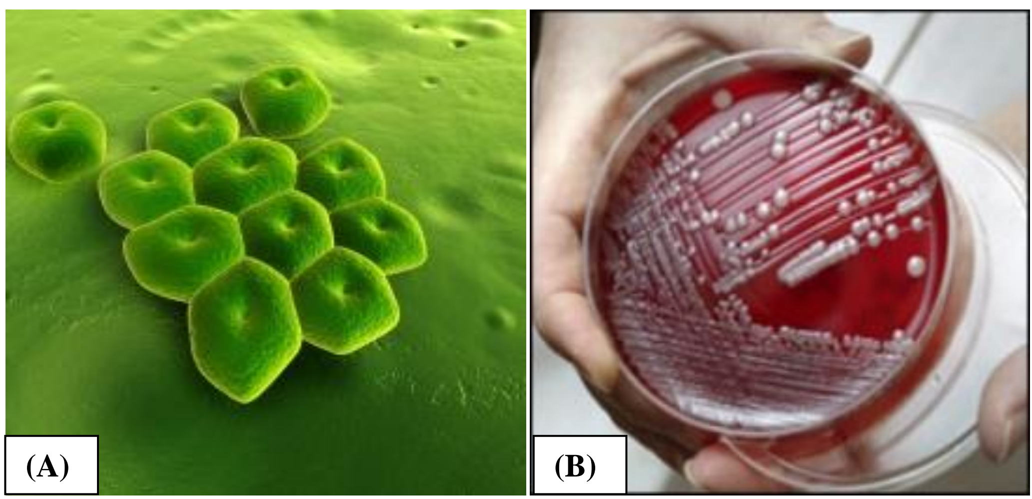


Figure 2.3: ***Acinetobacter baumannii***

(A) Microscopic image of *A. baumannii* (Bioquell 2013)

(B) Colonial growth of *A. baumannii* on the agar plate (Marshall 2011)

2.4.2 Clinical Diseases and Pathogenesis

Acinetobacter species are skin, oral cavity and respiratory tract colonizers and appear to be less significant causative agent of infection (Sebeny et al., 2008). However, *A. baumannii* is low virulence but capable of causing infection. It is the opportunistic pathogen that commonly targets the vulnerable hospitalized patients, those who are critically ill with breaches in skin integrity and airway protection,

immunocompromised, cancer patients and Human Immunodeficiency Virus (HIV) infected patients (Peleg 2008; CDC 2010). The infection sites usually involve organ systems that have a high fluid content such as respiratory tract, cerebrospinal fluid (CSF), peritoneal fluid and urinary tract (Cunha 2013). Pneumonia is the most common infection, followed by the urinary tract and soft tissue infections (CDC 2010; Ryan and Ray 2010). Mode of transmission normally involves open wounds and invasive devices like urinary catheters. Choi et al. (2005) reported that *A. baumannii* is able to induce cell apoptosis to human laryngeal epithelial HEP-2 cells due to the contribution of outer membrane protein 38 (Omp38) that present in it. Besides, Omp38 that localized to mitochondria can led to a release of proapoptotic molecules such as cytochrome c and apoptosis-inducing factor (AIF) into epithelial cells, resulting in DNA degradation (Choi et al., 2005)

2.4.3 Antibiotic Susceptibility

The multidrug resistant *A. baumannii* is highly resistant to more than three classes of antibiotics since more than half of the *A. baumannii* isolates were resistant to imipenem, amikacin, and ampicillin-sulbactam (Dent at al., 2010). Besides, commonly used antibiotics including aminoglycosides, cephalosporins, carbapenems, extended spectrum penicillins, and quinolones hardly exert the antimicrobial effect on multidrug resistant *A. baumannii* (Towner 2009; Dent et al., 2010; Ryan and Ray 2012). However, polymyxins, sulbactam, glycylcyclines

have potential activity against *A. baumannii* strains. New class of tetracycline-related antimicrobial agents, the glycylcyclines was found to have good antimicrobial activity against some carbapenem-resistant *Acinetobacter* (Henwood et al., 2002; Ibanez et al., 2004; Towner 2009).

2.5 *Escherichia coli*

2.5.1 Morphology and Structure

Escherichia coli is a short, non-spore forming, motile Gram-negative rod which belongs to family Enterobacteriaceae. The size is at the range of 0.1-0.5 µm in diameter and 1-2 µm in length as shown in Figure 2.4. It is facultative anaerobic that live in the intestinal tracts of human. *Escherichia coli* usually produce dry and pinkish colony on MacConkey agar. They show positive result for some biochemical tests such as indole test, catalase test and methyl red test, however, negative result is obtained for Voges-Proskauer test, oxidase test and citrate test (Huang et al., 2001; Brooks et al., 2004).

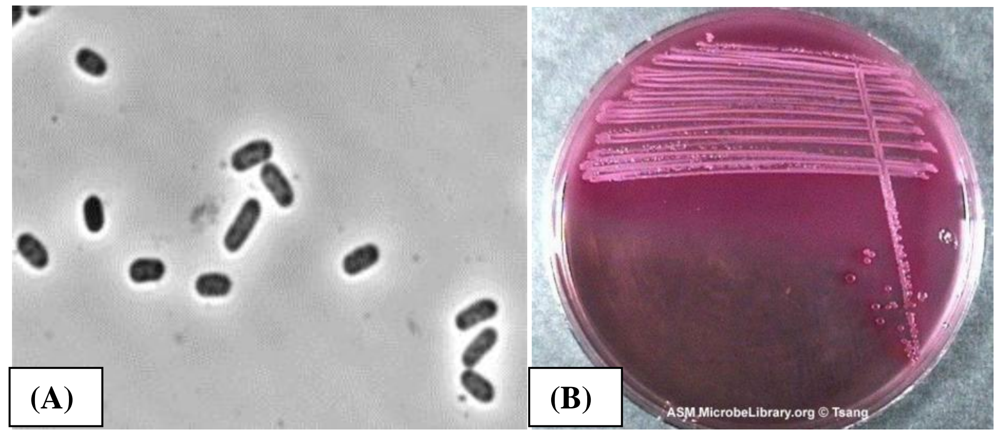


Figure 2.4: ***Escherichia coli***

(A) Phase contrast image of *E. coli* (Graham2010)

(B) Colonial growth of *E. coli* on MacConkey agar plate (Graham 2010)

2.5.2 Clinical Diseases and Pathogenesis

Escherichia coli is one of the most common inhabitants of human intestinal tract. However, there are certain strains which can cause intestinal and extraintestinal infection in individuals. Three most common types of infections caused by pathogenic strains of *E. coli* are urinary tract infections (UTI), neonatal meningitis, and intestinal diseases (Johnson et al., 2003). Factors such as the distribution and expression of the virulence determinants, including adhesins, invasins, toxins, and abilities to endure with host defenses system of *E. coli* strains influence their ability to cause disease (Todar 2012b). *Escherichia coli* accounts for more than 90% of 7 million cases of cystitis and 250000 of pyelonephritis estimated to occur in otherwise healthy individuals every year in United States (Ryan and Ray 2010). UTIs are much more common in women, 40% of whom have an episode in their

life time, usually when they are sexually active. Uropathic *E. coli* (UPEC) is one of the pathogenic strains which enhanced potential to cause UTI because UPEC possess P fimbria or Type 1 pili which are important for periurethral colonization and for attachment to epithelial cells in bladder. Besides, the production of LPS and α-hemolysin can lead to LPS-induced shock and lyses of erythrocytes respectively (Ryan and Ray 2010).

Escherichia coli is one of the common causes of neonatal meningitis and 75% of cases are caused by the strains possessing K1 capsular antigens. It inhibits phagocytosis, complement, and responses from the host's immunological mechanisms. Besides, the pathogenesis also involves colonization of vaginal *E. coli* in infants via ruptured amniotic membranes or during childbirth and failure of protective maternal Ig M antibodies to cross the placenta (Ryan and Ray 2010; Todar 2012b).

Diarrheal-causing *E. coli* are classified into five classes which are enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) and enteroaggregative *E. coli* (EAEC) based on their properties (World Health Organization 2011; CDC 2012). ETEC strains produce heat-labile enterotoxin (LT) and heat stable toxin (ST) in proximal small intestine, causing watery diarrhea (Nataro and Kaper 1998; World Health Organization 2011; Todar 2012b). Moreover, pathogenicity of EIEC is primarily due to its ability to invade and

destroy colonic tissue. The recognition of EHEC is resulted from two key epidemiologic observations which are hemorrhagic colitis (HC) or bloody diarrhea, and fatal hemolytic uremic syndrome (HUS) (Riley et al., 1983; Nataro and Kaper 1998; Wang et al., 2006). EHEC are characterized by the production of verotoxin or Shiga toxins (Stx) which can cause capillary thrombosis and inflammation of colonic mucosa (O'Brien et al., 1986; Todar 2012b). Besides, EHEC can also cause the attachment and effacing (A/E) lesion on the enterocyte cell surface which then enhanced the severity of the disease. EPEC induce a profuse watery and bloody diarrhea via attaching-and-effacing (A/E) mechanism in which the microvilli is attached to epithelial cell surface (Nataro and Kaper 1998; Todar 2012b). EAEC is associated with protracted watery diarrhea with blood and mucus. Its pathogenesis involves the formation of a thick mucus-bacteria biofilm on the intestinal surface (Ryan and Ray 2010).

2.5.3 Antibiotic Susceptibility

Escherichia coli showed an increasing trend in resistance to older drugs such as tetracycline, sulfonamide, streptomycin and ampicillin (Tadesse et al., 2012). The emergence of strains that producing extended spectrum β-lactamases (ESBLs) is multiply resistant to non-β-lactam antibiotics, including trimethoprim, ciprofloxacin and gentamicin (Potz et al., 2006; Public Health Agancy of Canada 2012). However, *E. coli* was showed to have high susceptibility toward tigecycline, imipenem, nitrofurantoin and amikacin (Al-Assil et al., 2013).

