PRELIMINARY SCREENING OF CRUDE EXTRACTS OF CALOPHYLLUM SPECIES FOR CYTOTOXICITY, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES

By

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ABSTRACT

PRELIMINARY SCREENING OF CRUDE EXTRACTS OF CALOPHYLLUM SPECIES FOR CYTOTOXICITY, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES

Beulah Yeap Yian Ran

Calophyllum andersonii and Calophyllum gracilentum are both from the family of Calophyllaceae. These plants are native to Malaysia, Thailand and Brunei. Traditionally, Calophyllum species were used to treat burns, skin diseases, internal haemorrhage, eye ailments and orchitis. Ethyl acetate, dichloromethane and methanol stem bark extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* were evaluated for their antioxidant activity using DPPH assay, antibacterial activity against *Bacillus subtilis* subsp. *spizizenni*, *Bacillus cereus*, *Escherichia coli*, *Salmonella enterica* Thyphimurium and Methicillin-resistant *Staphylococcus aureus* using MIC and MBC assays and cytotoxic effect against MDA-MB-231 cancer cell line using MTT assay. In phytochemical screening, most of the crude extracts of Calophyllum species showed the presence of alkaloids, terpenoids, saponins, phenols, flavonoids, tannins, quinone, and glycosides. These metabolites exhibited widest range of R_f values based on TLC profile indicating the presence of both non-polar and polar compounds. In DPPH assay, ethyl acetate extract of *Calophyllum andersonii* and methanol extract of *Calophyllum gracilentum* exhibited the highest radical scavenging activity of

80.4±0.001% (EC_{50}=0.025 mg/mL) and 80.0±0.000% (EC_{50}=0.025 mg/mL), respectively. Ascorbic acid exhibited radical scavenging activity of 81.1±0.002% with EC_{50} of 0.050 mg/mL. In antibacterial assay, all the crude extracts showed MIC and MBC values in the range of 0.250 to 1.000 mg/mL, respectively against tested bacteria. Gentamycin sulfate exhibited MIC and MBC values of 0.040 mg/mL and 0.080 mg/mL, respectively. Among all the tested bacteria, methanol extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* showed bactericidal effect at 0.250 mg/mL against *Escherichia coli*. In MTT assay, the highest cytotoxic effect was shown by dichloromethane extract of *Calophyllum andersonii* and ethyl acetate and dichloromethane extracts of *Calophyllum gracilentum* against MDA-MB-231 cells with IC_{50} of 20.00 µg/mL, respectively. Meanwhile, doxorubicin hydrochloride exhibited IC_{50} of 4.00 µg/mL. The crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* are potential sources of antioxidant, antibacterial and cytotoxic agents. Thus, further investigations using pure compounds should be carried out to discover its potential as pharmaceutical drugs.

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DECLARATION

I hereby declare that the dissertation is based on my original work except for the quotations and citations which have been dully acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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(BEULAH YEAP YIAN RAN)

APPROVAL SHEET

This project report entitled "**PRELIMINARY SCREENING OF CRUDE EXTRACTS OF CALOPHYLLUM SPECIES FOR CYTOTOXICITY, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES**" was prepared by BEULAH YEAP YIAN RAN and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **BEULAH YEAP YIAN RAN** (ID No: 14ADB06400) has completed this final year "**PRELIMINARY SCREENING OF CRUDE EXTRACTS OF CALOPHYLLUM SPECIES FOR CYTOTOXICITY, ANTIOXIDANT AND ANTIBACTERIAL ACTIVITIES**" under the supervision of **Ms. SANGEETHA A/P ARULLAPPAN** (Supervisor) from the Department of Biomedical Science, Faculty of Science.

I hereby give permission to the University to upload the softcopy of my final year project in PDF format into the UTAR Institutional Repository, which may be made accessible to the UTAR community and public.

Yours truly,

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(BEULAH YEAP YIAN RAN)

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LIST OF ABBREVATIONS

AP1	Activator protein-1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetracetic acid
ERK	Extracellular signal-regulated kinase
FRAP	Ferric reducing anti-oxidant power
FBS	Fetal bovine serum
GC-MS	Gas chromatography-mass spectrophotometer
HCT-116	Human colorectal carcinoma cell line
HPLC	High performance liquid chromatography
IC_{50}	Half-maximal inhibitory concentration
INT	2-(4-iodo-phenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium
MAPK	Mitogen-activated protein kinase
MBC	Minimum bactericidal concentration
MDA-MB-231	Breast cancer cell line
MIC	Minimum inhibitory concentration
MOH	Ministry of Health
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth

MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCI	National Cancer Institute
NMR	Nuclear magnetic resonance
PBS	Phosphate buffer saline
R_f	Retention factor
ROS	Reactive oxygen species
TEAC	Trolox equivalent anti-oxidant capacity
TLC	Thin layer chromatography
TRAP	Total radical trapping anti-oxidant potential
UV	Ultraviolet
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Natural products have been the most successful source of potential drug in medicines for the treatment of a wide spectrum of diseases with minimal side effects. Natural products are chemical compounds that are derived from living organisms, including plants, animal, insects and microorganisms (Dias, Urban and Roessner, 2012). Plant constituents comprise a wide variety of organic substances which can be categorized as primary and secondary metabolites. The primary metabolites are protein, carbohydrates, lipids, nucleic acids and enzymes which play a vital role in life functions of plants such as photosynthesis and respiration. These metabolites are commonly present in all organisms in large amounts. On the other hand, secondary metabolites are not essential for organism's development, growth or reproduction, but are needed by the plants as a defense system. These metabolites are pharmacologically active natural plant products and synthesize in small quantities for the purpose of survival (Dewick, 2009).

To date, natural products still represent over 50% of all drugs in clinical use (Balandrin, Kinghorn and Farnsworth, 1993). Malaysia is one of the 12 mega biodiversity countries in the world that has approximately 19.12 million hectares of rainforest with 8100 plant species (Wong, 2009). However, only less than 5% of these plants were reported to show medicinal values (Jantan, 2004). According to Kashman, et al. (1992), the active compound calanolide

A, which was isolated from *Calophyllum lanigerum* is able to prevent HIV-1-induced cytopathic effect in human T-lymphoblastoid cells, and thus inhibiting HIV replication. Therefore, the National Cancer Institute in the United States has selected calanolide A as a drug candidate for early clinical trials (Fabricant and Farnsworth, 2001).

According to World Health Organization (WHO) (2017a), cancer is a disease characterized by the abnormal growth of cells beyond their boundaries that can invade and spread to other parts of the tissues. Cancer is the second leading cause of death globally and accounted for 8.8 million deaths in 2015. Breast, colorectal, lung, stomach and cervix cancer are the most common types of cancer in women, while lung, prostate, colorectal, stomach and liver cancer are the most common among men (WHO, 2015). According to The Star Online (2016), about 100,000 Malaysians suffer from cancer each year and by year 2020, the number of cancer cases is expected to increase by 15%.

In spite of the availability of a large number of anticancer drugs and various chemotherapy options, these therapies have many severe side effects such as anemia, alopecia, fatigue, thrombocytopenia, nausea and short-term memory loss (Singh and Singh, 2012). Thus, natural products which are easily accessed, lesser side effects and possess active metabolites with cytotoxic effects on cancer cells, would be a better option as a source of medicines for humans (Siddiqui, 1993).

According to WHO (2017b), infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, parasites or fungi and can be spread directly or indirectly from one person to another. Infectious diseases caused by multidrug-resistant (MDR) bacteria have become a public health concern all over the world. These bacteria drastically reduced the efficacy of antibiotics, consequently, increasing the therapeutic failure and mortality rate. It is estimated that every year, around 25,000 patients die due to infections with MDR bacteria (Fankam, Kuiate and Kuete, 2017).

Due to these problems, many scientists are searching for new antimicrobial substances. The screening of plant extracts and natural products for their antimicrobial activity has shown a potential source of novel antibiotic prototypes (Dzotam, Touani and Kuete, 2016). Many studies reported that plants and their active metabolites possess antimicrobial activity and resistance modifying effects. Thus, with increased incidence of resistance to antibiotics, medicinal plants could be an alternative treatment for infectious diseases (Seukep, et al., 2016). Aminudin, et al. (2016), reported that chromanone acids from *Calophyllum inophyllum* showed antibacterial activity towards *Staphylococcus aureus* in disc diffusion assay.

The present study evaluates the antioxidant, antibacterial and cytotoxic effect of the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum*. Calophyllum species that are native to Malaysia have been reported to be rich in secondary metabolites responsible for various biological activities (Mah, et al., 2015). However, there are still lacking of

phytochemical and biological studies on *Calophyllum andersonii* and *Calophyllum gracilentum*. Hence, preliminary screening for both species was carried out in this study.

The objectives of this research are:

1. To detect the presence of metabolites using thin layer chromatography and qualitative phytochemical screening,
2. To determine the percentage of radical scavenging activity of the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* via DPPH assay,
3. To determine the antibacterial activity of the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* via MIC and MBC assays,
4. To determine the cytotoxicity of the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* in MDA-MB-231 cells via MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Calophyllum Species

2.1.1 Botanical Description

Calophyllum andersonii and *Calophyllum gracilentum* are both originated from the genus Calophyllum. Calophyllum belongs to the Guttiferae or Clusiaceae family, which comprised of approximately 200 species (Nasir, et al., 2013). Generally, these species are found in tropical rain forest and locally known in Malaysia as "bintangor" (Aminudin, Ahmad and Taher, 2015). The taxonomical classification of *Calophyllum andersonii* and *Calophyllum gracilentum* is as shown in **Table 2.1**.

Calophyllum species is a medium-sized evergreen tree, growing up to 25 to 35 m in height. It has a thick trunk covered with a rough, greyish-brown, fissured bark. The outer bark with a characteristic of diamond to boat-shaped fissures secretes a yellowish-white resin, whereas the inner bark which is pink to red in color and darkens to brownish on sun exposure is usually thick, firm, fibrous and laminated (Orwa, et al., 2009). The leaves of the trees are dark green in color, elliptic to ovate in shaped with a blunt end, 8 cm long and 5 to 7 cm wide. There are many distinct parallel veins perpendicular to the mid vein of the leaves. The flowers are white, 2.5 cm wide and have a very strong sweet fragrance (Gilman and Watson, 1993). The flower of Calophyllum species displays the properties of hermaphroditic with an ovary, basal and anatropous

ovule (Orwa, et al., 2009). The fruits of Calophyllum species is in green color, has the appearance of a spherical drupe-like berry and contains seed with a nutlike kernel that may be poisonous. **Figure 2.1** shows the tree and fruits of Calophyllum species.

Table 2.1: Taxonomical classification of *Calophyllum andersonii* and *Calophyllum gracilentum* (Global Biodiversity Information Facility, 2016).

Rank	**Taxonomical Classification**	
Kingdom	Plantae	Plantae
Phylum	Tracheophyta	Tracheophyta
Class	Magnoliopsida	Magnoliopsida
Order	Malpighiales	Malpighiales
Family	Calophyllaceae	Calophyllaceae
Genus	Calophyllum	Calophyllum
Species	*Calophyllum andersonii*	*Calophyllum gracilentum*

Figure 2.1: Calophyllum tree (left) and its fruits (right) (Orwa, et al., 2009).

2.1.2 Habitat and Geographical Distribution

Calophyllum andersonii and *Calophyllum gracilentum* are mainly distributed in Sarawak, Brunei, West and East Kalimantan (Lee, et al., 2017). However, other species of Calophyllum can also be found in Australasia, Madagascar, Eastern Africa, South and Southeast Asia, the Pacific islands, the West Indies and Latin America (Taher, et al., 2010). Calophyllum species is normally found in swamp forests from sea level to the peak of hills (Lee, et al., 2017).

2.1.3 Traditional and Medicinal Uses

Previous reports indicated that the Calophyllum species are widely used in traditional medicine to treat ailments. Its seed oil is employed as a remedy for rheumatism and ulcer ailments (Díaz, 2013). An infusion of the leaves is used to treat inflamed eyes. Besides, the mature fruits can be burned as a mosquito repellent. Since the wood of the tree is hard, it is used to build boats, flooring, furniture and made into plywood (Díaz, 2013).

Calophyllum is rich in coumarins, xanthones, flavonoids, and triterpenes. These compounds have been reported to have antioxidant, anti-inflammatory, cytotoxic, anti-HIV and antimicrobial activities (Lee, et al., 2017).

2.1.4 Pharmacological Studies

2.1.4.1 Antioxidant Activity

The bioactive compounds isolated from Calophyllum species demonstrated antioxidant properties specifically by inhibiting lipid peroxidation. Based on Taher, et al. (2010), dichloromethane extract of *Calophyllum rubiginosum* showed high antioxidant activity with an IC_{50} value of 0.11 mg/mL, followed by methanol and hexane extracts with IC_{50} values of 0.23 and 4.5 mg/mL. The antioxidant activity was reported due to the presence of xanthones and coumarins.

2.1.4.2 Anticancer Activity

Alkhamaiseh, et al. (2015) reported that the hexane and dichloromethane fractions of *Calophyllum rubiginosum* showed inhibition against lung cancer cell line (A-549) with IC_{50} values of 24.2 and 26.5 μg/mL respectively. These fractions inhibited at lower cell viability of 35 and 39.8% respectively. However, methanol fraction showed no effect against A-549 cells.

Furthermore, coumarin isolated from Calophyllum species has also received significant attention following the discovery of anti HIV RT-1 as cytotoxic agents (Mckee, et al., 1996). Aminudin, et al. (2016) reported that coumarins exhibited cytotoxic effect against KB cell line, K562, U251 and PC3 human tumor cell lines.

2.1.4.3 Antibacterial Activity

The hexane and dichloromethane fractions of *Calop*hyllum *canum* stem bark inhibited *Bacillus cereus* and *Staphylococcus aureus* with minimum inhibitory concentration (MIC) value of 12.5 μg/mL and 25 μg/mL, respectively. However, these fractions showed no activity against *Escherichia coli, Psedomonas aeruginosa, Candida albicans* and *Cryptococcus neoformans* (Alkhamaiseh, et al., 2012).

Aminudin, et al. (2016) proved that inophyllum D, inophyllum H, calanone and chromanone acid isolated from *Calophyllum symingtonianum* showed anti-bacterial activity towards *Staphylococcus aureus, Bacillus subtilis, Pseudomonas aeruginosa and Escherichia coli*. However, isocordato-oblongic acid showed moderate antibacterial activity against *S. aureus and B. subtilis* at 125 μg/mL and 62.5 μg/mL, respectively (Aminudin, et al., 2016).

2.2 Cancer

Cancer or neoplasm is a collection of diseases affecting any parts of the body and often characterized by the uncontrolled growth and invasion (metastasis) of cells via blood circulation and lymphatic system (World Health Organization [WHO], 2017a). Normally, healthy cells grow and replicate in a controlled way to increase cell number. However, when genetic material of a cell is mutated, damaged or changed, the growth and division of the healthy cells will become uncontrolled and hence forming mass of tissue known as tumor (Crosta, 2015). There are two types of tumor which are the rapid-

growth, invasive malignant tumor and slow-growth, non-invasive benign tumor (Crosta, 2015).

Cancer is one of the leading causes of death in Malaysia. According to National Cancer Registry in the year 2007 to 2011, there were a total of 103,507 new cancer cases been diagnosed in Malaysia which increased five times from 2003 (21,464 cases of cancer) (Periasamy, et al., 2017). It is estimated that the annual incidence of cancer is 30 000 (Lim, 2002). The three most common cancers in Malaysia from 2007 to 2011 were breast, colorectal and lung cancer. The most common cancer among males and females were colorectal and breast cancer respectively (National Cancer Institute [NCI], 2015). In a recent update, it is estimated that incidence rate of cancer will rise to approximately 56,000 cases in 2025 with the ratio of one out of four Malaysian (National Cancer Society of Malaysia, 2016).

The conventional treatment for cancer includes chemotherapy, radiotherapy and surgery, which have contributed to the increase of survival rates in cancer patients (Periasamy, et al., 2017). However, these treatments can cause damaging psychological effects such as depression, anxiety and poor quality of life in cancer patients (Singh and Singh, 2012). Besides, other common side effects are anemia, alopecia, thrombocytopenia, nausea and vomiting (NCI, 2015). Thus, medicinal plants act as a potential source of drugs to cure cancer as these metabolites are relatively safer with minimal side effects than synthetic drugs (Khan, et al., 2008).

2.3 Cancer Cell Line

Various cancer cell lines were obtained from cell culture techniques. Cell culture refers to a method of growing cells *in vitro* in a favorable artificial environment. The cells may be removed from the tissues directly before cultivation or derived from a cell line or cell strain that has already been established (Thermo Fisher Scientific, 2017). Cell culture technique is important in cellular and molecular biology, providing a model system for studying the normal physiology and biochemistry of cells. Besides, it also helps in studying the effect of drugs and toxic compounds on the cells, mutagenesis and carcinogenesis. The advantages of cell culture are the applications are consistency and reproducibility of results using a batch of clonal cells (Thermo Fisher Scientific, 2017).

In this research, MDA-MB-231 cell line was used. MDA-MB-231 is an estrogen receptor alpha negative human breast epithelial carcinoma cell line which was derived from mammary gland of a 51-year-old Caucasian female with breast adenocarcinoma (American Type Culture Collection, 2014). These adherent monolayer cells grow in spindle-shape as well as fibroblast-like shape and adapt to culture conditions due to its polyclonal cell characteristics (Gozgit, et al., 2006; Yin, 2011). MDA-MB-231 cells are highly aggressive, invasive and poorly differentiated human breast cancer cell line. These cells are characterized by their significant metastatic pattern that involves the routes of lymph nodes, bone marrow, liver and lung (Müller, et al., 2001). MDA-MB-231 cells mediate proteolytic degradation of the extracellular matrix through increased expression of matrix metalloproteinases (Yin, 2011). MDA-

MB-231 cells may develop resistance to several anticancer agents (Zordoky, et al., 2014).

2.4 Infectious Diseases

Infectious diseases are caused by pathogenic microorganisms such as bacteria, viruses, parasites or fungi that can be spread from one person to another (WHO, 2017b). According to Doughari and Manzara (2008), approximately 50,000 deaths due to infectious diseases are reported all around the world every year. In 2013, Malaysia has reported to have a high incidence of tuberculosis, dengue and hand-foot-and-mouth diseases with a rate of 78.28, 143.27 and 78.52 per 1,000 live births, respectively.

Infectious diseases also can be transmitted through ingestion of contaminated food or drinks and through zoonotic transmission (Mayo Clinic, 2014). Since our immune system acts as a protective barrier, thus most of the infectious agents are harmless. However, under certain conditions, some are capable of breaching the immune system, evading defense mechanisms and cause disease. Signs and symptoms of infectious diseases often include fever and fatigue but they also vary depending on the causative agents (Mayo Clinic, 2014).

The discovery of antibiotics, antiviral, antifungal and antiparasitic drugs in pharmaceutical industries are important breakthrough in medical history. Unfortunately, bacteria are very adaptable and the misuse or overuse of

antibiotics has made many bacteria resistant to antibiotics. Antibiotic resistance occurs when bacteria are no longer sensitive to drugs that should eliminate an infection (Melinda, 2017; Robert, 2016). Antibiotic-resistant bacterial infections are potentially very dangerous and increase the risk of death. About 2 million people in the U.S. suffer from antibiotic resistant infections each year and 23,000 die due to the condition (Robert, 2016). Therefore, medicinal plants can be a potential source of drug to cure infectious disease without side effects (Pan, et al., 2013).

2.5 Bacteria

Bacteria are simple and microscopic single-cell microorganisms that are found in diverse environments. Bacterial DNA floats freely in a thread-like mass called the nucleoid (Tortora, Funke and Case, 2013). Bacteria can be classified based on cell walls, shape, or the differences in their genetic makeup. The Gram stain method is used to identify bacteria by the composition of their cell walls. Gram-positive bacteria will be stained purple because of their thick peptidoglycan cell, whereas Gram-negative bacteria whose cells walls have two layers take on a red coloring (Vidyasagar, 2015).

2.5.1 *Escherichia coli*

Escherichia coli or *E. coli* is a Gram-negative, rod-shaped facultative anaerobic bacterium, which possesses metabolism that is both fermentative and respiratory. *E.coli* is a non-spore forming, motile bacterium that is 0.1 to

0.5 μm in diameter and 1.0 to 2.0 μm in length. *E. coli* showed indole-positive, catalase-positive, oxidase-negative and citrate-negative in the biochemical analysis (Huang, Chang and Chang, 2001). *E. coli* is commonly found as the normal flora of the gut and produce vitamin K2 that is benefit to their hosts and prevent the colonization of pathogenic pathogens within the intestine. However, certain strains of *E.coli* are pathogenic, which can cause various diseases, for instance, urinary tract infection (UTI), bacteremia and traveler's diarrhea (Madappa, 1994).

2.5.2 *Bacillus cereus*

Bacillus cereus or *B. cereus* is a Gram-positive, rod-shaped facultative anaerobe. *B. cereus* is a motile and spore-forming bacterium that is approximately 5 to 10 μm in length and 1 μm wide. *B. cereus* usually arranged singly or in short chains and found naturally in soil, water and dust. *B. cereus* possesses flagella that aid in motility (Todar, 2012a). *B. cereus* infection causes food borne illness that is caused by enterotoxins produced during vegetative growth in the small intestine and emetic toxin is produced by growing cells in the food (Granum and Lund, 1997).

2.5.3 *Bacillus subtilis* subsp. *spizizenii*

Bacillus subtilis or *B. subtilis* is Gram-positive, rod-shaped bacteria that are naturally found in soil. *B. subtilis* is an aerobe organism that requires oxygen to grow and unable to undergo fermentation. *B. subtilis* is also an endospore

forming bacterium whereby the endospore allows it to withstand extreme temperatures and dry environments (Kirk, 2009). *B. subtilis* are non-pathogenic and can contaminate food, but seldom resulting in food poisoning. This bacterium is used as fungicide for soybean seeds and vegetables as a fungicide. Some strains of *B. subtilis* can cause rots in potatoes (Ucar, 2015).

2.5.4 *Staphylococcus aureus*

Staphylococcus aureus or *S. aureus* is a Gram-positive that are non-motile and non-spore forming bacteria. It is a coccus shaped bacteria with approximately 0.5 to 1.5 μm in diameter and arranged in a grape-like cluster. *S. aureus* is also a facultative anaerobe that forms yellow colony on rich medium and often hemolytic on blood agar (Loir, et al., 2003; Turnidge, et al., 2008; Todar, 2012b). Today, methicillin-resistant *Staphylococcus aureus* (MRSA) strains are found worldwide and most are multidrug resistant (Appelbaum, 2006; Appelbaum, 2007; Ryan and Ray, 2010). Although *S. aureus* is naturally found in nasopharynx and skin, however, it can also cause bacteremia (sepsis), pneumonia and endocarditis which can lead to heart failure or stroke (Centers for Disease Control and Prevention, 2012). Besides, the production of exfoliating toxins A and B causes scalded skin syndrome. Certain strains of *S. aureus* are able to produce toxic shock syndrome toxin (TSST-1), which are responsible for toxic shock syndrome cases (Parsonnet, et al., 2008; Ryan and Ray, 2010; Todar, 2012b).

2.5.5 *Salmonella enterica* Typhimurium

Salmonella enterica Typhimurium or *S. Typhi* is a rod-shaped, Gram-negative, flagellated facultative anaerobe that is mostly found in the mammalian gastrointestinal tract. It is a non-lactose fermenting bacteria and produces no gas when grown in triple sugar iron agar (TSI) medium (Pollack, 2003). *S. Typhi* causes typhoid fever in human and transmitted via fecal-oral route from the infected individuals to healthy ones. This disease is characterized by the sudden onset of a systemic fever, severe headache, diarrhea, nausea and loss of appetite. Untreated typhoid fever cases resulting in 12 to 30% of mortality rates while treated cases allow for 99% survival (Curio, et al., 2016; Pollack, 2003). The virulence factors of *S. Typhi* are the production of endotoxin and Vi antigen. Besides, *S. Typhi* also produces and excretes a protein known as "invasin" that enables non-phagocytic cells to take up the bacterium in order for it to live intracellularly (Pollack, 2003).

2.6 Thin Layer Chromatography

Chromatography is a technique that enables separation, identification, and purification of the components in a mixture. Chromatography is widely used in both qualitative and quantitative analysis based on the differential adsorption between the stationary and mobile phases (Coskun, 2016). Stationary phase in chromatography is either solid or liquid phases coated on the surface of a solid phase. Whereas, mobile phase is either gaseous or liquid phase flowing over the stationary phase (Coskun, 2016).

Thin layer chromatography (TLC) is used to separate mixture, as it is simple, fast and relatively cheap method. TLC allows the researchers to determine the presence of bioactive compounds and its polarity based one retention factor (R_f) values (Sasidharan, et al., 2011; Busia, 2016). TLC comprises of stationary and mobile phases in which the separation is based on the relative affinity of compounds towards the phases. Stationary phase in TLC is a thin aluminium plate coated with silica gel, whereas the mobile phase is an organic solvent (University of Leeds, 2016). The tested samples that contain various polarities of compounds will travel at different rates depending on their degree of adsorption on the stationary phase (Busia, 2016).

2.7 Bioassays

2.7.1 DPPH Assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is a commonly used assay to study the free radical scavenging activity in natural products. DPPH is a stable free radical and thus in the presence of antioxidant agents, the purple color DPPH radical will be reduced to yellow product by accepting a hydrogen atom from the antioxidants (Kedare and Singh, 2011). Antioxidant activity can be measured spectrophotometrically and the absorbance is directly proportional to concentration and color intensity.