2.6 *Klebsiella pneumoniae*

2.6.1 Morphology and Structure

Klebsiella pneumoniae is a non-motile Gram-negative bacterium that belonging to the family Enterobacteriaceae. It is a straight rod with the size of 1-2 μm as shown in Figure 2.5, containing a thick and prominent polysaccharide capsule that enclosed the entire cell surface and resistant against host defense system (Ryan and Ray 2010; Umeh 2011). It is a facultative anaerobe and produces colonies which have circular, glistening viscous or mucoid appearance when cultured on medium (Umeh 2011). This species produces a distinctive yeasty odor as well. *Klebsiella pneumoniae* is a glucose and lactose fermenter, showing positive result in catalase test, nitrate reduction, lysine decarboxylase test and Voges-Proskauer test, however, it shows negative in indole test and unable to hydrolyze starch as well as does not produce amylase (Hansen et al., 2004).

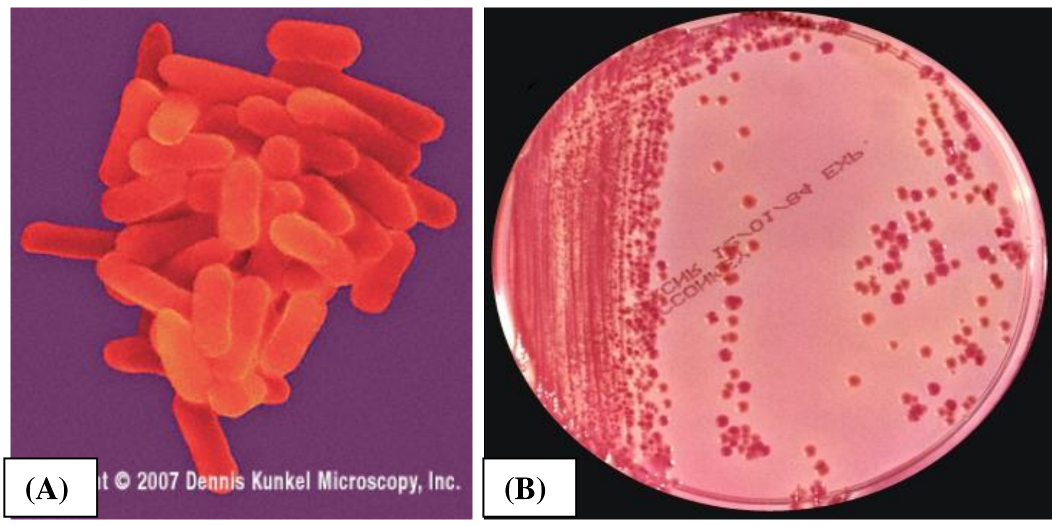


Figure 2.5: ***Klebsiella pneumoniae***

(A) **Microscopy image of *K. pneumoniae*** (Kunkel 2007)

(B) **Colonial growth of *K. pneumoniae* on MacConkey agar plate** (Vastag 2012)

2.6.2 Clinical Diseases and Pathogenesis

Klebsiella pneumoniae is commonly found in the human gastrointestinal tract as part of the natural microflora. It is an opportunistic pathogen which causes nosocomial infections involving the urinary and pulmonary systems, especially since it is able to adapt to an existence in an oxygenated or deoxygenated environment (Podschun and Ullmann 1998). Immunocompromised individuals such as AIDS or cancer patients infected with *K. pneumoniae* usually develop respiratory tract infections such as pneumonia, other spectrum of clinical syndromes such as blood infections (septicemia), wound or surgical site infections, urinary tract infection (UTI), osteomyelitis and meningitis. Besides, patients

whose care requires devices like ventilators or intravenous catheters, and patients who are taking long courses of certain antibiotics are at high risk for *Klebsiella* infections (CDC 2012). Clinically, infection by *K. pneumoniae* is a sudden onset, characterized by fever, pleuritic pain, coughing with thick and red currant jelly-like sputum. Necrosis, inflammation, and hemorrhage occur within lung and eventually lead to death.

This pathogen possesses many virulence factors that allow it to overcome the host's immune system and cause infection in a variety of ways. Firstly, its thick polysaccharide capsule protects the bacteria from phagocytosis by polymorphonuclear granulocytes and prevents their somatic antigens from being detected by the host's antibodies (Smit et al., 1985; Podschun and Ullmann 1998). Nevertheless, this species uses ferric-siderophore receptors of the host to activate their enterobactin-mediated iron-sequestering system, allowing for bacterial growth. Besides, it also produces adhesions such as Type 1 pili and Type 3 pili which aid the microorganism to adhere to host cells (Podschun and Ullmann 1998; Damian et al., 2009). This prevents opsonization and membrane attack complex (MAC) insertion, which leads to lysis of the bacterium (Umeh 2011).

2.6.3 Antibiotic Susceptibility

Among all species in Enterobacteriaceae, *Klebsiella* species are now among the most resistant to antimicrobics. *Klebsiella pneumoniae* produces β-lactamases at

low level which are sufficient enough to protect them against ampicillin, amoxicillin, carbenicillin, and ticarcillin (Livermore 1995). Furthermore, extensive use of broad-spectrum antibiotics in hospitalized patients results in the development of multidrug-resistant strains that produce extended-spectrum beta-lactamase (ESBL) in *K. pneumoniae*. It is also carbapenem-resistant bacteria due to the ability to produce carbapenemase (Umeh 2011; CDC 2012).

2.7 *Pseudomonas aeruginosa*

2.7.1 Morphology and Structure

Pseudomonas aeruginosa as shown in Figure 2.6 is an aerobic Gram-negative rod that belongs to the family Pseudomonadaceae. Their size is approximately 0.5 to 0.8 µm by 1.5 to 3.0 µm and almost all strains are motile by means of a single polar flagellum. Its most striking bacterial feature is the production of colourful pigment including the fluorescent pigment pyoverdin and the blue pigment pyocyanin (Ryan and Ray 2010; Todar 2012c). It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. Confluent growth produces colonies with a spreading border and emits an intense “fruity” odor and produce hemolysis on blood agar. The positive oxidase reaction distinguishes it from other Enterobacteriaceae (Ryan and Ray 2010; Todar 2012c).

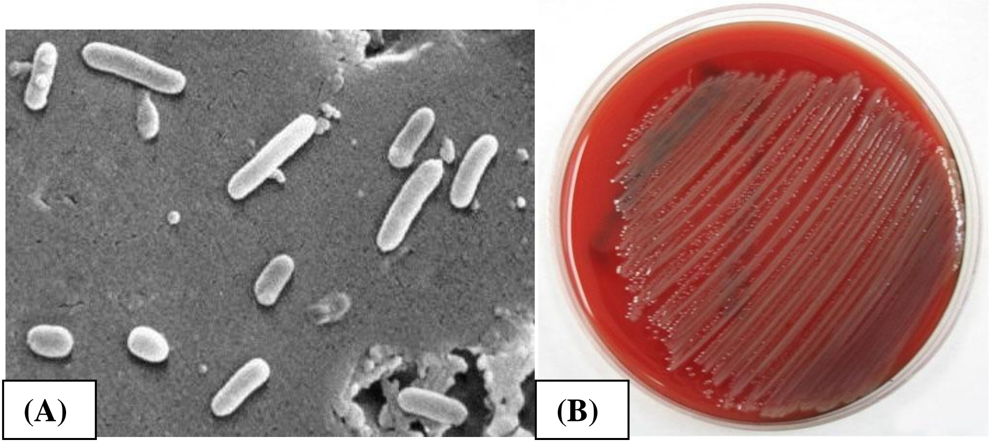


Figure 2.6: ***Pseudomonas aeruginosa***

(A) Electron micrograph of *P. aeruginosa* (Todar 2012c)

(B) Colonial growth of *P. aeruginosa* on Tryptic Soy Agar (TSA) (Putty 2007)

2.7.2 Clinical Diseases and Pathogenesis

Pseudomonas aeruginosa is an opportunistic pathogen which usually requires a significant break in first-line defenses, for example, wound or a route such as intratracheal tube to initiate the infection. First step of infection involves the attachment of bacteria to epithelial cells through pili, flagella and the extracellular polysaccharide slime (Ryan and Ray 2010; Todar 2012c). Attachment is aided by production of a protease enzyme that degrades fibronectin in order to expose the underlying pilus receptors on the epithelial cell surface. Besides, elastase and phospholipase are produced to cleave collagen, IgG, IgA, and some complement component. Elastase attacks elastin and disrupts the respiratory epithelium and interferes with ciliary function. Hemorrhagic destruction, including the walls of

blood vessels becomes histologic hallmark of *Pseudomonas* infection (Ryan and Ray 2010; Todar 2012c).

Serious infection with *P. aeruginosa* is more commonly seen in the context of immune-compromising conditions, especially in Cystic Fibrosis (CF) patients (Feldman et al., 1998; Ryan and Ray 2010). The cells from CF patients are less highly sialylated than normal epithelial cells and therefore, increase availability of bacterial receptors for attachment. Formation of biofilm in bronchi prevents the activation of host immune system and antimicrobial agents (Davies 2002; Ryan and Ray 2010). *Pseudomonas aeruginosa* pneumonia is a rapid, destructive infection which associated with alveolar necrosis, vascular invasion, infarcts, and bacteremia. Intravenous drug users and patients on hemodialysis are at risk for *P. aeruginosa* endocarditis (Hassan and Riyami 2011). Nevertheless, it causes bacteremia primarily in immunocompromised patients. Moreover, it is the common causes of bacterial keratitis and neonatal ophthalmia (Hancock and Speert 2000; Ryan and Ray 2010; Todar 2012c).

2.7.3 Antibiotic Susceptibility

Pseudomonas aeruginosa is resistant to many antimicrobials due to the porins that restrict the entry of antimicrobial agents. It regularly resistant to penicillin, ampicillin, cephalothin, tetracycline, chloramphenicol, sulfonamides, and aminoglycosides (streptomycin and kanamycin). The newer aminoglycosides such

as gentamicin, amikacin, and tobramycin are actively against *P. aeruginosa* (Henwood et al., 2001; Lambert 2002; Ryan and Ray 2010). Besides, third generation cephalosporin such as ceftazidime, cefoperazone and cefepime can be used as single agent against *P. aeruginosa*. Moreover, carbapenems such as imipenem, meropenem give broad spectrum activity against Gram-negative bacteria including *P. aeruginosa*. Furthermore, extended-spectrum penicillins such as carbenicillin and ticarcillin act synergistically with aminoglycosides against *P. aeruginosa* (Banerjee and Stableforth 2000; Hancock and Speert 2000; Ryan and Ray 2010).

2.8 *Bacillus cereus*

2.8.1 Morphology and Structure

Bacillus cereus is a non-motile spore-forming Gram-positive rod that belongs to the family Bacillaceae. It is a facultative anaerobe with nonfastidious growth requirements and has an optimum growth temperature ranging from 25 to 37°C. Most strains of *B. cereus* are motile and they are roughly 5 µm to 10 µm long by 1 µm wide, and arranged singly or in short chains as shown in Figure 2.7 (Todar 2012d). *Bacillus cereus* colonies appear to large sphere, dull gray and opaque with a rough matted surface and surrounded by wide zone of hemolysis when grown under aerobic conditions on 5% sheep blood agar at 37°C (Garofalo 2011). Colony perimeters are irregular and represent the configuration of swarming from the site of initial inoculation due to *B. cereus* swarming motility (Senesi et al.,

2002; Bottone 2010). They are able to ferment glucose and positive for nitrate reductase test, Voges–Proskauer test and catalase test (Wong et al., 1987). This organism is naturally found in soil, but also exists as harmless microflora in the intestinal tract of many soil-dwelling invertebrates.

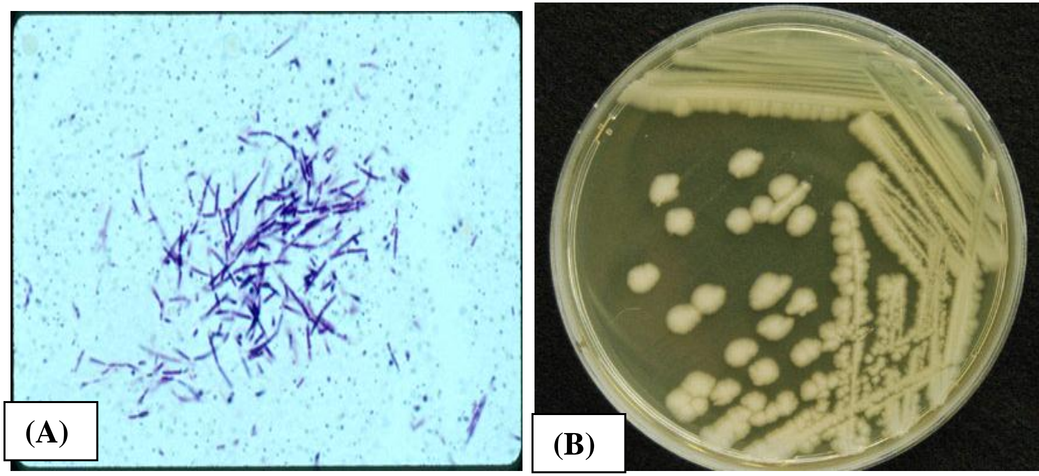


Figure 2.7: ***Bacillus cereus***

(A) Gram stain of *B. cereus* (Bottone 2010)

(B) Colonial growth of *B. cereus* on the agar plate (ASM MicrobeLibrary 2012)

2.8.2 Clinical Diseases and Pathogenesis

Bacillus cereus is most likely to cause opportunistic infection and as a causative agent for food poisoning due to its ability to produce endospores and enterotoxins as well as emetic toxins (Phelps and McKillip 2002; Ryan and Ray 2010). Since the endospores are heat-resistant, the vegetative form starts growing when cooked food is exposed to warm temperatures over a prolonged period of time (Heyndrickx 2011). There are two types of clinical syndromes associated with

each toxin, namely, rapid-onset emetic syndrome and slow-onset diarrheal syndrome. The diarrheal type which characterized by abdominal cramps with watery diarrhea is caused by protein enterotoxins which composed of haemolysin BL (HBL) and non-hemolytic enterotoxin (NHE) (Loir et al., 2003; Granum et al., 2004; Bottone 2010). HBL and NHE proteins form pores on the cell membrane of target cells, resulting in the loss of cellular membrane potential and eventually cell death. These enterotoxins can also activate the adenylyl cyclase pathway, leading to intestinal fluid secretion (Ryan and Ray 2010). Whereas the emetic type is which characterized by nausea and vomiting is caused by a heat-stabile toxin called cereulide which mainly found in dairy products (Loir et al., 2003; Bottone 2010).

Other than food poisoning, *B. cereus* also causes a number of systemic and local infections in both immunologically compromised and immunocompetent individuals such as neonates, intravenous drug abusers, traumatic patients and those with indwelling catheters. The spectrum of infections includes fulminant bacteremia, central nervous system (CNS) involvement such as meningitis and brain abscesses, endophthalmitis, pneumonia, and gas gangrene-like cutaneous infections (Drobniewski 1993; Bottone 2010).

2.8.3 Antibiotic Susceptibility

Since *B. cereus* is able to produce broad-spectrum β-lactamase, therefore it is resistant to penicillins, ampicillin and cephalosporins (Badarau 2007; Fenselau 2008). *Bacillus cereus* is resistant to trimethoprim while susceptibility to clindamycin, erythromycin, chloramphenicol, vancomycin, the aminoglycosides, and tetracycline is usually observed (Coonrod et al., 1971; Turnbull et al., 2004; Savani et al., 2009; Bottone 2010). Susceptibility to ciprofloxacin was uniform, and it has been shown to be highly effective in the treatment of *B. cereus* wound infections. However, a case of fulminant septicemia with *B. cereus* is resistant to carbapenem (Kiyomizu et al., 2008).

2.9 *Staphylococcus aureus*

2.9.1 Morphology and Structure

Staphylococcus aureus as shown in Figure 2.8 is a non-flagellate, non-motile, and non-spore forming Gram-positive spherical cell that belongs to family Staphylococcaceae. It is approximately 0.5-1.5 µm in diameter that arranged in a grape-like cluster. They are facultative anaerobes that form round, smooth and yellow colony on rich medium and often hemolytic on blood agar (Loir et al., 2003; Turnidge et al., 2008; Ryan and Ray 2010; Todar 2012e). The most important test that can be used to distinguish *S. aureus* from other Staphylococci such as *S. epidermidis* is the coagulase test. Nearly all strains of *S. aureus* produce the enzyme coagulase which activates prothrombin to form a fibrin clot. Besides,

S. aureus can be also distinguished from *Streptococci* through catalase test in which former one are vigorous catalase-producers (Foster 1996; Ryan and Ray 2010; Katz 2013). Thus, they are catalase-positive but oxidase-negative bacteria. *Staphylococcus aureus* colonizes mainly the nasal passages, oral cavity, gastrointestinal tract and skin due to their ability which can withstand NaCl concentrations as high as 15% (Ryan and Ray 2010; Todar 2012e).

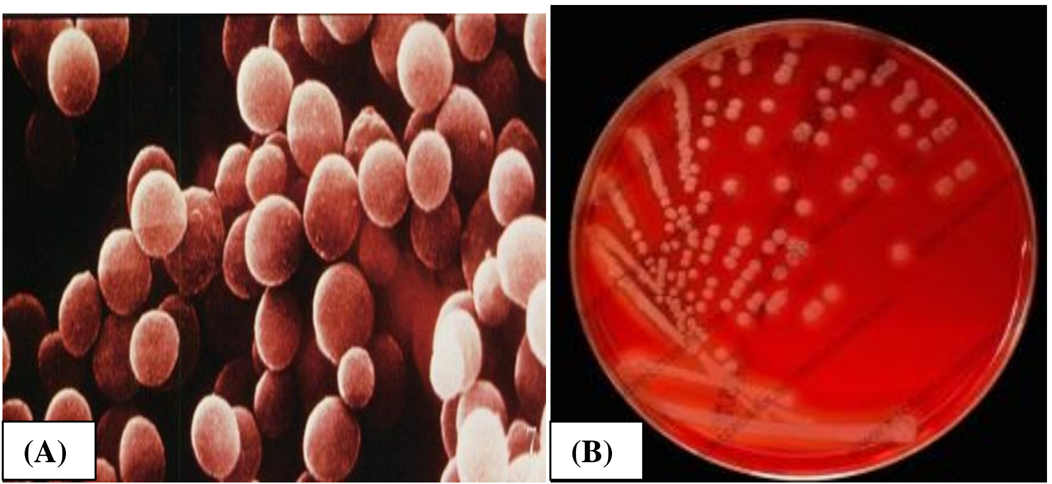


Figure 2.8: *Staphylococcus aureus*

(A) Electron micrograph of *S. aureus* (Todar 2012e)

(B) Colonial growth of *S. aureus* on the agar plate (Microbiology Lab 2013)