2.7.2 MIC and MBC Assays

Minimum inhibitory concentration (MIC) is defined as the lowest concentration of an antimicrobial agent that required to inhibit the visible growth of a microorganism (Andrews, 2001). INT (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride) dye is an indicator for inhibition activity in MIC assay by observing its color changes. The inhibition activities of the antimicrobial agents are measured by the dehydrogenase activity in mitochondria of bacteria. Dehydrogenase in bacteria is able to reduce the colorless tetrazolium chloride salt in INT dye to pink color formazon. Therefore, viable cells will give pink color precipitate in the solution, whereas the dead cells will appear as colorless (Mwinyihija, 2011).

Minimum bactericidal concentration (MBC) is the lowest concentration of antimicrobial agent that required to kill the microorganism by 99.9% on antibiotic-free media (Andrews, 2001). The sample in MIC wells that shows inhibition will be inoculated onto the agar plate and the growth of the bacteria will be observed. MBC can be used to determine the bactericidal and bacteriostatic effect of the antimicrobial agents at each concentration (Pankey and Sabath, 2004). Bacteriostatic indicates agents that are able to prevent the growth of bacteria and bactericidal indicates agents that are able to kill bacteria (Pankey and Sabath, 2004).

2.7.3 MTT Assay

MTT (3- [4, 5-dimethylthiazol-2-yl]-2,5diphenyltetrazolium bromide) assay is a colorimetric assay used to determine viability of cancer cells upon treatment with natural products. The yellow color MTT is reduced to insoluble purple formazon crystals by metabolically active cells through the action of dehydrogenase to generate reducing equivalents such as NADH and NADPH. Dead cells lost the ability to convert MTT into purple formazon, thus color formation serves as a useful and convenient marker of the viable cells (Riss, et al., 2016). The insoluble purple formazon crystals are dissolved using dimethyl sulfoxide (DMSO) and the absorbance of the samples are measured spectrophotometrically (Hamid, et al., 2004).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

The stem-bark extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* were given by Dr. Lim Chang Kiang, Associate Professor, Department of Chemical Science, Universiti Tunku Abdul Rahman. These plants were collected in Sarawak in 2016.

3.1.2 Cancer Cell Line

Human breast epithelial carcinoma cell line (MDA-MB-231) (ATCC® HTB-26TM) was used in this research. The cryopreserved cells were cultured in Dulbecco's Modified Eagle medium. The cells were observed regularly using an inverted phase contrast microscope.

3.1.3 Test Bacteria

Glycerol stocks of *Bacillus subtilis* subsp. *spizizenii* (ATCC6633), *Bacillus cereus* (ATCC13061), *Escherichia coli* (ATCC25922), *Salmonella enterica* Thyphimurium (ATCC14028) and *Methicillin-resistant Staphylococcus aureus* (ATCC 29213) were obtained from -80°C. These cells were cultured and maintained on Mueller Hinton agar.

3.1.4 Chemicals and Solvents

The list of chemicals and solvents used throughout the research is shown in **Table 3.1**.

Table 3.1: List of chemicals and solvents used with respect to their brand and manufacturer.

Chemicals and Solvents	Brand/ Manufacturer
Ammonia	SYSTERM, Malaysia
Ascorbic acid	Gene Chem, Canada
Bromine water	Bendosen, Malaysia
Chloroform	QRëC^{TM} Grade AR
DPPH reagent	Calbiochem®, USA
DMEM	Capricorn Scientific, Germany
Dimethyl sulfoxide	Merck, Germany
Doxorubicin hydrochloride	Fisher Scientific, New Jersey
Ethyl acetate (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Ferric chloride	SYSTERM, Malaysia
Fetal bovine serum	JR Scientific, Inc, USA
Gelatin	Biobasic, Canada
Gentamycin sulfate	Bio Basic Inc, Canada
Hexane (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Methanol (Industrial grade)	QRëC^{TM} Grade AR
MTT reagent	Merck, Germany
Mueller Hinton agar	HiMedia Laboratories, India
Mueller Hinton broth	Scharlau Chemical, Spain
Penicillin-streptomycin	Bio Basic Inc, Canada
Potassium dihydrogen phosphate	SYSTERM, Malaysia
Potassium chloride	SYSTERM, Malaysia
Silica gel	Merck, Germany
Silica-coated aluminum sheets	Merck, Germany
Sodium chloride	SYSTERM, Malaysia
Sodium dihydrogen phosphate	SYSTERM, Malaysia
Sulfuric acid	QRëC^{TM} Grade AR
95% ethanol (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
0.4% Trypan blue dye	Life Technologies, USA
0.25% Trypsin-EDTA	Biowest, USA

3.1.5 Equipment

Table 3.2 shows the list of equipment used throughout this research.

Table 3.2: List of equipment used with respect to their brand and manufacturer.

Equipment	**Manufacturer**
Autoclave	Hirayama, Japan
Centrifuge	Thermofisher, Malaysia
Electronic balance	Kern, ABJ, Australia
Freezer (-20°C)	Pensonic, Malaysia
Freezer (-80°C)	Thermo Scientific, USA
Fume hood	Qinos, Malaysia
Hemocytometer	Hecht-Assistant, Germany
Inverted phase contrast microscope	Olympus, Japan
Laminar flow	Edamix, Malaysia
Refrigerator (4°C)	Samemax, Malaysia
Sonicator	Elmasonic, USA
Vortex	Stuart, United Kingdom
Water bath	SASTEC, Malaysia
5% CO_2 incubator	BINDER, Germany

3.2 Phytochemical Screening

3.2.1 Phenols

Five hundred microliters of respective crude extract was treated with five drops of 5% ferric chloride in a test tube. The formation of black color indicates the presence of alkaloids (Wintola and Afolayan, 2015).

3.2.2 Terpenoids

Five hundred microliters of respective crude extract was added with 1 mL of chloroform in a test tube, followed by five drops of sulfuric acid (1M). Formation of reddish brown precipitate indicates the presence of terpenoids (Wintola and Afolayan, 2015).

3.2.3 Saponins

Six milliliters of distilled water was added to 500 µL of the respective crude extract in a test tube. The tube was closed with stopper and the mixture was shaken vigorously. Persistent foamy froth formation indicates the presence of saponins (Wintola and Afolayan, 2015).

3.2.4 Alkaloids

Five drops of Wagner's reagent (iodine and potassium iodide) were added to 500 µL of the respective crude extract. The presence of reddish brown precipitate indicates the presence of alkaloids (Wintola and Afolayan, 2015).

3.2.5 Tannins

Five drops of 1% gelatin was added to 500 µL of the respective crude extract in a test tube. Formation of white precipitate indicates the presence of tannins (Wintola and Afolayan, 2015).

3.2.6 Quinones

Formation of yellow precipitate indicating the presence of quinones upon the addition of five drops of sulfuric acid (1M) into 500 µL of the respective crude extract in a test tube (Wintola and Afolayan, 2015).

3.2.7 Glycosides

Five drops of 1% bromine water were added into 500 µL of the respective crude extract. The formation of yellow precipitate indicates the presence glycosides (Wintola and Afolayan, 2015).

3.2.8 Flavonoids

Five hundred microliters of ammonia and five drops of sulfuric acid (1M) were added into 500 µL of the respective crude extract. Formation of yellow precipitate confirms the presence of flavonoids (Wintola and Afolayan, 2015).

3.3 Thin Layer Chromatography

Silica coated aluminium thin layer chromatography (TLC) plates were cut in width of 5.0 cm and length of 10.0 cm. A baseline of 1.0 cm from the bottom and a frontline with 1.0 cm from the top were drawn on the plate using a pencil as shown in **Figure 3.1**. A tiny and concentrated spot of the extracts were spotted on the baseline using a capillary tube. This plate was then placed in an airtight chamber saturated with various mixtures of organic solvents. Once the solvent reached the frontline, the plate was removed and all the visible spots were marked immediately using a pencil. The plate was then further visualized under an ultraviolet lamp at short (254 nm) and long (365 nm) wavelengths, respectively (Kaur and Arora, 2009). The retention factor R_f value of each spot on the TLC plate was calculated by dividing the distance travelled by the compound (cm) with the distance of the solvent front (cm) (University of Colorado, 2015).

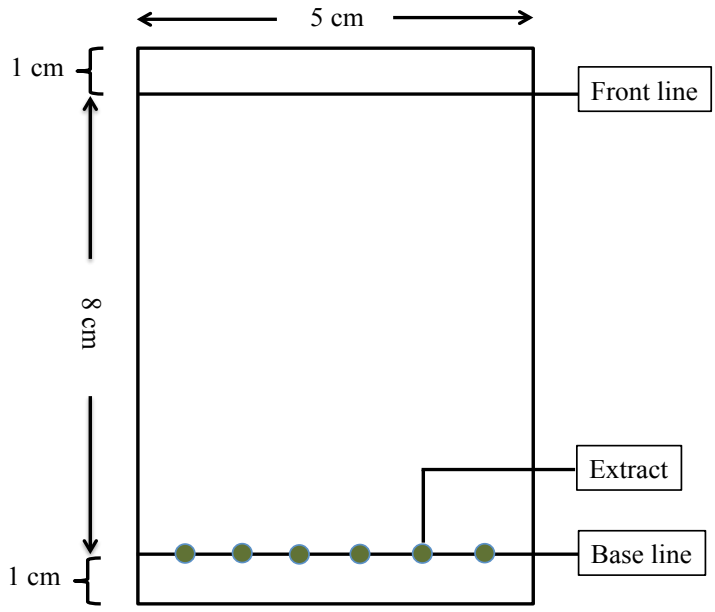

Figure 3.1: Design of TLC plate.

3.4 Preparation of Extracts for Bioassays

In DPPH assay, a stock solution of each extract was prepared at 10 mg/mL by dissolving 10 mg of the extract in 1 mL of methanol. The stock solutions were further sonicated for complete solubilization.

In MTT assay, a stock solution of each extract was prepared by dissolving 10 mg of extract in 1 mL of 100% dimethyl sulfoxide (DMSO). The prepared stock solution was then vortexed and sonicated for solubilization. The stocks were further diluted using basic DMEM by adding 100 µL of the stock solution into 900 µL of basic DMEM, to obtain 1 mg/mL working solution.

In MIC assay, a stock solution of 2 mg/mL for each extract was prepared by dissolving 2 mg of extract in 1 mL of 100% dimethyl sulfoxide (DMSO). The stocks were sonicated and were further diluted using Mueller Hinton broth. All the stocks and working solutions were kept at -20°C for further analysis.

3.5 Preparation of Positive and Negative Controls

Ascorbic acid was used as positive control in DPPH assay. The 10 mg/mL stock solution was prepared by dissolving 10 mg of ascorbic acid powder in 1 mL of distilled water. Meanwhile, negative control used was only methanol.

In MIC assay, the positive control used was gentamycin sulfate. About 10 mg of gentamycin sulfate powder was dissolved in 1 mL of distilled water to obtain a concentration of 10 mg/mL. The stock solutions were further diluted

using Mueller Hinton broth (MHB) in a 96-well plate. The negative control used was 20% DMSO and was prepared by adding 1 mL of 100% DMSO into 4 mL of MHB in an Eppendorf tube.

Doxorubicin hydrochloride was used as positive control in MTT assay. The working solution of 100 µg/mL was prepared by diluting 10 µL of stocks solutions (10 mg/mL) into 990 µL of basic DMEM. The working solutions were then further diluted using basic DMEM in the range of 3.13 to 100.00 µg/mL. The negative control used 1% DMSO and was prepared by diluting 10 µL of 100% DMSO with 990 µL of basic DMEM medium.

3.6 Preparation of Reagents and Chemicals

DPPH reagent at concentration of 0.2 mM was prepared by dissolving 11.83 mg of DPPH powder in a Scott bottle containing 150 mL of methanol. DPPH reagent is light sensitive, thus was prepared in dark and the bottle was wrapped with aluminium foil. The reagent was incubated in dark for 30 minutes before the assay or stored at 4°C.

INT dye at concentration of 0.4 mg/mL was prepared by dissolving 16 mg of INT powder in 40 mL of distilled water. The solution was filtered using 0.20 µm cellulose filter membrane. INT dye is light sensitive, thus the tube containing INT dye was wrapped with aluminium foil and the preparation was done in dark. The INT dye was stored in -20°C.

Phosphate buffered saline (PBS) was prepared by adding 16.00 g of sodium chloride (NaCl), 0.04 g of potassium chloride (KCl), 0.29 g of disodium phosphate (Na_2HPO_4) and 0.05 g of monopotassium phosphate (KH_2PO_4) into an autoclaved Schott bottle containing 160 mL of distilled water. The solution was adjusted to pH of 7.4 and autoclaved at 121°C for 15 to 20 minutes. The sterile PBS was stored in 4°C.