2.9.2 Clinical Diseases and Pathogenesis

Staphylococcus aureus is one of the most common causes of acute purulent infection world widely. Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) which can cause diarrhea and vomiting when ingested and are

the causative agents of staphylococcal food poisonings (Loir et al., 2003; Turnidge et al., 2008). The symptoms of staphylococcal food poisoning involve abdominal cramps, nausea, vomiting, sometimes followed by diarrhea (Loir et al., 2003). Besides, *S. aureus* can cause scalded skin syndrome due to the production of its exfoliating toxins A and B which secreted and bind to a specific cell membrane ganglioside found only in the keratinized epidermis of young children and rare adults (Ryan and Ray 2010). Other skin conditions caused by *Staphylococcal* exfoliative toxins include blisters, skin loss, pimples, furuncles, impetigo, folliculitis, abscesses, poor temperature control, fluid loss, and secondary infection. Certain strains of *S. aureus* are able to produce toxic shock syndrome toxin (TSST-1) which expressed systemically and responsible for 75% of toxic shock syndrome cases (Parsonnet et al., 2008; Ryan and Ray 2010; Todar 2012e). Toxic shock syndrome (TSS) is an acute disease characterized by fever, rash, hypotension, multiple-organ-system dysfunction, and desquamation and associated with vaginal colonization with toxin-producing *S. aureus* during menstruation (Reingold et al., 1999; Parsonnet et al., 2008). Almost all strains of *S. aureus* are able to secrete cytotoxin, α-Toxin which forms pores that lyses the cytoplasmic membrane of host cells through direct insertion into lipid bilayer membrane, leading to cell death (Ryan and Ray 2010).

2.9.3 Antibiotic Susceptibility

In the 1940s, penicillin was introduced to treat *S. aureus* infection and all strains were highly susceptible. However, emergence of *S. aureus* strains which able to produce penicillinase, an enzyme that opens up the β-lactam ring, making the drug unable to bind with its target (Turnidge et al., 2008; Appelbaum 2007). The introduction of methicillin in 1961 was rapidly followed by reports of methicillin resistance in *S. aureus*. Today, methicillin-resistant *Staphylococcus aureus* (MRSA) strains are found worldwide, and most are multidrug resistant (Appelbaum 2006; Appelbaum 2007; Ryan and Ray 2010). *Staphylococcus aureus* strains are highly resistant to ampicillin, tetracycline, chloramphenicol and erythromycin, but they are vancomycin susceptible (Turnidge et al., 2008, Kitara et al., 2011; Duran et al., 2012). Kitara et al. (2011) has proposed several alternative drugs which are gentamicin, clindamycin, streptomycin, neomycin and kanamycin for the treatment of MRSA infection.

2.10 Minimum Inhibitory Concentration (MIC) Assay

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that required to inhibit the visible growth of a microorganism. Microorganisms are usually considered susceptible to an antimicrobial agent if the MIC value of the antimicrobial agent is lower than levels of that agent achieved in the blood using appropriate parenteral dosages and

implies that an infection can be appropriately treated with that antimicrobial agent (Andrew 2001; Ogle 2011).

2.11 Minimum Bactericidal Concentration (MBC) Assay

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of antimicrobial that required to kill the growth of a microorganism by 99.9% after subculture onto antibiotic-free media (Andrew 2001). To carry out MBC test, the sample in MIC wells which shows no growth is taken and transferred to agar plate. After overnight incubation, bacteria growth on the subcultures will be observed and the lowest concentration of antimicrobial drug that show no growth on subculture is the MBC value (Bauman 2009).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Materials

The plant sample, *Lobelia chinensis* was obtained from a herb garden in Endau, Johor on 30th September, 2012. The aerial parts of *Lobelia chinensis* were used in this study. The plant was identified by Professor Ong Hean Chooi of University of Malaya. Besides, the extracts of *Ipomoea batatas* were also studied and provided by the supervisor, Dr. Sit Nam Weng.

3.1.2 Bacterial Strains

Six types of bacteria were used in this study as listed in Table 3.1. All of these bacteria were cultured and maintained on Mueller-Hinton agar (MHA).

Table 3.1: Details of tested bacterial strains.

Bacterial Species	ATCC Number
Gram-negative	
Acinetobacter baumannii	18606
Escherichia coli	35218
Klebsiella pneumoniae	13883
Pseudomonas aeruginosa	27853
Gram-positive	
Bacillus cereus	11778
Staphylococcus aureus	6538

ATCC: American Type Culture Collection

3.1.3 Chemical Reagents

All the chemicals and solvents used are listed in Table 3.2.

Table 3.2: List of chemicals and solvents.

Chemical/ Solvent	Manufacturer
n-Hexane	PROCHEM Chemicals, North Carolina, USA
Chloroform	PROCHEM Chemicals, North Carolina, USA
Ethyl acetate	PROCHEM Chemicals, North Carolina, USA
Ethanol 95%	PROCHEM Chemicals, North Carolina, USA
Methanol	PROCHEM Chemicals, North Carolina, USA
Mueller-Hinton Agar (MHA)	Media Laboratories Pvt.Lth, India
Mueller-Hinton Broth (MHB)	Scharlau Microbiology, Spain
Chloramphenicol	Duchefa Biochemie
Tetracycline	Sigma Aldrich, China
Iodonitrotetrazolium chloride	Sigma, USA

3.1.4 Equipment and Labwares

The instruments and labwares used are listed in Table 3.3.

Table 3.3: List of equipment and labwares.

Equipments/Labwares	Brand/Model
Aluminium foil	DIAMOND
Autoclave machine	HICLAVE™HVE-50, HIRAYAMA
Blender	MJ-68M, National
Cotton swab	Premier Diagnostics
Ductless fume cabinet	ESCO
Microcentrifuge tubes	Axygen Scientific
Evaporating flask	Schott Duran 1000 ml
Freeze drier	Scanvac Cool Safe
Incubator	Memmert, Germany
Laboratory film	Parafilm "M" ®, Pechiney Plastic Packaging
Laboratory oven	Memmert, Germany
Laminar flow cabinet	Edamix series
Micropipette	ViPRΩ
Micropipette tips	AXYGEN, INC., Union City, USA
Multichannel pipette	Gilson, France
Orbital shaker	ORBITLS, Labnet International, Inc.

Table 3.3 (continued): List of equipment and labwares.

Equipments/Labwares	Brand/Model
Petri dishes	Greiner bio-one
Polystyrene round bottom tube	Greiner bio-one
Portable bunsen	CAMPINGAZ® LABOGAZ 206
Refrigerator and Freezer	Juscool
Rotary evaporator set	BÜCHI Rotavapor R-20
Sample vial	S Murray & Co
Glass bottle	DURAN, Germany
Sonicator	S 100H Elmasonic
Spectrophotometer	GENESYS 20, Thermo Spectronic
Syringe (3 mL/5 mL)	Cellotron
Syringe filter (0.45 μm)	Sartorius Minisart®
U-shaped polystyrene 96-well plate	Cellstar® Greiner bio-one
Vacuum concentrator	Eppendorf, Concentrator plus, Hamburg, Germany
Vortex mixer	VELP® SCIENTICA, Europe

3.2 Methodology

3.2.1 Plant Preparation

The plant sample, *Lobelia chinensis* was obtained from Endau, Johor. The stems, flowers and leaves were cleaned under running tap water to remove dirt and soil. Then, the plant sample was air-dried within the laboratory under room temperature. The sample was weighed by using a digital weighing scale and then cut into smaller pieces. Finally, it was blended into rough powder form and placed in conical flasks (1 L) for soaking procedure. For *Ipomoea batatas,* the plant extracts were provided by the supervisor.

3.2.2 Vouchering of Plant

Before plant preparation, the entire plant of *Lobelia chinensis* was placed on paper towels and stacked in between newspaper sheets, which then pressed by heavy object for several weeks at room temperature until the plant was completely dried. The dried plant was then flatly mounted on a piece of A3-size mounting board by strapping and gluing. A label with information about the plant, location of collecting site, collector, date of collection, genus and species of the plant, and the family to which the plant belongs was placed at the back of the mounting board. In order to avoid damage to the dried, mounted specimen, the board was wrapped with transparent plastic wrapper.

3.2.3 Sequential Extraction of Plant Sample

In this study, sequential extraction method was applied to extract the plant sample by using increasing polarity solvents from non polar to polar namely hexane, chloroform, ethyl acetate, ethanol, methanol and distilled water. After the plant sample was blended, hexane was firstly added into the conical flasks until it fully covered the entire plant sample. Then, conical flasks with plant samples and solvent inside were placed on an orbital shaker (speed = 140 rpm) at room temperature for overnight. After one day, filtrate was collected and stored in glass bottles for evaporation and the plant samples were further macerated in solvent with the sequence mentioned above. Plant sample was soaked inside each solvent for three cycles, with one day for each cycle.

3.2.4 Evaporation of Plant Extracts

Rotary evaporator was used to evaporate the plant extracts. This step is important to remove the respective solvents and concentrate the extracts. During evaporation, the temperature was set to 40 °C and pressure required for different solvent is shown in Table 3.4. The evaporated extracts were then transferred to pre-weighed and labeled empty vials. The vials were placed in the oven at 37°C for further evaporation until the weight of extracts become constant. Meanwhile, vacuum concentrator was used to concentrate the distilled water extract. Dry weight of each extract was measured and the extracts were stored in a -20 °C freezer pending for the screening assay.

Table 3.4: Pressure used for different extracts during rotary evaporation.