Fetal bovine serum (FBS) was filtered using 0.20 μm cellulose filter membrane and aliquoted in 15 mL centrifuge tubes. The centrifuge tubes were sealed with parafilm aseptically and stored in -20°C.

3.7 Preparation of Media

Mueller Hinton agar (MHA) was prepared by dissolving 20.4 g of MHA powder in 600 mL of deionized water, whereas Mueller Hinton broth (MHB) was prepared by dissolving 8.4 g of MHB powder in 400 mL of deionized water. These medium were autoclaved at 121°C for 15 minutes. MHA was poured into sterile petri dishes aseptically in a laminar hood to prevent contamination. Once the agar solidifies, the petri dishes were then sealed with parafilm and kept at room temperature. Autoclaved MHB broth was sealed with parafilm and kept at room temperature.

Complete DMEM was prepared by adding 20 mL of 10% FBS and 2 mL of 1% of Penicillin-Streptomycin into 178 mL of basic DMEM. The medium was sealed with parafilm and then stored in refrigerator at 4°C.

3.8 Culture and Subculture of Cells

3.8.1 MDA-MB-231 Cells

MDA-MB-231 cell line was taken out from liquid nitrogen tank and was thawed immediately in water bath at 37°C for one minute. The cells were transferred into a 75 cm^2 culture flask containing 5 mL of complete DMEM medium. The cells were observed under an inverted microscope and incubated in 5% CO_2 incubator at 37°C. The cells were observed regularly to ensure no contamination. Once the cells reached 80 to 90% confluency, subculture was performed.

The old medium was discarded and the cells were rinsed with 10 mL of PBS twice. Approximately 2 mL of 0.25% trypsin-EDTA solution was added to the flask and incubated for 10 minutes. The cells were then viewed under inverted microscope to ensure detachment and 3 mL of complete medium was added immediately into the flask to stop trypsinization. The cells were centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and the pellet was suspended with 1 mL of complete medium. The cells were transferred to a new flask containing 4 mL of complete medium. All the flasks were labeled and incubated in 5% CO_2 humidified incubator at 37°C.

3.8.2 Bacterial Cells

The glycerol stocks of bacteria were taken from -80°C freezer and were thawed in water bath at 37°C. The bacterial suspension was streaked on MHA aseptically in a laminar hood using inoculating loop. The plates were then

sealed with parafilm and incubated overnight in 37°C incubator. One to two bacterial colonies from the plates were transferred to a centrifuge tube filled with 30 mL of fresh MHB using inoculating loop. The cultures were then incubated in 37°C shaking incubator at 200 rpm.

3.9 Cell Counting

3.9.1 Cell Line

Cancer cells were counted using hemocytometer in which the cells were stained with 0.4% trypan blue dye. Approximately 100 µL of the cell suspension was mixed with 100 µL of the 0.4% trypan blue dye in an Eppendorf tube. Twenty microliters of the mixture was transferred to hemocytometer through capillary action. The total number of viable cells were observed and counted under 100x magnifications using an inverted microscope. The average number of viable cells in the four main counting grids and the concentration of cells were calculated using following formula (Katsares, et al., 2009).

Concentration of cells = average viable cells × dilution factor × 10^4 x volume of original cell suspension

3.9.2 Bacteria

The 10^6 CFU/mL bacteria were obtained by diluting the original bacterial suspension with MHB. Approximately 1 mL of the diluted bacterial

suspension was then transferred to cuvette to obtain absorbance ranging from 0.08 to 0.10 at 600 nm wavelength. The absorbance is equivalent to 0.5 McFarland standards which proportionate to 10^6 CFU/mL bacteria (Donay, et al., 2007).

3.10 Bioassays

3.10.1 DPPH Assay

DPPH assay was carried out using U-bottom 96-well plate. About 50 µL of methanol was added to all the wells except row H. One hundred microliters of 10 mg/mL of the extracts and ascorbic acid were added to rows A and H, respectively as shown in **Figure 3.2**. These samples were serially diluted with methanol to obtain concentrations ranging from 0.04 to 5.00 mg/mL. Approximately 20 µL of DPPH reagent was added to each well and the plate was incubated in dark for 30 minutes. The absorbance was read using microplate reader at 517 nm. The data were tabulated and EC_{50} values were determined based on the graph of percentage of radical scavenging activity against concentration of extracts. The percentage of radical scavenging activity was calculated based on the following formula (Basma, et al., 2011):

Percentage of radical scavenging (%) = (A0 – A1) x 100% / A0

Where,

A0 = Absorbance of negative control

A1 = Absorbance of sample

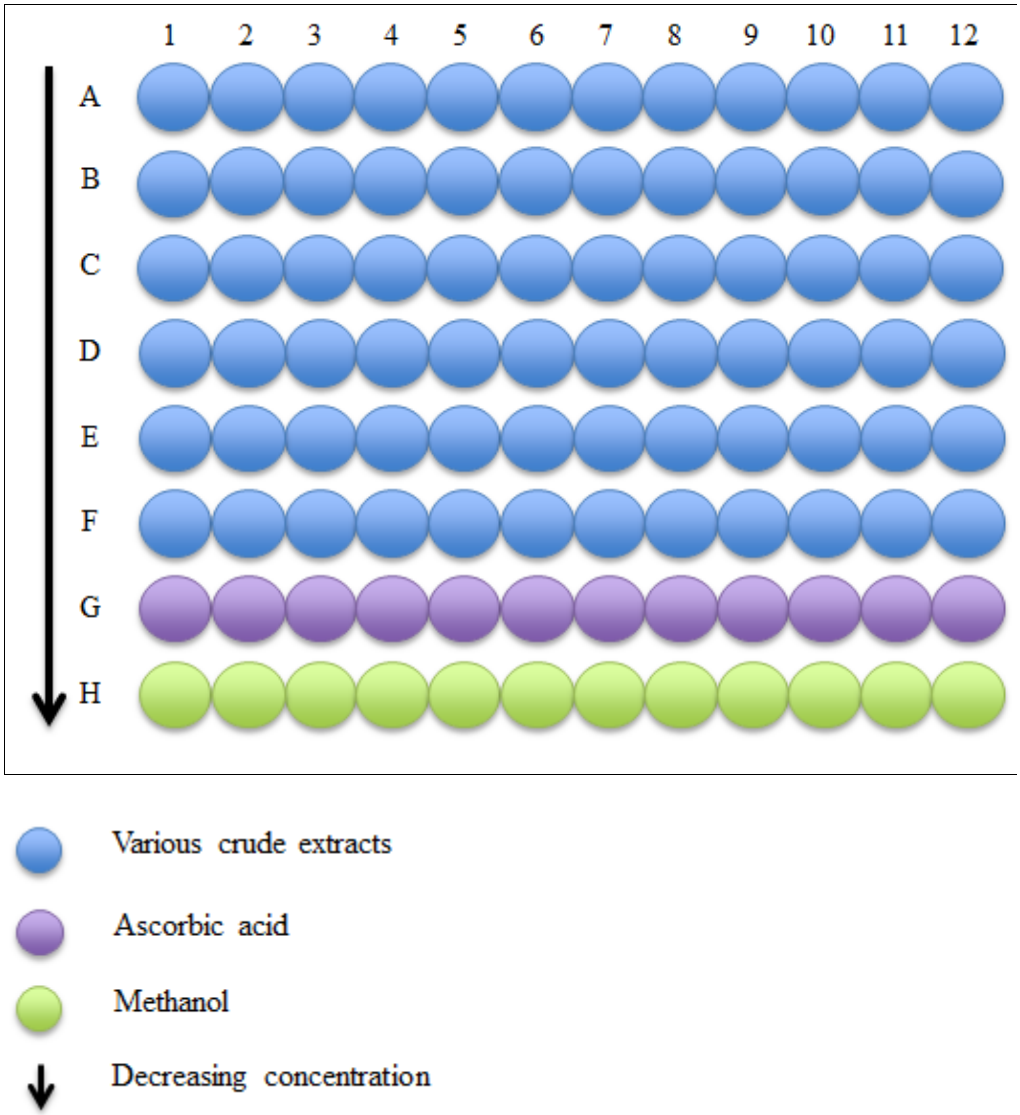

Figure 3.2: The design of 96-well plate used in DPPH assay.

3.10.2 MTT Assay

A flat-bottom 96-well plate was used in MTT assay. Fifty microliters of cells at concentration of 1×10^4 cells per well were seeded into all the wells. The wells were observed under an inverted microscope to ensure equal amount of cells were transferred into the wells. The plates were then incubated overnight in 5% CO_2 incubator at 37°C for attachment of cells. The medium was

discarded in rows A and G and 100 µL of the respective samples were added accordingly. About 50 µL of 1% DMSO was added to row H and the plate was incubated in humidified 5% CO_2 incubator at 37°C for 72 hours. **Figure 3.3** shows the design used in MTT assay.

After 72 hours of incubation, 20 µL of MTT solution was added into all the wells. The plate was then wrapped with aluminium foil and further incubated in 5% CO_2 incubator at 37°C for 4 hours. The samples were removed and 100 µL of 100% DMSO was added to the wells to dissolve the crystals. The absorbance was then measured at 570 nm using microplate reader.

The percentage of cell viability was calculated using the following formula (Mahavorasirikul, et al., 2010). Graphs of percentage of cell viability against concentration of various extracts were plotted and the IC_{50} values were determined.

$$\text{Percentage of cell viability} = \frac{\text{optical density of extracts}}{\text{optical density of negative control}} \times 100\%$$

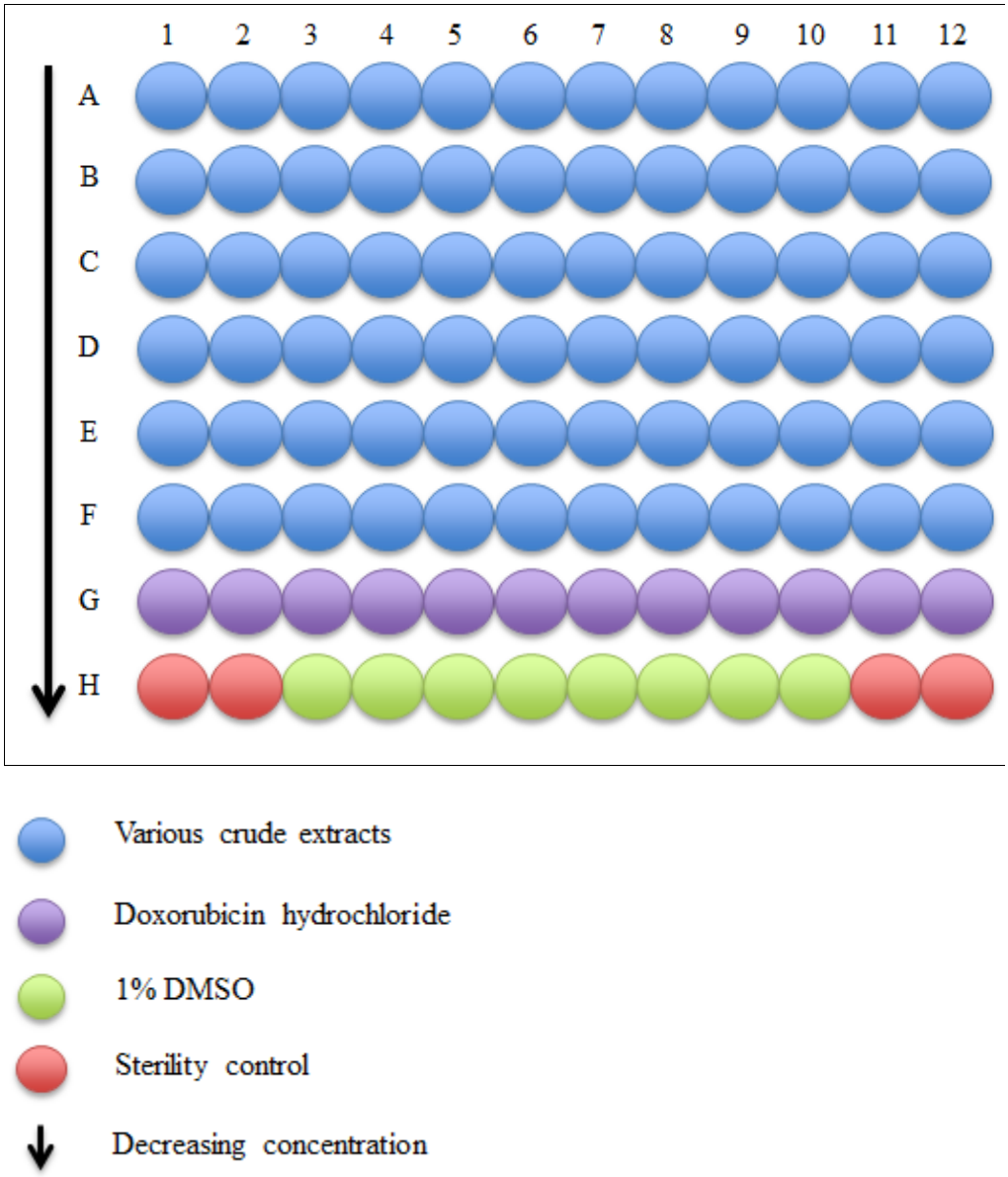

Figure 3.3: The design of 96-well plate used in MTT assay.

3.10.3 MIC Assay

The design of a U-bottom 96-well plate used in MIC assay is as shown in **Figure 3.4**. A volume of 50 µL of MHB was added into each well except row A. One hundred microliters of crude extracts, gentamycin sulfate and DMSO were added in row A, respectively. These samples were serially diluted to

obtain concentrations ranging from 0.03 to 1.00 mg/mL. About 50 µL of adjusted bacteria cells were added into each well and were incubated in 37°C incubator for 24 hours. Approximately, 20 µL INT dye was added into each well in dark and incubated at 37°C for 30 minutes. The MIC value of the extracts and gentamycin sulfate were determined by observing the color changes in each well.

3.10.4 MBC Assay

The lowest concentration based on the color changes of samples in MIC assay were streaked onto MHA. The agar plates were further incubated in 37°C incubator for 24 hours. The plates were then observed for any growth of bacterial colonies and the MBC values were tabulated. The sample with the lowest concentration and no growth of bacteria will be reported as MBC, less colonies indicate higher MBC value and can be considered as an effective antibacterial agent.

3.11 Data Analysis

DPPH, MIC and MTT assays were repeated thrice. The data obtained were expressed as mean±standard deviation and data were calculated using Microsoft Office Excel 2013.

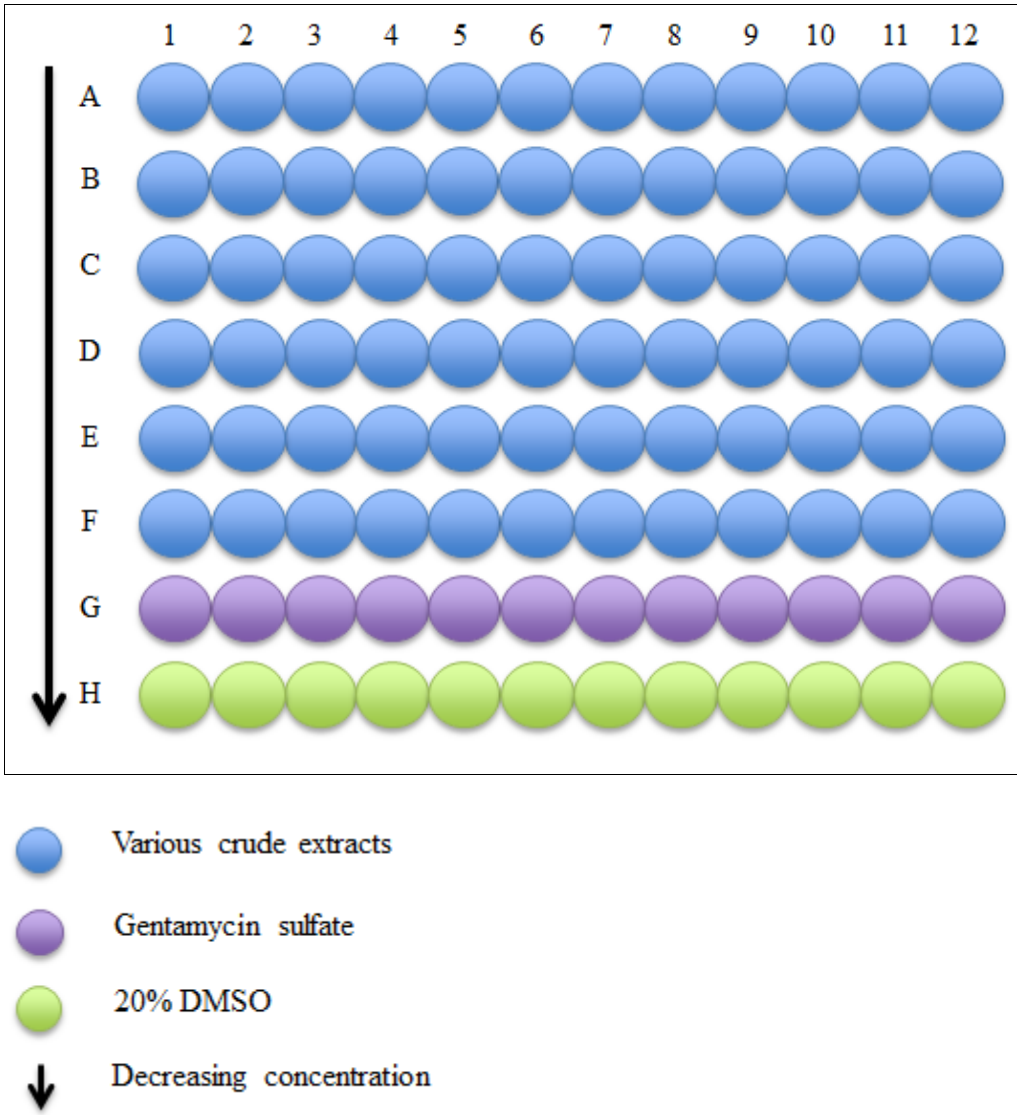

Figure 3.4: The design of 96-well plate used in MIC assay.

CHAPTER 5

DISCUSSION

5.1 Phytochemical Screening

The qualitative phytochemical screening of plant's crude extracts is an important step in the detection of bioactive compounds that may lead to novel drug discovery. In this study, various secondary metabolites were detected in the crude extracts of both Calophyllum species. Initial screening of bioactive is crucial to determine the bioactvity of the plants. There are many possible reasons for the detection of secondary metabolites in the Calophyllum species. Methods of extraction, type of solvents used, parts of plants used and geographical distribution of plants could be attributed in the isolation of bioactive compounds.

Two classical methods used in the extraction of bioactive compounds are Soxhlet extraction and cold solvent extraction (Azmir, et al., 2013). Cold extraction involves soaking the plant sample in selected solvents and allowed to stand at room temperature for a period time with frequent agitation. This method does not require heating. The longer the soaking period, the more compounds could be isolated from the plant which possesses the medicinal properties (Azwanida, 2015). Arya, Takhur and Kashyap (2012) showed that extraction of *Psidium guajava* L. leaves using cold extraction method resulted in highest extraction yield with maximum presence of phytochemicals such as alkaloids, saponins, tannins and flavonoids.

On the other hand, Soxhlet extraction has been widely used for extracting bioactive compounds from various natural sources using thermal heat. In this method, finely grounded sample is heated to obtain the crude extracts (Azwanida, 2015). However, the disadvantage of this method is the poor extraction of polar compounds and the possibility of thermal decomposition of the target compounds as the extraction usually occurs at the boiling point of the solvent for a long time (Sukri, 2012). Vongsak, et al. (2013) showed that Soxhlet extraction in *Moringa oliefera* leaves resulted in a lower yield of polar compounds such as phenolics and flavonoids. Besides, another study by Anuradha (2010) demonstrated the absence of anthocyanin from the *Clitorea ternate* flowers due to the Soxhlet method. The author suggested this method could possible oxidize and degrade the bioactive compounds.

Another reason for the successful extraction and isolation of bioactive compounds from plants is due to the type of solvent used in the extraction. Different polarities of solvents were used during extraction of bioactive compounds. These solvents are capable to formed noncovalent bonds with functional groups of secondary metabolite and thus solubilizing and extracting molecules. For example, polar solvent such as methanol was used to isolate polar compounds, whereas intermediate polar solvents like ethyl acetate and dichloromethane can be used for extraction of both polar and nonpolar compounds (Sasidharan, et al., 2011).

In this research, ethyl acetate crude extract of *Calophyllum andersonii* and *Calophyllum gracilentum* and dichloromethane crude extract of *Calophyllum*

andersonii showed presence of the most bioactive compounds. This is due to the nature of ethyl acetate and dichloromethane solvents that are able to extract both polar and non-polar compounds. Furthermore, phytochemical analysis also demonstrated the presence of same secondary metabolites present in ethyl acetate and dichloromethane extract of *Calophyllum andersonii*. For example alkaloids, tannins, terpenoids, quinones, glycosides and flavonoids, indicating the rate of extraction for both is the same. Both of the solvents can isolate out equal amount of secondary metabolites because of their nature of extracting both polar and non-polar compounds.

On the other hand, methanol crude extract of *Calophyllum andersonii* and *Calophyllum gracilentum* both showed the least presence of secondary metabolites in phytochemical analysis. This is because methanol is a polar solvent due to the presence of hydroxyl group that has high electronegative oxygen atom which allows hydrogen bonding with other molecules thus attracting only polar compounds (Schiller, 2010). Therefore, methanol crude extracts of both species may contain only metabolites with higher polarity such as phenols, terpenoids, saponins and tannins.

The crude extracts from stem barks of *Calophyllum andersonii* and *Calophyllum gracilentum* were used in this study. This could also be another reason for the presence of many secondary metabolites. Many studies proved that the stem bark may contain more secondary metabolites than leaves due to translocation. Plant translocation is the transport of materials from leaves to

other parts of the plant such as stem bark, resulting in accumulation of secondary metabolites in the stem bark (Crafts, 1938).

Different bioactivities of *Calophyllum andersonii* and *Calophyllum gracilentum* may be due to the difference in phytochemical profiles across geographical regions. The variations in the growing conditions are major contributors to the differences in secondary metabolite profile. Different stressors may result in the production of one metabolite over another in response to the various needs of the plant. Variations in altitude, carbon dioxide levels, insect and pathogenic presence are also factors that will affect the composition of the metabolite constituents (Buhian, et al., 2016). Malaysia is a country where a tropical rainforest climate is apparent all year round and rich in natural resources. The Calophyllum plants were collected from wild and do not contain any herbicides. Thus, many secondary metabolites could have been present in Malaysian species of Calophyllum.

5.2 Thin Layer Chromatography

Various polarity solvents were optimized in thin layer chromatography (TLC) for mobile phase such as hexane and ethyl acetate. According to Lee, et al. (2017), Calophyllum species contain both polar and non-polar compounds and hence the choice of solvent system must include polar and non-polar solvents that could isolate various compounds. Furthermore, phytochemical analysis also demonstrated the presence of polar and non-polar compounds in this research. Hexane and ethyl acetate mixture at 10:3 ratios was selected as the

mobile phase in TLC after a few trial and error methods. This two solvent combination produced many separated spots to indicate the polarity of the compounds (Visht and Chaturvedi, 2012).

Aluminium plate coated with silica gel acted as the stationary phase. Silica gel consists of surface silanol groups with exposed hydroxyl group which are able to form strong hydrogen bonds with other compounds (Sarker, Latif and Gray, 2005). Polar compounds tend to interact strongly with the silica surface, thus compounds will move up the plate slower and giving smaller R_f values. On the other hand, nonpolar compounds which have less affinity towards silica surface will move up the plate faster compared to polar compounds resulting in wider R_f values (University of Colorado, 2015).

Based on **Table 4.2**, dichloromethane crude extract of *Calophyllum gracilentum* showed the widest range of R_f values and was isolated using mixture of hexane and ethyl acetate. Hexane is a non-polar solvent whereas ethyl acetate is an intermediate polar solvent, this combination produced the widest range of R_f value deduced that dichloromethane crude extract of *Calophyllum gracilentum* contains compounds with different polarities (nonpolar to polar).

The crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* were dissolved in DMSO. DMSO is a universal solvent that is commonly used to solubilize both polar and non-polar samples in bioassays. Since crude extract contains multiple compounds, hence a universal solvent is needed to

dissolve all the compounds to produce a homogenized solution (Timm, et al., 2013). However, studies have shown that more than 20% of DMSO is toxic to the cells as it damages the integrity of the cell membrane (Da Violante, et al., 2002). Hence, in the present study, a lesser percentage of DMSO was used in bioassays to ensure that the cytotoxic and antibacterial activities are exhibited by phytochemicals in the plants rather than DMSO.

5.3 Bioassays

5.3.1 DPPH Assay

Oxidative stress occurred when there is an imbalance between free radicals and antioxidants. This phenomenon eventually resulting in biological damages and increase the risk of developing cancers (Leibfritz, et al., 2007). Natural antagonists to this process are antioxidants (Hassanbaglau, et al., 2012). Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as "free radical scavengers" (National Cancer Institute, 2014). Recent researches have shown that the antioxidants originate from plants possess free-radical scavenging properties and could have great importance as therapeutic agents in several diseases caused by oxidative stress. Plant phytochemicals are found effective as radical scavengers (Sen, et al., 2010).

Based on the results, all the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* exhibited antioxidant activitiy. According to Sultana, Anwar and Ashraf (2009), the amount of antioxidant components that

can be effectively extracted from a plant is strongly dependent on the nature of extracting solvent, different chemical characteristics and polarities of different antioxidant compounds that may or may not be soluble in a particular solvent.