Solvents	Pressure (mbar)
Hexane	335
Chloroform	474
Ethyl acetate	240
Ethanol	175
Methanol	337

3.2.5 Preparation of Plant Extracts

Fifty milligrams of each extract was dissolved in 5 mL of methanol and water mixture (2:1, v/v) to obtain a concentration of 10 mg/mL. In order to ensure all the extracts were dissolved completely in the mixture, sonication process was carried out. The extract solutions were then filtered by using 0.45 µm syringe filter into sterile labeled microcentrifuge tubes. All the filtered extracts were stored in a -20 °C freezer pending for the assay.

3.2.6 Preparation of Reagents

3.2.6.1 Iodonitrotetrazolium Chloride (INT)

Iodonitrotetrazolium chloride (INT) solution was prepared by dissolving 40 mg of INT powder into 100 mL of distilled water in order to achieve the concentration of 0.4 mg/mL. The solution was filtered using 0.45 µm syringe filter into sterile polystyrene tubes and stored at -20 °C prior usage.

3.2.6.2 Chloramphenicol

Chloramphenicol solution was prepared by dissolving 51.2 mg of chloramphenicol powder into 100 mL of distilled water and 95% ethanol mixture (9:1, v/v) to achieve the concentration of 512 µg/mL. The solution was filtered using 0.45 µm syringe filter into sterile polystyrene tubes and stored at -20 °C prior usage.

3.2.6.3 Tetracycline

Tetracycline solution was prepared by dissolving 3.2 mg of tetracycline powder into 100 mL of distilled water to achieve the concentration of 32 µg/mL. The solution was filtered using 0.45 µm syringe filter into sterile polystyrene tubes and stored at -20 °C prior usage.

3.2.6.4 Mueller-Hinton Agar (MHA)

MHA agar was used to culture and maintain all bacteria tested in this study. For MHA preparation, 19 grams of MHA powder was weighed and transferred into a 1 L glass bottle, then 500 mL of distilled water was added to dissolve the MHA powder. The glass bottle was then autoclaved. After that, the sterilized medium was immediately poured into blank petri dishes and allowed to be solidified inside the laminar flow cabinet. Next, MHA plates were covered and sealed properly and then stored at room temperature prior usage.

3.2.6.5 Mueller Hinton Broth (MHB)

MHB was used for broth microdilution assay in this study. For MHB preparation, 11 grams of MHB powder was weighed and transferred into a 1 L glass bottle, then 500 mL of distilled water was added to dissolve the MHB powder. The broth was then autoclaved. After that, the glass bottle contained sterilized broth was sealed with laboratory film and stored at room temperature prior usage.

3.2.7 Preparation of Bacterial Culture

To prepare bacteria culture, the bacteria was obtained from the master plate and then subcultured on Mueller-Hinton agar (MHA). The subcultured plate with bacterial species was incubated at 37˚C for 24 hours. After 24 hours, a few single colonies was chosen from the subcultured plate and transferred into a sterile round

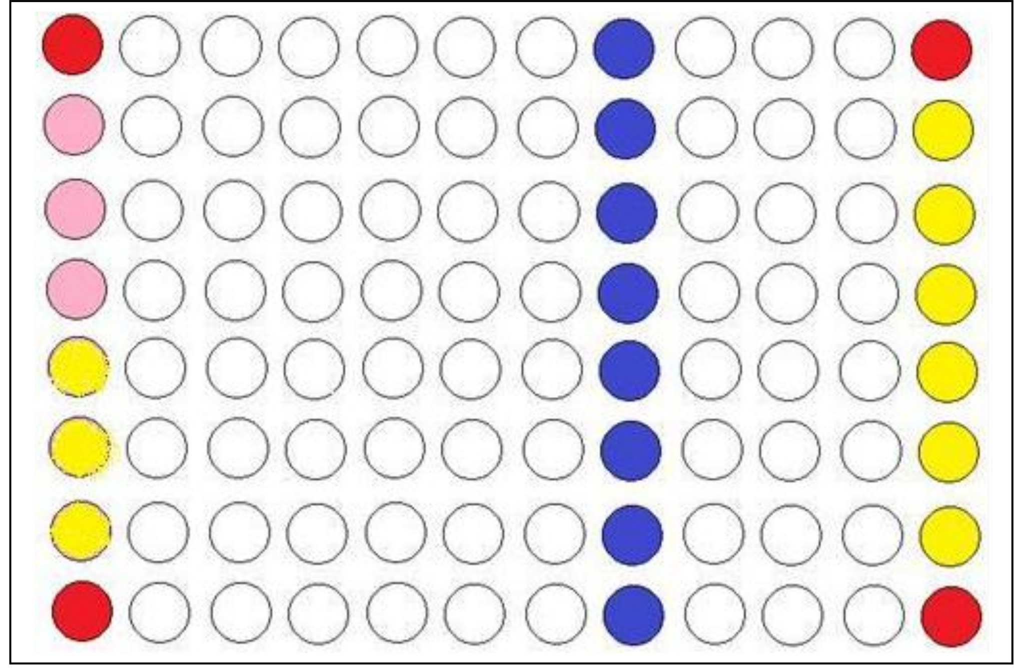
bottom tube containing 3 mL of Mueller-Hinton broth (MHB). Spectrophotometer was set to blank using MHB at 625 nm wavelength and turbidity of bacterial suspension in the round bottom tube was adjusted until it falls within the absorbance range of 0.08-0.10, which is equivalent to $1x10^8$ CFU/mL. Then, 50 µL of respective bacterial suspension was diluted to achieve a concentration of $1x10^6$ CFU/mL by pipetting into a sterile centrifuge tube which containing 4950 µL of MHB. Then, 50 µL of this diluted bacterial suspension was pipetted into sterile 96-well plates within 30 minutes. Final concentration in the well was $5x10^5$ CFU/mL.

3.2.8 Minimum Inhibitory Concentration (MIC) Assay

In order to prevent any contamination from happening, MIC assay was performed in the laminar flow cabinet. All sterile 96-well plates were labeled correctly as shown in Figure 3.1. First of all, 100 µL of MHB was pipetted into the four corners of 96-well plates which serve as the sterility control. Then, 50 µL of MHB was pipetted into each testing well and growth control wells, except the four corner wells. After that, 75 µL of MHB was pipetted into negative control wells. Then, 50 µL of each extract was pipetted into the first row of testing wells accordingly while 25 µL of extracts was added into negative control wells accordingly. After that, 50 µL of antibiotic was pipetted into first well of positive control column and followed by a serial two fold dilution using a multi-channel micropipette. After serial dilution, 50 µL of solution was discarded from last row

of the plates. Finally, 50 µL of prepared bacterial suspension was added into each well except sterility control and negative control wells. The final concentrations of extracts in different wells were 2.50, 1.25, 0.63, 0.31, 0.16, 0.08, 0.04 and 0.02 mg/mL respectively. Besides, the concentration of first well of the positive control column was 128 µg/mL, followed by 64, 32, 16, 8, 4, 2 and 1 µg/mL respectively. Sterility control wells contain 100 µL of MHB. Negative control wells contained 75 µL of MHB and 25 µL of extracts while growth control wells contained 50 µL of MHB and 50 µL of bacterial suspension. All plates were covered and sealed properly with parafilm and incubated at 37 °C for 24 hours.

After incubation, 20 µL of INT solution was added into each well and followed by orbital shaking of 60 rpm for 10 minutes. After 10 minutes, the plates were incubated at 37 °C for 15 minutes for color development. Results and observations were recorded. The wells which remained yellowish color indicate that the extracts inhibited bacterial growth, while the wells which showed pink or red color indicate the growth of bacteria. This antibacterial assay was carried out in triplicate for each bacterial strain.



100 µL of MHB acts as sterility control

75 µL of MHB + 25 µL of extract acts as negative control

50 µL of MHB + 50 µL of bacteria acts as growth control

50 µL of MHB + 50 µL of antibiotic acts as positive control

Foot note: Hex=Hexane, $CHCl_3$= Chloroform, EA= Ethyl acetate, EtOH= Ethanol, Met= Methanol, Aq= Distilled water.

Figure 3.1: Design of a 96-well plate for MIC assay

3.2.9 Minimum Bactericidal Concentration (MBC) Assay

Based on MIC result, 20 µL of medium was pipetted out from the wells which remained yellowish color after adding INT into labeled MHA plates and spread evenly with a sterile cotton bud. The plates were sealed with parafilm and incubated at 37 °C for 24 hours. After incubation, the number of colony was counted and recorded.

3.3 Calculation

3.3.1 Percentage of Yield of Each Extract

The percentage of yield of each plant extract was calculated by using the formula shown below:

$$\text{Percentage of yield of each extract} = \frac{\text{Dry weight of each extract}}{\text{Fresh weight of plant sample}} \times 100\%$$

3.3.2 Total Activity

Total activity of an extract is refers to the efficacy of the active constituents present in 1 g which can be diluted and still able to inhibit the growth of tested bacteria (Eloff 2004).

$$\text{Total activity} = \frac{\text{Quantity of material extracted from 1g of plant material}}{\text{Minimum Inhibitory Concentration (g/mL)}}$$

3.3.3 Bacterial Susceptibility Index (BSI)

Bacterial Susceptibility Index (BSI) which expressed in percentage is used to compare the relative susceptibilities among the bacterial strains. BSI can be ranged from 0% (bacterial strain is resistant to all extracts) to 100% (bacterial strain is susceptible to all extracts) (Bonjar 2004).

$$\text{BSI (\%)} = \frac{\text{100x number of extracts effective against each bacterial strain}}{\text{Number of total extracts}}$$

CHAPTER 4

RESULTS

4.1 Extraction of Plant

Table 4.1 shows the fresh weight of plant sample, the appearance and color, dry weight as well as percentage of yield (%) for each extract of *Lobelia chinensis*. According to Table 4.1, the total dry weight of the plant extracts was 15.07 g and the total percentage of yield of all extracts was 18.58%. The ethanol extract of *Lobelia chinensis* gave the highest percentage of yield which was 9.82% among all extracts. The second highest percentage of yield was aqueous extract that gave 4.98%, followed by the methanol extract, ethyl acetate extract, chloroform extract and the least hexane extract which gave 1.99%, 0.75%, 0.65% and 0.39% only respectively. From Table 4.1, it clearly indicates that most of the chemical compounds in *Lobelia chinensis* dissolved better in polar solvents especially in ethanol than non-polar solvents. Figure 4.1 shows the color of filtered extracts of *Lobelia chinensis*.