In this study, ethyl acetate and methanol extracts exhibited highest scavenging activity. This shows that extraction using polar and intermediate solvents exhibited higher antioxidant activity. These findings are in agreement with previous research by Aminudin, Ahmad and Taher (2015). The authors revealed that the ethyl acetate and methanol extracts of *Calophyllum symingtonianum* and *Calophyllum depressinervosum* possessed strong antioxidant activity with IC_{50} values comparable to ascorbic acid compared to dichloromethane extracts which showed moderate activity. The contributor for the higher radical scavenging activity in Calophyllum species could be due to the presence of phenolic compounds and xanthones.

Phenolic compounds such as flavonoids and tannins are always associated with strong antioxidant properties due to their rich in hydroxyl groups which are excellent hydrogen donors. Flavonoids and tannins act as major free radical scavengers in a termination reaction and thus hindering the production of new reactive oxygen species (Aminudin, Ahmad and Taher, 2015; Brewer, 2011). Moreover, phenolic compounds were suggested might be a contributor to the antioxidant activity by terminating reactive oxygen species and scavenge free radicals (Shoeb, Madkour and Refahy, 2014). Based on phytochemical screening, tannins and phenols were present in both ethyl acetate crude extracts of *Calophyllum andersonii* and methanol crude extract

of *Calophyllum gracilentum.* Although xanthones were not screened in this study but this bioactive compounds that are commonly isolated from Calophyllum species (Negi, et al., 2013). Studies have reported that xanthones displayed antioxidant, antimicrobial, anticarcinogenic, antileprosy and radioprotective activity (Negi, et al., 2013).

One of the important parameters of this assay is the determination of the concentration of the crude extracts to obtain a 50% of free radical scavenging activity (EC_{50}). Lower EC_{50} value indicates a higher antioxidant activity (Sowndhararajan and Kang, 2013). Based on the result, ethyl acetate crude extract of *Calophyllum andersonii* and methanol crude extract of *Calophyllum gracilentum* showed lowest EC_{50} value which is 0.24 mg/mL as compared to other crude extracts and ascorbic acid. The reactive metabolites in the samples contributed to the antioxidant activity.

Ascorbic acid was used as the positive control in DPPH assay. Ascorbic acid is well known antioxidant agents that help to reduce cell damages caused by reactive species such as oxygen and nitrogen by acting as reducing agent, free radical scavenger and detoxifying agent in humans (National Cancer Institute Thesaurus, 2016). Ascorbic acid interacts with plasma membrane by donating electrons to the α-tocopheroxyl radical and thus converts into ascorbate radical. Recycling of α-tocopherol by ascorbate helps to protect membrane lipids from peroxidation (May, 1999).

There is a strong correlation between antioxidant activity and cytotoxic effect of *Calophyllum andersonii* and *Calophyllum gracilentum* crude extracts. High level of reactive oxygen species (ROS) in human body promotes the development and progression of tumor (Liou and Storz, 2010). ROS causes oxidative damage to host cellular DNA leading to gene mutation causing activation of oncogenes that may be the initiation step of carcinogenesis (Waris and Ahsan, 2006). Therefore, compounds with good antioxidant property will be able to exert antiproliferative effect towards cancer cells. Hence, the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* that demonstrated higher antioxidant activity with lower EC_{50} values showed higher cytotoxic effect in this research.

5.3.2 MTT Assay

In this study, MDA-MB-231 cancer cell line was incubated for 72 hours of treatment. From the perspective of toxicology, this incubation period was selected because the membrane permeability greatly affects the efficacy of a drug in absorption, metabolism, distribution and elimination (Katzung and Trevor, 2015). The rate of diffusion of molecules is dependent on the molecules size and strength of hydrophobicity. Hence, phospholipid bilayer is less permeable to polar compounds but hydrophobic or nonpolar compounds are readily across the bilayer (Fulton, 2016). This statement correlates with the present study, in which the dichloromethane crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* and ethyl acetate crude extract of *Calophyllum gracilentum* exhibited a higher cytotoxic effect and lower IC_{50}

value (0.02 mg/mL) against MDA-MB-231 cells than the methanol crude extracts. Dichloromethane and ethyl acetate are intermediate polar solvents, thus the compounds extracted from these solvents contain both polar and nonpolar metabolites.

Besides, the higher cytotoxic effect of crude extracts of Calopyllum species could be attributed to the presence of active compounds such as saponins and tannins. These metabolites were detected in dichloromethane crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum*, and ethyl acetate crude extract of *Calophyllum gracilentum*.

Many studies have reported that saponins isolated from plants have shown to specifically inhibit the growth of cancer cells *in vitro*. Saponins are phytochemicals with soap-like properties that forms stable foams in aqueous solutions, thus they are often used as natural surfactants in cleansing products (Marrelli, et al., 2016). Saponins exert their cytotoxic by stimulating the disintegration of the microtubular network or actin filaments of cancer cells, which then lead to further nonapoptotic cell death (Yu, et al., 2015).

Apart from the strong antioxidant and antibacterial activity, tannins also participate in biological and physiological functions which are predominantly related to the modulation of carcinogenesis (Gulecha and Sivakuma, 2011). Tannins are capable of inducing caspase-3-dependent apoptosis in cancer cell lines through cell cycle arrest, extracellular signal-regulated kinase (ERK) and P38 mitogen-activated protein kinase (MAPK) pathway blockage, inhibiting

transcription factors activation such as activator protein-1 (AP1), protein kinase C and growth factor-mediated pathways suppression (Dai and Mumper, 2010).

However, methanol crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* showed lower cytotoxic effect compared to ethyl acetate and dichloromethane crude extracts. This might be due to the antagonism effect exhibited by the polar bioactive compounds in the crude extracts. According to Basri, et al. (2014), a lower cytotoxicity was observed against HCT-116 cancer cells using methanol extract of *Canarium ododntophyllum* due to the presence of polar compounds that may act antagonistically. Meanwhile, nonpolar and intermediate solvents are able to isolate various polarities of secondary metabolites as compared to methanol. Methanol is able to isolate only polar compounds, hence the number of compounds extracted using methanol is lesser and this could be the reason for its lower cytotoxicity.

Furthermore, higher cell viability was observed when the concentration of the crude extracts is increased. This scenario could also be explained with the antagonism of multiple phytoconstituents present in the crude extracts which cause them to act differently. The interaction of phytochemicals in the crude extracts resulting in the decrease of biological effects rather than enhancing the effects (Milugo, et al., 2013). In some cases, crude extracts did not show any activity but their pure compounds were proved to be active. The reason could be due to the presence of certain compounds which may act to inhibit

the activity of others in a crude extract. Such antagonistic interactions will therefore reduce the cytotoxic effect of the crude extracts (Schinor, et al., 2007; Mazza, Shi and Le Maguer, 2002). This can be used to explain the results obtained in the present study. At lower concentration of crude extracts, lesser phytoconstituents are present to cause antagonistic effect towards those displaying cytotoxic effect. However, at higher concentration, the cytotoxic effects of certain phytoconstituents towards MDA-MB-231 cells might be repressed by higher number of compounds which act as an antagonist in the crude extracts, reducing the overall cytotoxicity and hence resulting in an increase in the percentage of cell viability (Odhiambo, et al., 2009).

Doxorubicin hydrochloride was used as positive control in the study. This broad spectrum intercalating anticancer agent is commonly used in the cancer therapy (Sadeghi-aliabadi, Minaiyan and Dabestan, 2010). Doxorubicin hydrochloride exhibits toxicity on cancerous cells by intercalating into DNA, disrupt the topoisomerase-II-mediated DNA repair and generating free radical in which any of this mechanism leads to cell death (Thorn, et al., 2011).

A crude extract is said to be a potent anticancer drug if the IC_{50} value is l20.00 µg/mL or less and this assessment is based on the criteria established by National Cancer Institute (Reddy, et al., 2012). In present study, the IC_{50} values of the crude extracts were 20.00 µg/mL and thus are potential as cytotoxic agents based on the criteria.

5.3.3 MIC and MBC Assays

All the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* showed antibacterial activity against all the five tested bacterial strains which include Gram-positive and Gram-negative bacteria with MIC values of 0.25 mg/mL. This may be due to the presence of broad-spectrum antibacterial compounds in the crude extracts that contribute to their ability in inhibiting both Gram-positive and Gram-negative bacteria (Kandhasamy, Arunachalam and Thatheyus, 2008).

Phytochemical screening of crude extracts revealed the presence of flavonoids, phenols, saponins, and tannins in most of the crude extracts which could be responsible for the observed antibacterial activity. These metabolites could be possibly exerting antibacterial activity by inhibiting various cellular processes, increasing in plasma membrane permeability and ion leakage from the cells (Khan, et al., 2009).

Tannins might play a major role in the antibacterial activity showed by the crude extracts as they are the only bioactive compound that were present in all of the crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum*. Tannins are complex polyphenolic compounds that are widely found in higher plants. Similar to many polyphenols, tannin has been shown to possess antibacterial activities (Sung, 2012). Several previous studies have suggested that the antibacterial effect of tannins may be due to their ability to inhibit bacterial growth and protease activity by damaging the cell wall and cytoplasm causing rapid structural destruction (Sung, 2012).

Terpenoids are active against bacteria through mechanism involving the disruption of membrane by lipophilic compounds (Tiwari, et al., 2011). Terpenoids readily interact with membrane proteins and increase the permeability of the membranes, which lead to uncontrolled efflux of ions and metabolites and even to cell leakage, resulting in necrotic or apoptotic cell death (Khan, et al., 2009).

Saponins are glycosylated phytoanticipins that are found in a wide range of plant species (González-Lamothe, 2009). The natural role of saponins in plants is to confer protection against potential pathogens as saponins have potent antimicrobial activities which can cause leakage of proteins and certain enzymes from the cell (Shihabudeen, Priscilla and Thirumurugan, 2010).

Flavonoids are hydroxylated phenolic substances that are commonly found in fruits, vegetables, seeds, stems, flowers, tea, wine and honey (Yadav and Agarwala, 2011). Flavonoids are known to be synthesized by plants in response to microbial infection and have been found *in vitro* to be effective antimicrobial substances against a broad spectrum of microorganisms. The activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Shihabudeen, Priscilla and Thirumurugan, 2010).

The results of the study showed that among the five tested bacterial strains, Gram-negative bacteria, *Escherichia coli* was the most susceptible bacteria towards the crude extracts compared to other bacteria. The result is

contradictory to previous studies which reported that Gram-positive bacteria that have only one outer peptidoglycan layer (Karou, et al., 2006) were more susceptible to plant extracts than Gram-negative bacteria that have an outer phospholipidic membrane that makes the cell wall impermeable to lipophilic solutes (Islam, 2008). This may be due to the synergistic effects of the mixture of natural compounds found in the crude extracts that might be responsible in the antibacterial activity against Gram-negative bacteria (González-Lamothe, 2009). This synergistic effect is due to one metabolite enhancing the effect of another metabolite resulting in increasing overall efficacy of antibacterial activity (Engleberg, et al., 2007).

Gentamycin sulfate was used as the positive control in this assay and showed lowest MIC and MBC value of 0.04 and 0.08 mg/mL respectively. Gentamycin sulfate is a type of aminoglycoside antibiotic and is potent broad-spectrum antibiotics that have been widely used for the treatment of Gram-positive and Gram-negative infections. Aminoglycosides exert their bactericidal effect by inhibiting protein synthesis via binding to the 16S rRNA and by disrupting the bacterial cell membrane integrity (Kotra, Haddad and Mobashery, 2000).

5.4 Limitations of Study

The limitations of this study are more focused on the methodology of the experiment. The crude extracts were dissolved in DMSO and some crude extracts were very difficult to dissolve. Thus, these crude extracts were

sonicated longer to ensure solubility. Complete solubilization is important to prevent the loss of some bioactive compounds which are essential in biological activities, as the loss of the bioactive compounds may result in inaccuracy of the result.

Besides, improper pipetting techniques as well as uneven cell seeding into the 96-well plates led to imprecision and inconsistent absorbance readings for the assays. Hence, in order to obtain a reliable and significant result, the assays were repeated for at least thrice and the average of the absorbance was calculated.

5.5 Future Studies

Further studies are needed in order to investigate more detail bioactive compounds present in *Calophyllum andersonii* and *Calophyllum gracilentum* crude extracts. Techniques such as high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) can be employed to isolate and purify individual constituents from mixture of compounds in *Calophyllum andersonii* and *Calophyllum gracilentum* crude extracts. Besides, nuclear magnetic resonance (NMR) can be used to elucidate the structure of the isolated pure bioactive compounds (Sasidharan, et al., 2011).

Furthermore, other bioassays can be conducted to obtain more reliable results because single assay is very difficult to conclude the potential activity exerted

by the crude extracts due to different mechanisms and actions involved in anticancer and antioxidant activities. Antioxidant assays such as total radical trapping antioxidant parameter (TRAP) method, ferric reducing antioxidant power (FRAP) assay, nitric oxide scavenging activity and Trolox equivalent antioxidant capacity (TEAC) assay can be carried out to further evaluate the antioxidant activity of the crude extracts (Prior, Wu and Schaich, 2005). Meanwhile, to further evaluate the cytotoxicity of the crude extracts towards cancer cells, *in vitro* assays such as neutral red assay, protease viability marker assay, DNA fragmentation assay and ATP assay can be conducted (Riss, et al., 2013).

Besides, to evaluate the efficacy and validate the *in vitro* findings, studies using animal models such as mice and rodents should be carried out. Analysis on mechanism of action, dosage efficacy, therapeutic index and toxicity of isolated active compounds can be done as well to provide the researcher better and promising evidence on the activities posed by the plant.

Cytotoxicity of *Calophyllum andersonii* and *Calophyllum gracilentum* crude extracts were evaluated using only one cancer cell line (MDA-MB-231). To have a better assessment on the anticancer effect, more monolayer and suspension cell lines should be tested. Normal cell lines should also be tested to ensure isolated compounds cause no or minimal side effects on normal healthy cells besides killing the cancer cells.

CHAPTER 6

CONCLUSIONS

Phytochemical screening of crude extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* showed the presence of phenols, alkaloids, flavonoids, glycosides, quinones, saponins, tannins and terpenoids. In DPPH assay, ethyl acetate extract of *Calophyllum andersonii* and methanol extract of *Calophyllum gracilentum* exhibited the highest radical scavenging activity of 80.4±0.001% and 80.0±0.000%, respectively in which the EC_{50} values for both were 0.025 mg/mL.

In MTT assay, dichloromethane extract of *Calophyllum andersonii*, ethyl acetate and dichloromethane extracts of *Calophyllum gracilentum* showed the highest cytotoxic effect against MDA-MB-231 cells with IC_{50} of 20.00 µg/mL, whereas doxorubicin hydrochloride exhibited IC_{50} of 4.00 µg/mL. In antibacterial assay, all the crude extracts from both Calophyllum species showed MIC and MBC values in the range of 0.250 to 1.000 mg/mL against tested bacteria. Methanol extracts of *Calophyllum andersonii* and *Calophyllum gracilentum* showed the lowest MIC of 0.250 mg/mL and MBC of 0.500 mg/mL against *Escherichia coli,* respectively.

In conclusion, further analysis on Malaysian species of Calophyllum should be carried out due to its potential as cytotoxic, antibacterial and antioxidant agents.

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APPENDIX A

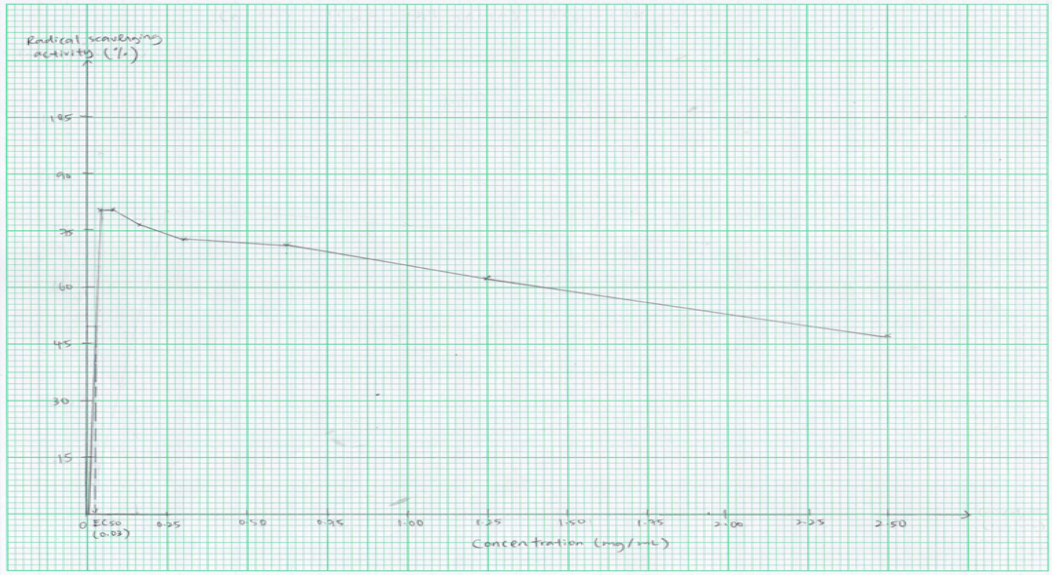

Figure A: The percentage radical scavenging activity of ethyl acetate extract of *Calophyllum andersonii* at various concentrations.

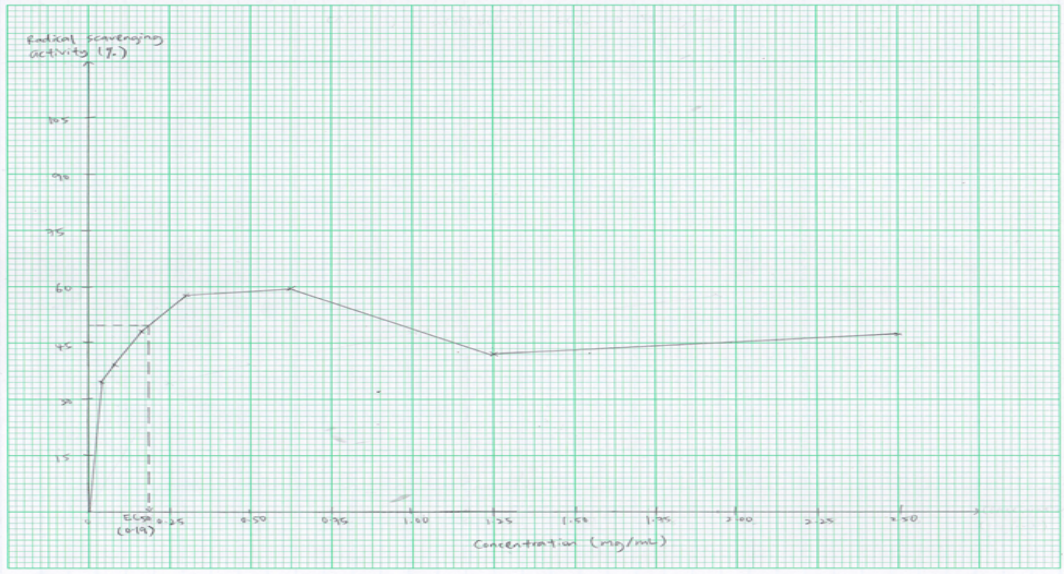

Figure B: The percentage radical scavenging activity of dichloromethane extract of *Calophyllum andersonii* at various concentrations.

APPENDIX B

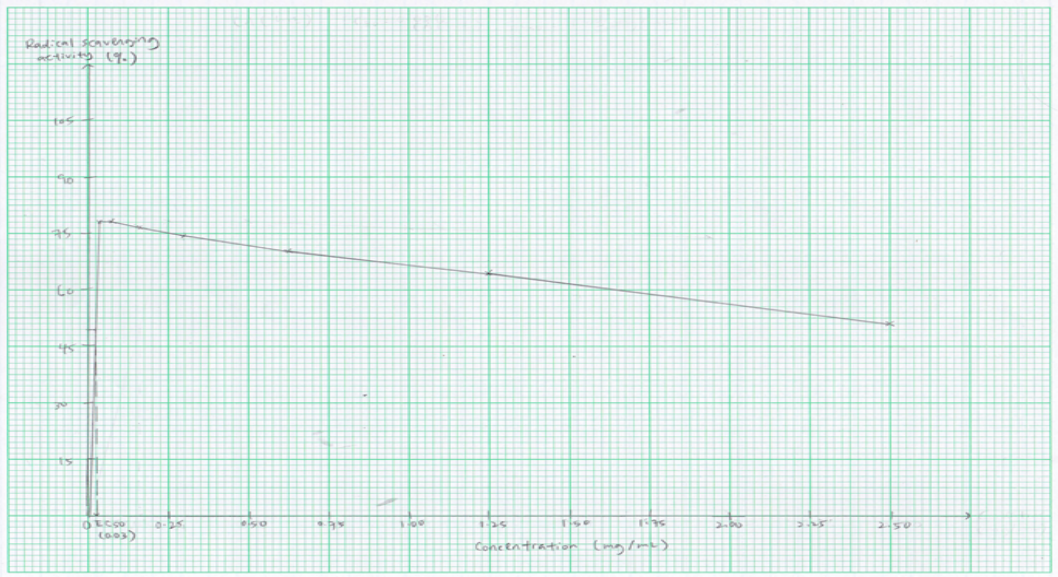

Figure C: The percentage radical scavenging activity of methanol extract of *Calophyllum andersonii* at various concentrations.

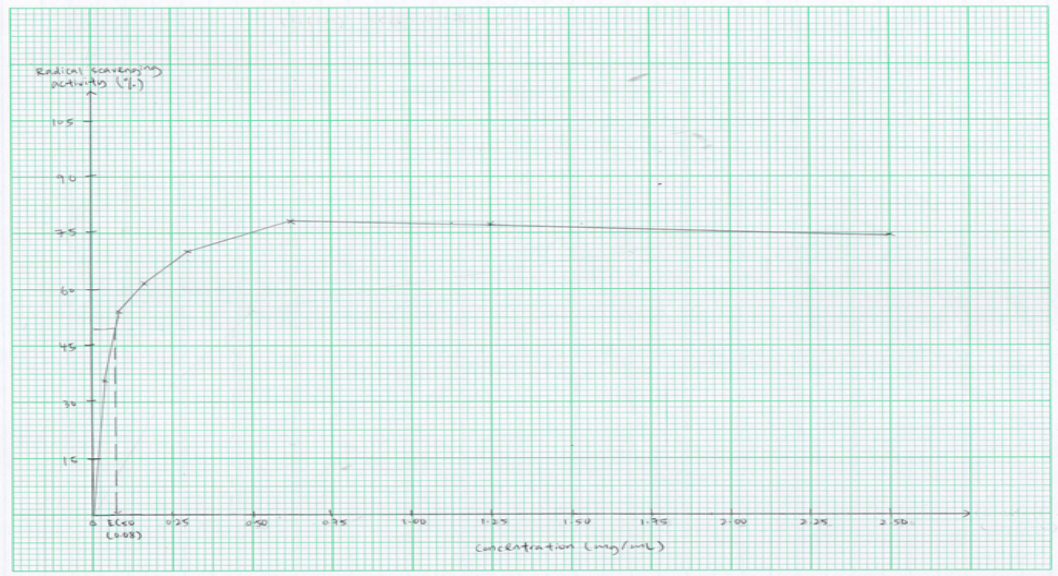

Figure D: The percentage radical scavenging activity of ethyl acetate extract of *Calophyllum gracilentum* at various concentrations.

APPENDIX C

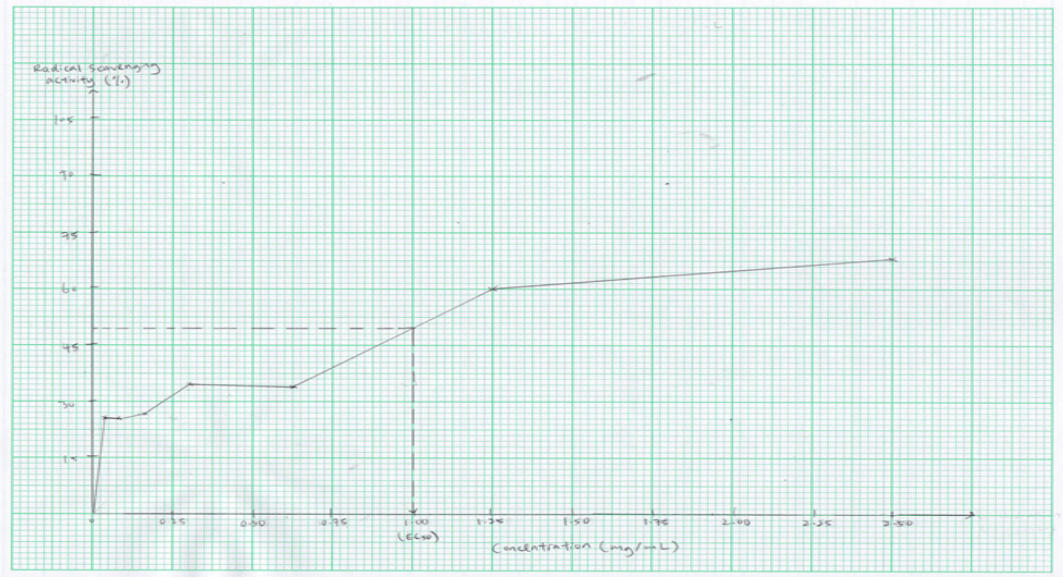

Figure E: The percentage radical scavenging activity of dichloromethane extract of *Calophyllum gracilentum* at various concentrations.