Table 4.1: Fresh weight of plant sample, appearance, dry weight and percentage of yield (%) of each extract for *Lobelia chinensis*.

Plant name	Fresh Weight of Plant (g)	Solvent	Appearance and color of extract	Dry weight of extract (g)	Percentage of yield (%)
Lobelia chinensis	81.10	Hexane	Clear, pale yellow	0.32	0.39
		Chloroform	Clear, dark yellow	0.53	0.65
		Ethyl acetate	Clear, yellow	0.61	0.75
		Ethanol	Clear, Yellow	7.96	9.82
		Methanol	Clear, light yellow	1.61	1.99
		Aqueous	Clear, Light orange	4.04	4.98
			Total	15.07	18.58

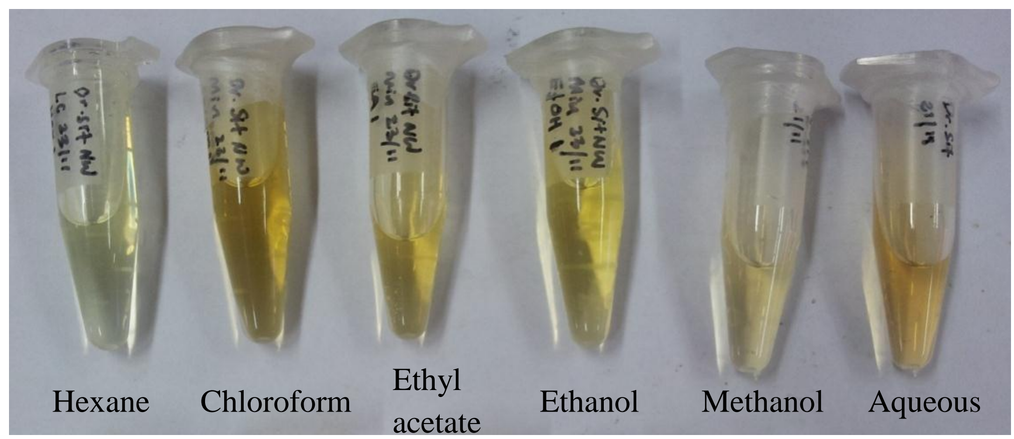


Figure 4.1: Color of the extracts of *Lobelia chinensis.*

4.2 Minimum Inhibitory Concentration (MIC) Assay

Tables 4.2 and 4.3 show the MIC (mg/mL) value of each plant extract on Gram-negative bacteria, *Acinetobacter baumannii*, *Escherichia coli*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* as well as Gram-positive bacteria which were *Bacillus cereus* and *Staphylococcus aureus*. All bacterial strains were susceptible to both antibiotics that used in this study which served as positive control, chloramphenicol and tetracycline.

From Table 4.2, all of the six extracts of *Lobelia chinensis* did not show inhibitory activity against *Acinetobacter baumannii*, *Escherichia coli*, and *Pseudomonas aeruginosa*. *Klebsiella pneumoniae* and *Bacillus cereus* were susceptible to five extracts of *Lobelia chinensis*, except for the aqueous extract with the MIC value ranged from 0.42-2.50 mg/mL and 0.13-1.67 mg/mL respectively. For *Staphylococcus aureus*, four extracts were able to exert inhibitory activity against

it except the methanol and aqueous extract with MIC value ranged from 0.31-2.50 mg/mL.

From Table 4.3, all of the six extracts of *Ipomoea batatas* did not show inhibitory activity against *Acinetobacter baumannii* and *Escherichia coli*. For *Klebsiella pneumoniae* and *Staphylococcus aureus*, only hexane extract was able to inhibit both bacterial strains with the MIC values of 0.31 mg/mL and 0.63 mg/mL respectively. Four extracts except hexane and chloroform extracts showed inhibitory activity against *Pseudomonas aeruginosa* with the MIC value ranged from 1.25-1.67 mg/mL. *Bacillus cereus* was susceptible to hexane, ethanol and methanol extracts only with MIC values of 0.16, 2.08 and 2.08 mg/mL respectively.

Table 4.2: Minimum inhibitory concentration value of *Lobelia chinensis* on six bacterial strains.

[1]Extracts	Bacterial Strains					
	A. baumannii	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***B. cereus***	***S. aureus***
Hexane	NA	NA	0.42±0.18	NA	0.13±0.05	0.31±0
Chloroform	NA	NA	1.04±0.36	NA	0.31±0	1.04±0.36
Ethyl acetate	NA	NA	1.25±0	NA	0.63±0	1.25±0
Ethanol	NA	NA	1.67±0.72	NA	0.63±0	2.50±0
Methanol	NA	NA	2.50±0	NA	1.67±0.72	NA
Aqueous	NA	NA	NA	NA	NA	NA
*Chloramphenicol	-	-	2.00±0	-	4.00±0	4.00±0
*Tetracycline	1.34±0.58	2.00±0	-	4.00±0	-	-

Mean ± Standard deviation, n=3

[1] Concentration of extracts is in mg/mL; *Concentration of Chloramphenicol or Tetracycline is in µg/mL

"-": The particular antibiotic was not used in the MIC assay; NA: No activity

Table 4.3: Minimum inhibitory concentration value of *Ipomoea batatas* on six bacterial strains.

[1]**Extracts**	**Bacterial Strains**					
	A. baumannii	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***B. cereus***	***S. aureus***
Hexane	NA	NA	0.31±0	NA	0.16±0	0.63±0
Chloroform	NA	NA	NA	NA	NA	NA
Ethyl acetate	NA	NA	NA	1.25±0	NA	NA
Ethanol	NA	NA	NA	1.25±0	2.08±0.72	NA
Methanol	NA	NA	NA	1.25±0	2.08±0.72	NA
Aqueous	NA	NA	NA	1.67±0.72	NA	NA
*Chloramphenicol	-	-	2.00±0	-	4.00±0	4.00±0
*Tetracycline	1.34±0.58	2.00±0	-	4.00±0	-	-

Mean ± Standard deviation, n=3

[1] Concentration of extracts is in mg/mL; *Concentration of Chloramphenicol or Tetracycline is in µg/mL

"-": The particular antibiotic was not used in the MIC assay; NA: No activity

4.3 Minimum Bactericidal Concentration (MBC) Assay

By referring to MIC result, only broth that remained as yellowish color in the well after INT addition was pipetted into MHA plates for MBC assay. Therefore, determination of MBC value was done by observing the presence of bacteria colonies growth on MHA plate. The lowest concentration of extract which show no colony growth or less than or equal to eight colonies was considered as the MBC value.

As shown in Table 4.4, five extracts of *Lobelia chinensis* excluded the aqueous extract showed bactericidal activity against *Klebsiella pneumoniae* with the MBC value ranged from 0.42-2.50 mg/mL. The hexane, chloroform and ethyl acetate extracts showed MBC value against *Bacillus cereus* which was 0.13, 0.31 and 0.63 mg/mL respectively. For *Staphylococcus aureus*, only the hexane, chloroform and ethyl acetate extracts were able to show MBC value of 0.63, 2.50 and 2.50 mg/mL respectively.

From Table 4.5, only the hexane extract of *Ipomoea batatas* was able to show bactericidal activity against *Klebsiella pneumoniae* and *Staphylococcus aureus* with the MBC value of 0.31 mg/mL and 1.25 mg/mL respectively. Besides, both ethanol and methanol extracts gave the same MBC value against *Pseudomonas aeruginosa* which was 2.50 mg/mL. The hexane, ethanol and methanol extracts showed MBC value against *Bacillus cereus* which was 0.16, 2.08 and 2.50 mg/mL respectively.

Table 4.4: Minimum bactericidal concentration value of *Lobelia chinensis* extracts on six bacterial strains.

[1]Extracts	Bacterial Strains					
	A. baumannii	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. cereus*	*S. aureus*
Hexane	-	-	0.42±0.18	-	0.13±0.05	0.63±0
Chloroform	-	-	1.04±0.36	-	0.31±0	2.50±0
Ethyl acetate	-	-	1.25±0	-	0.63±0	2.50±0
Ethanol	-	-	1.67±0.72	-	NA	NA
Methanol	-	-	2.50±0	-	NA	-
Aqueous	-	-	-	-	-	-

Mean ± Standard deviation, n=3

[1]Concentration of extracts is in mg/mL

NA: No activity

"-": MBC assay was not carried out since the extract did not show MIC result.

Table 4.5: Minimum bactericidal concentration value of *Ipomoea batatas* extracts on six bacterial strains.

[1]Extracts	Bacterial Strains					
	A. baumannii	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. cereus*	*S. aureus*
Hexane	-	-	0.31±0	-	0.16±0	1.25±0
Chloroform	-	-	-	-	-	-
Ethyl acetate	-	-	-	NA	-	-
Ethanol	-	-	-	2.50±0	2.08±0.72	-
Methanol	-	-	-	2.50±0	2.50±0	-
Aqueous	-	-	-	NA	-	-

Mean ± Standard deviation, n=3

[1]Concentration of extracts is in mg/mL

NA: No activity

"-": MBC assay was not carried out since the extract did not show MIC result.

4.4 Total Activity of Bioactive Extracts

According to Table 4.6, the ethanol extract of *Lobelia chinensis* has the highest magnitude of antibacterial activity, as the extract from this sample can be diluted in 155.79 mL of solvent and still can inhibit the growth of *Bacillus cereus*. Besides, the ethanol extract also showed the highest total activity of 58.77 mL/g and 39.26 mL/g against *Klebsiella pneumoniae* and *Staphylococcus aureus* respectively. On the other hand, the ethyl acetate extract has the lowest magnitude of antimicrobial activity and showed the same total activity of 6.02 mL/g against *Klebsiella pneumoniae* and *Staphylococcus aureus.*

According to Table 4.7, the ethanol extract of *Ipomoea batatas* has the highest magnitude of antibacterial activity, as the extract from this plant able to inhibit the growth of *Pseudomonas aeruginosa* and *Bacillus cereus* with the highest total activity of 30.22 mL/g and 18.16 mL/g respectively. However, the ethyl acetate extract has the lowest magnitude of antibacterial activity against *Pseudomonas aeruginosa* with a total activity of 1.45 mL/g only.

Table 4.6: Total activity of *Lobelia chinensis* extracts on six bacterial strains.

[1]Extracts	Bacterial Strains					
	A. baumannii	***E. coli***	***K. pneumoniae***	***P. aeruginosa***	***B. cereus***	***S. aureus***
Hexane	NA	NA	9.39	NA	30.35	12.73
Chloroform	NA	NA	6.28	NA	21.08	6.28
Ethyl acetate	NA	NA	6.02	NA	11.94	6.02
Ethanol	NA	NA	58.77	NA	155.79	39.26
Methanol	NA	NA	7.94	NA	11.89	NA
Aqueous	NA	NA	NA	NA	NA	NA

[1]Activity of extracts is in mL/g

NA: No activity

Table 4.7: Total activity of *Ipomoea batatas* extracts on six bacterial strains.

[1]Extracts	Bacterial Strains					
	A. baumannii	*E. coli*	*K. pneumoniae*	*P. aeruginosa*	*B. cereus*	*S. aureus*
Hexane	NA	NA	3.15	NA	6.11	1.55
Chloroform	NA	NA	NA	NA	NA	NA
Ethyl acetate	NA	NA	NA	1.45	NA	NA
Ethanol	NA	NA	NA	30.22	18.16	NA
Methanol	NA	NA	NA	4.22	2.54	NA
Aqueous	NA	NA	NA	2.71	NA	NA

[1]Activity of extracts is in mL/g

NA: No activity

4.5 Bacterial Susceptibility Index (BSI)

Table 4.8 indicates the BSI values for the six bacterial strains used for screening of 12 different extracts obtained from two plants. Based on Table 4.8, *Bacillus cereus* was the most susceptible bacteria among the six bacterial strains which showed BSI value of 66.67%, followed by *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* with BSI values of 50%, 41.67% and 33.33% respectively. However, *Acinetobacter baumannii* and *Escherichia coli* showed 0% of BSI which indicated that both strains were resistant to all the extracts of both plants.

Table 4.8: Bacterial Susceptibility Index (BSI) for different bacterial strains used for screening of 12 extracts of two plants.

Bacterial Strains	No. of active extracts	BSI values (%)
Acinetobacter baumannii	0	0
Escherichia coli	0	0
Klebsiella pneumoniae	6	50.00
Pseudomonas aeruginosa	4	33.33
Bacillus cereus	8	66.67
Staphylococcus aureus	5	41.67

CHAPTER 5

DISCUSSION

5.1 Plant Sample Preparation

During the plant preparation, the aerial parts including leaves, stems and flowers of *Lobelia chinensis* were washed under running tap water to remove the dirt and soil, air dried in room temperature and finally blended into rough powder form. Fresh or dried plant can be used as a source to obtain secondary plant components through varies of extraction methods (Olayinka and Anthony 2010; Tiwari et al., 2011). It is depends upon the unique biochemical, biophysical and energetic properties of the interested plant being extracted. Since the water content is different within different plant tissues, plants are usually air dried to a constant weight before extraction (Tiwari et al., 2011). However, a high yield of plant extract does not mean a higher antimicrobial activity (Armando and Dennis 2010).

The purpose of blending the plant sample into smaller pieces is to rupture its organ, tissue and cell structures so that its medicinal ingredients are exposed to the extraction solvent. Furthermore, size reduction maximizes the surface area, which in turn enhances the mass transfer of active principle from plant material to the solvent (Handa et al., 2008; Bart and Pilz 2011).

5.2 Plant Sample Extraction

A successful extraction of phytochemical compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure (Armando and Dennis 2010; Tiwari et al., 2011). Differences in polarity among various solvents have been reported to account for the differences in solubility of active plant components, hence different extracts and extract compositions will be yielded (Dellavalle et al., 2011). Solvents, such as methanol, ethanol, acetone, ethyl acetate, and their combinations have been used for the extraction of phenolics from plant materials (Dai and Mumper 2010). Besides chloroform, another most widely used solvent to extract edible oils or lipids from plant sources is hexane due to its high oil solubility and ease of recovery (Wang et al., 2005).

Sequential extraction method was performed in this study in which the plant samples were immersed in the solvent with increasing polarity in order to ensure that a wide polarity range of secondary metabolites can be extracted from plant samples as much as possible (Tiwari et al., 2011). This method has been reported as a useful and favorable technique for further study of plant extracts (Abubakar 2009). This method is essential to minimize the number of compounds in each solvent as compounds are dissolved in solvents according to their polarity. As the number of compounds increased in the extracts, the action of one compound can be interfered by another compounds present in the extract. This interfere effect is known as antagonistic effect which may occur in single solvent extraction

(Nanasombat and Lohasupthawee 2005). Therefore, the antagonistic effect will be at low level in the solvent extracts obtained by sequential extraction (Pathmanathan et al., 2010; Uthayarasa et al., 2010). In the sequential extraction method, partial separation of compounds will also facilitate further isolation and purification of active compounds (Pathmanathan et al., 2010; Uthayarasa et al., 2010).

5.3 Percentage of Yield for Bioactive Components

Based on Table 4.1 (page 53) it shows that the ethanol extract of *Lobelia chinensis* gave the highest percentage of yield which was 9.82% among all the extracts, then followed by 4.98% of aqueous extract and 1.99% of methanol extract. The result indicated that the percentages of yields of the polar solvents (ethanol, methanol and distilled water) from *Lobelia chinensis* were much higher if compared to the non-polar solvents (hexane and chloroform). Usually, higher percentage of yield was obtained in polar solvents than non-polar solvents in plant samples (Pathmanathan et al., 2010). This indicates that the test plant materials were found to possess number of polar compounds than non-polar compounds since the plant compounds are more likely to dissolve in the solvent with similar polarity (Singh et al., 2002; Pathmanathan et al., 2010; Tiwari et al., 2011).

Major constituents in *Lobelia chinensis* are various alkaloids such as lobeline, lobelane, lobelanine, and lobelanidine (Shibano et al., 2001; Neugebauer et al.,

2007). Shibano et al. (2001) isolated two new pyrrolidine alkaloids, radicamines A and B from *Lobelia chinensis*. Others include lobelidine, lobelanine, nor-lobelaine, lobelanidine, nor-lobelanidine, and isolobenine, as well as 14 pyridine alkaloids (Mary 2008). According to Cowan (1999), alkaloid can be extracted from plant sample by using organic solvent such as ethanol. Since these alkaloid compounds were highly soluble in polar solvent such as ethanol and methanol, it explained that higher yield of the extracts were obtained in polar solvent rather than non-polar solvent.

5.4 Antimicrobial Susceptibility Test

By comparing both Table 4.2 (page 56) and Table 4.3 (page 57) which showed the MIC values for both plant extracts, the extracts from the aerial part of *Lobelia chinensis* has a greater efficiency than the *Ipomoea batatas*'s leaves extract in inhibitory the growth of tested bacteria. This was showed by the MIC value given by the *Lobelia chinensis*'s extract (0.13 – 2.50 mg/mL) compared to the *Ipomoea batatas* extracts (0.16 – 2.08 mg/mL). Besides, *Lobelia chinensis* extracts were more effective in bactericidal property compared to *Ipomoea batatas* extracts. This is showed by the MBC value given by the *Lobelia chinensis*'s extract (0.13 – 2.50 mg/mL) compared to the *Ipomoea batatas*'s leaves extracts (0.16 – 2.50 mg/mL) in Table 4.4 (page 59) and Table 4.5 (page 60). The factors such as type, composition and concentration of the plant extracts are responsible to the tested

plant's ability for exerting antimicrobial activity (Marino et al., 2001; Karatas and Ertekin 2010).

The major constituents that can be found in *Lobelia chinensis* are alkaloids such as lobeline, lobelane, lobelanine, and lobelanidine (Shibano et al., 2001; Neugebauer et al., 2007). Alkaloids are organic bases containing nitrogen in a heterocyclic ring which mostly have pronounced pharmacological activity. Most alkaloids have a strong bitter taste and are very toxic, for these reasons they are used by plants to defend themselves against herbivory, and attacks by microbial pathogens and invertebrate pests (Thovhogi 2009). Currently, no study has been reported on the antimicrobial activity of these alkaloids. However, Rotimi et al. (1988) reported that alkaloid extracts from plants generally possess broad-spectrum *in vitro* activity against a wide variety of microorganisms. Kuo et al. (2011) reported that lobechine and scoparone isolated from *Lobelia chinensis* had both anti-viral and anti-inflammatory properties. Besides, scoparone isolated from *Lobelia chinensis* significantly inhibited free radicals, making lobelia an effective natural antioxidant (Kuo et al., 2011; Murgananthan and Pabbithi 2012).

Joshi et al. (2011) reported that the antimicrobial activity exerted by the extract from Lobelia genus is due to the presence of terpenes. Terpenes or terpenoids are active against bacteria through the mechanism that involve membrane disruption by the lipophilic compounds (Cowan 1999; Tiwari et al., 2011). Besides terpenes, Joshi et al. (2011) also isolated constituents such as perilla ketone, isophytol and

small amount of α-phellandrene, δ-carene, limonene and mycrene from the plant belongs to Lobelia genus. There are several studies reported that plant extract containing perilla ketone (Yu et al., 2011) and isophytol (Saidana et al., 2008) possess the antimicrobial activity against bacteria. In addition, the components present at lower amounts such as α-phellandrene, δ-carene, limonene, and mycrene could also contribute to the antimicrobial activity of plant extracts (Oliviera et al., 2007; Barros et al., 2009; Liolios et al., 2009). Although the plant components present at lower concentration, however, they act synergistically with other active components (Marino et al., 2001).

Pochapski et al. (2011) had reported the presence of triterpenes or steroids, alkaloids, anthraquinones, coumarins, flavonoids, saponins, tannins, and phenolic acids as secondary metabolites with potential biological activities in the leaves extract of *Ipomoea batatas* (Yadav and Agarwala 2011). Flavonoids are hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring (Dixon et al., 1983; Yadav and Agarwala 2011). Since they are known to be synthesized by plants in response to microbial infection it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya et al., 1996; Cowan 1999).

Islam (2008) found that polysaccharides (30%) and protein (70%) were the main components of the antibacterial extract from *Ipomoea batatas* and these components were active against pathogenic *E. coli*, O157:H7, *Bacillus cereus* and *Staphylococcus aureus*. Polysaccharides and protein are soluble in polar solvents such as ethanol and aqueous and therefore, they can be found in ethanol extract of plant (Pace et al., 2004; Cao et al., 2011).

5.5 Future Perspectives

In this study, only four Gram-negative bacteria and two Gram-positive bacteria were used for susceptibility test. Therefore, a further study which involves a broader range of bacterial strains and fungal species as well as virus species should be carried out.

Furthermore, active secondary metabolites can be purified using the high performance liquid chromatography, column chromatography and thin layer chromatography to discover the new antibacterial drugs.

As for the plant extracts which did not give the significant MIC values, other parts of the plant such as the roots of *Lobelia chinensis* and the tuber of *Ipomoea batatas* can be used for screening in antimicrobial activity assay in future.

Last but not least, the bioactive metabolites which isolated from plant should be subjected to animal testing in order to determine their efficacy, mechanism of action as well as the toxicity of the metabolite. It is important so that the drug containing these compounds is safe for use and benefits to human being.

CHAPTER 6

CONCLUSION

The total percentage of yield of extracts from aerial part of *Lobelia chinensis* was 18.58%. *Lobelia chinensis* showed highest percentage of yields from polar extract, ethanol which was 9.82% than non-polar extract, hexane which gave the lowest percentage of yields, 0.39%.

Among the 12 plant extracts screened, nearly all of these extracts were active against at least one bacterial strain tested, except the aqueous extract from *L. chinensis* and chloroform extract from *I. batatas.* Extracts from *L. chinensis* were found to be more effective against bacterial strains compared to the extracts from *I. batatas*'s leaves.

Lobelia chinensis's extracts showed the same MIC and MBC ranges which was 0.13-2.50 mg/mL. The ethanol extract showed inhibitory activity against *B. cereus* and *S. aureus* with the MIC values of 0.63 mg/mL and 2.50 mg/mL respectively, but it was unable to show bactericidal activity against both bacterial strains. Same goes to the methanol extract which inhibited *B. cereus* with MIC value of 1.67 mg/mL, however it was unable to kill the respective bacteria.

Ipomoea batatas's leaves extract gave the MIC values ranged from 0.16-2.08 mg/mL and MBC values ranged from 0.16-2.50 mg/mL. *Pseudomonas aeruginosa* was able to be inhibited by the ethyl acetate and aqueous extracts from *I. batatas* with MIC values of 1.25 mg/mL and 1.67 mg/mL respectively, but no bactericidal activity was shown by these two extracts.

Among all the bacterial strains tested, *B. cereus* was the most susceptible bacteria which showed BSI value of 66.67%, whereas *A. baumannii* and *E. coli* showed 0% of BSI which indicated that none of the extract can inhibit their growth.

Overall, this study had proved that medicinal plant extracts do displayed promising antimicrobial activity which may leads to discovery of new antimicrobial drugs in future. Thus, the exact bioactive compounds of plants should be further isolated and purified in order to determine and identify the plant phytochemistry as well as their pharmacological activity.

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APPENDIX A

UNIVERSITI TUNKU ABDUL RAHMAN

FACULTY OF SCIENCE

Jalan Universiti, Bandar Barat, Kampar,

31900 Perak.

COLLECTOR: Leong Wei Min

DATE COLLECTED: 30th September, 2012

FAMILY: *Campanulaceae*

SCIENTIFIC NAME: *Lobelia chinensis*

SYNONYMS: *Lobelia chinensis* Lour, *Lobelia radicans* Thunb

LOCALITY: Endau, Johor

VERBAL: Chinese Lobelia, Chinese cardinal flower

HABITAT: Wet places especially around paddy fields

OCCURRENCE: Common

PLANT HEIGHT: Approximately 10 cm

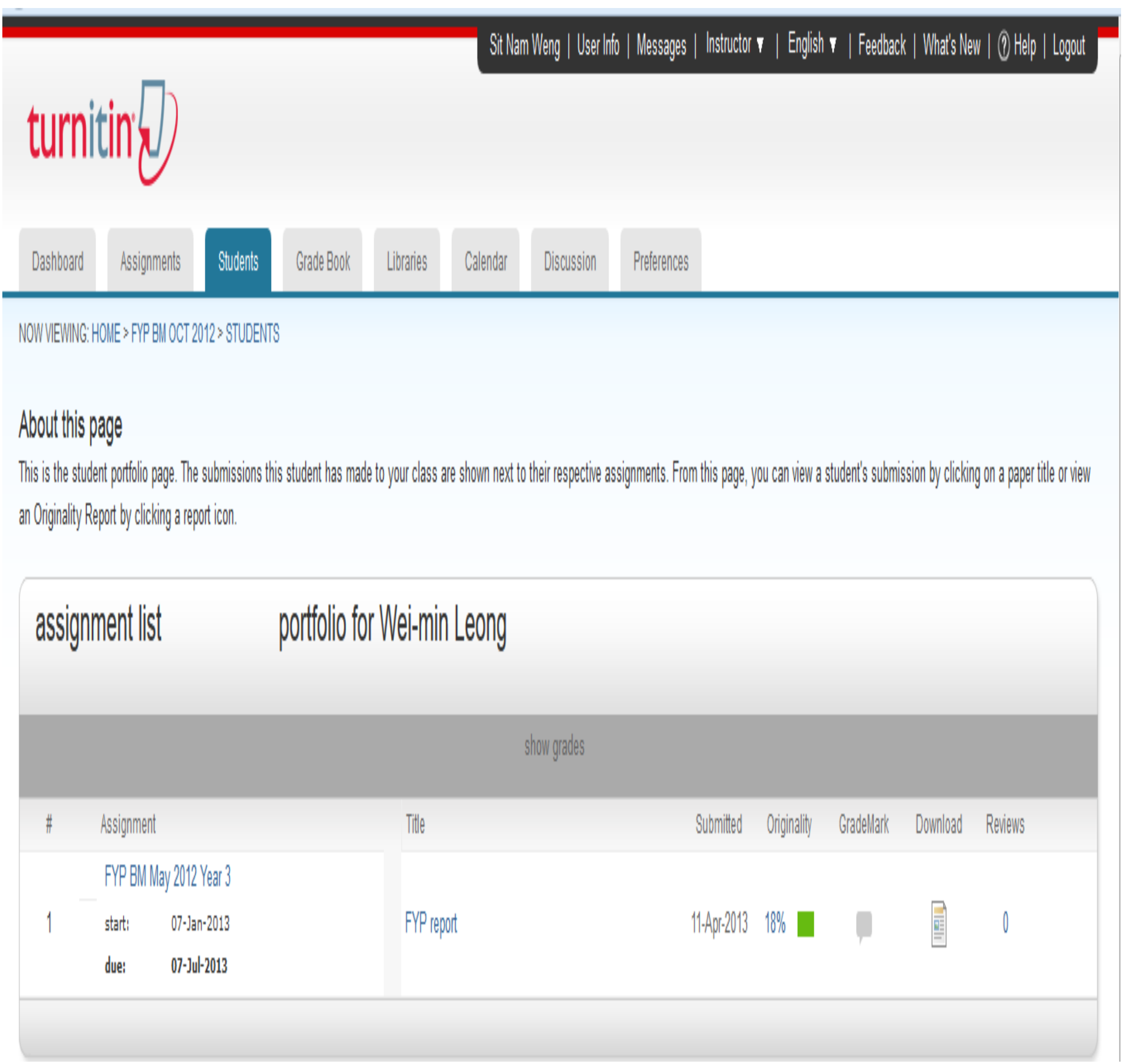
FLOWER COLOUR: Vary from white to rose-purple or bluish colour

FRUIT: Nil

EDIBLE / NON-EDIBLE: Non-edible

MEDICINAL PLANT (USAGE): Acts as an antidote, a hemostat, and as carcinostatic agents for stomach cancer in Chinese folk medicine. The whole plant can be used for anti-inflammatory, depurative and febrifuge purposes. The root is used for antisyphilitic, cathartic, and diuretic.

APPENDIX B



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