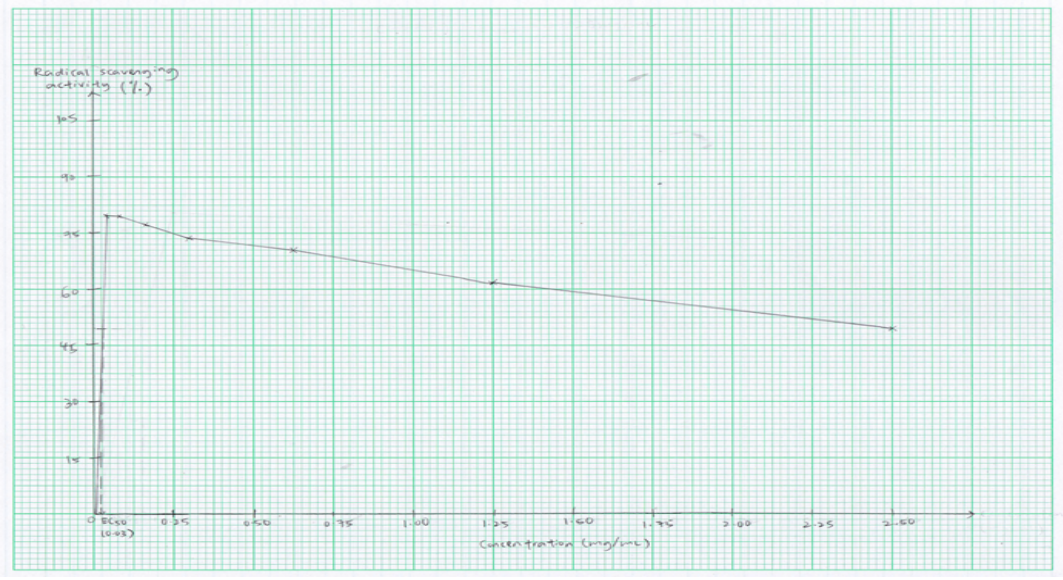

Figure F: The percentage radical scavenging activity of methanol extract of *Calophyllum gracilentum* at various concentrations.

APPENDIX D

Figure G: The percentage radical scavenging activity of ascorbic acid at various concentrations.

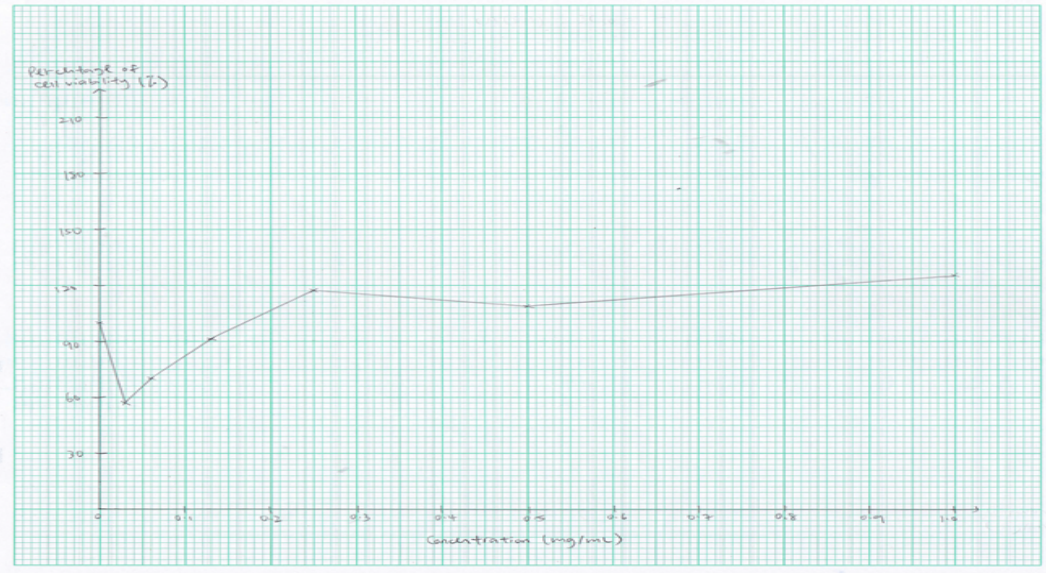

Figure H: The percentage viability of MDA-MB-231 cells against various concentrations of ethyl acetate extract of *Calophyllum andersonii.*

APPENDIX E

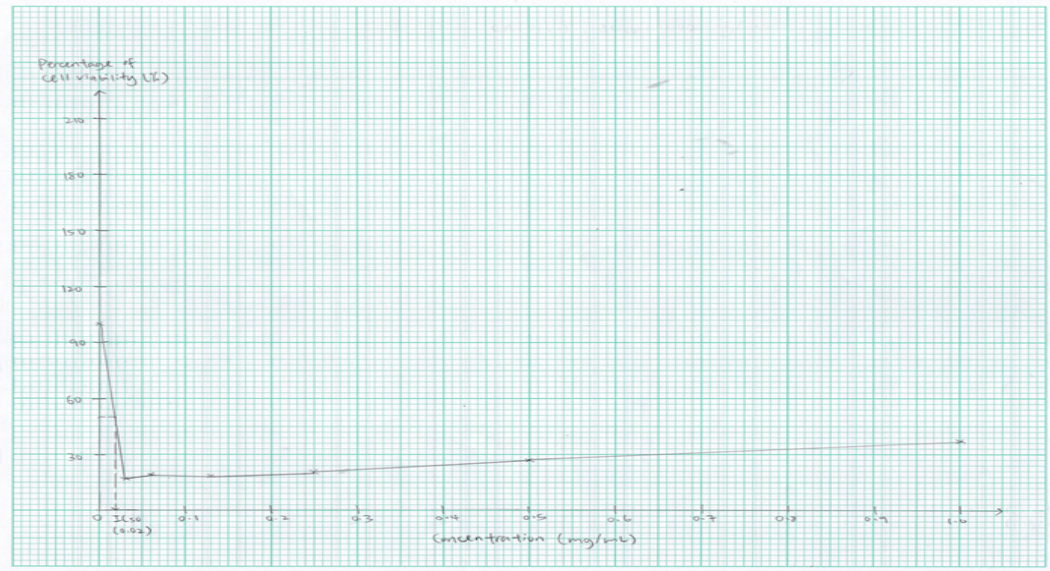

Figure I: The percentage viability of MDA-MB-231 cells against various concentrations of dichloromethane extract of *Calophyllum andersonii.*

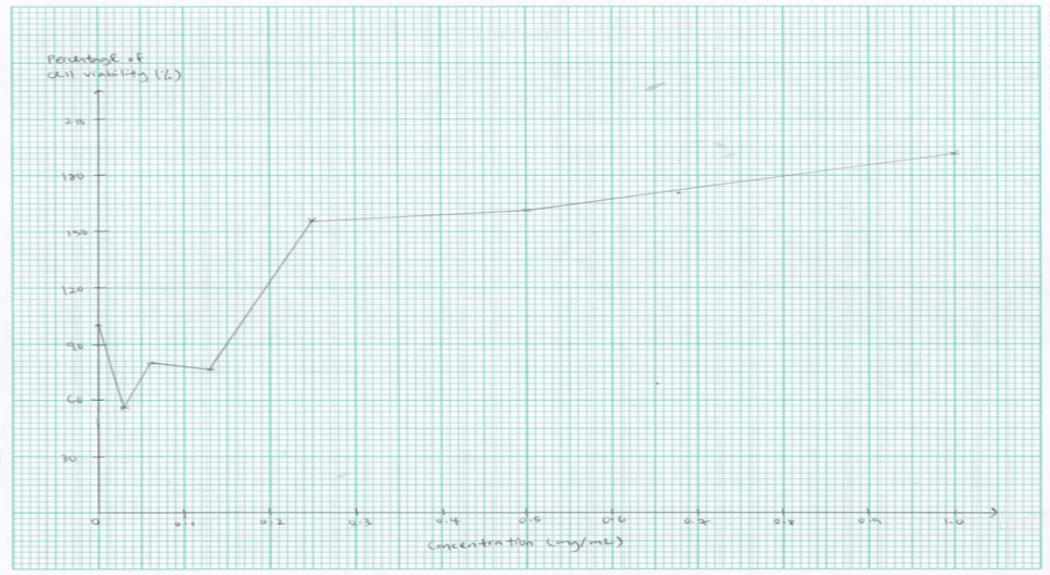

Figure J: The percentage viability of MDA-MB-231 cells against various concentrations of methanol extract of *Calophyllum andersonii.*

APPENDIX F

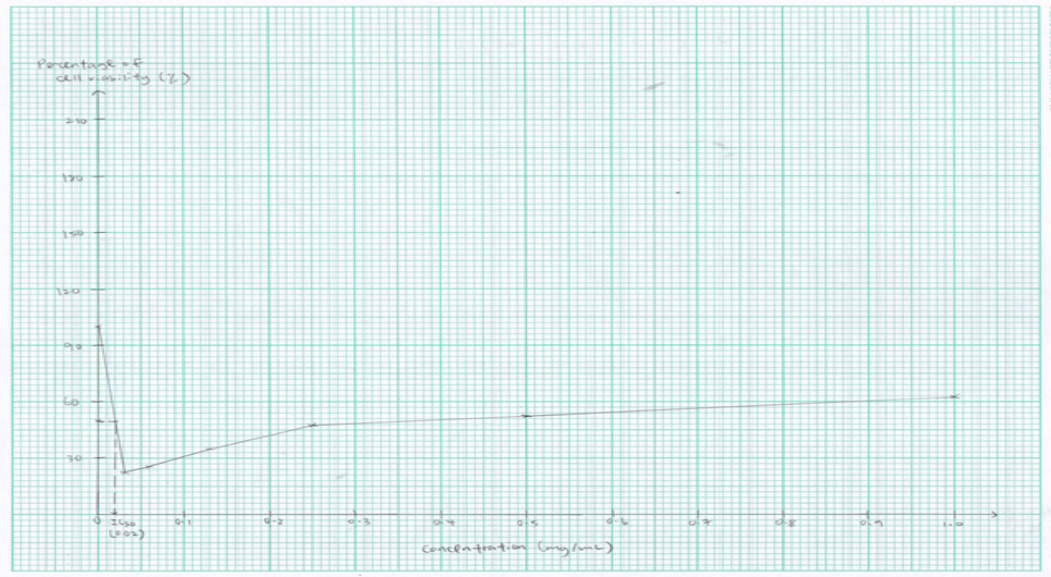

Figure K: The percentage viability of MDA-MB-231 cells against various concentrations of ethyl acetate extract of *Calophyllum gracilentum.*

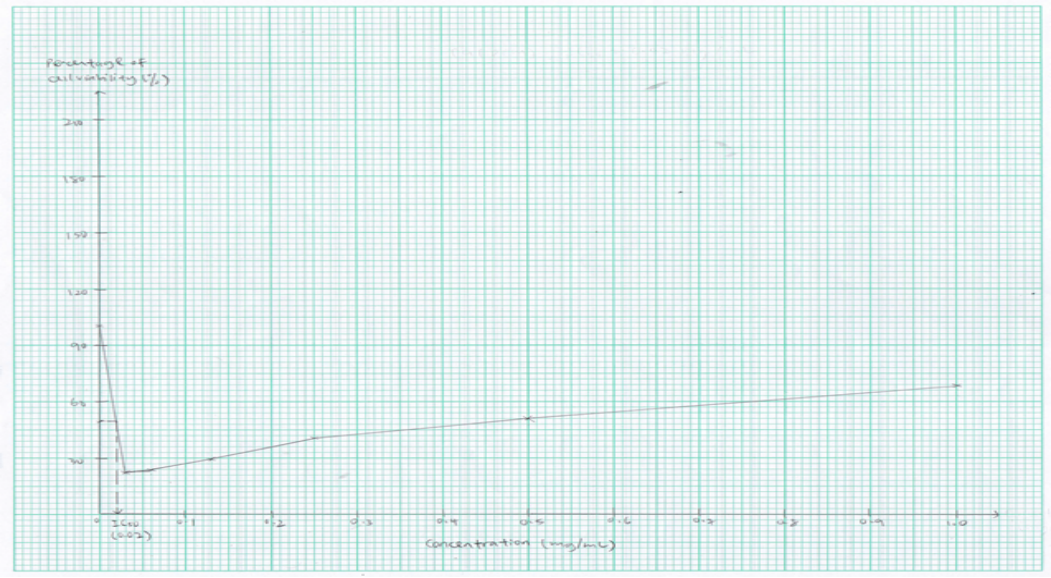

Figure L: The percentage viability of MDA-MB-231 cells against various concentrations of dichloromethane extract of *Calophyllum gracilentum.*

APPENDIX G

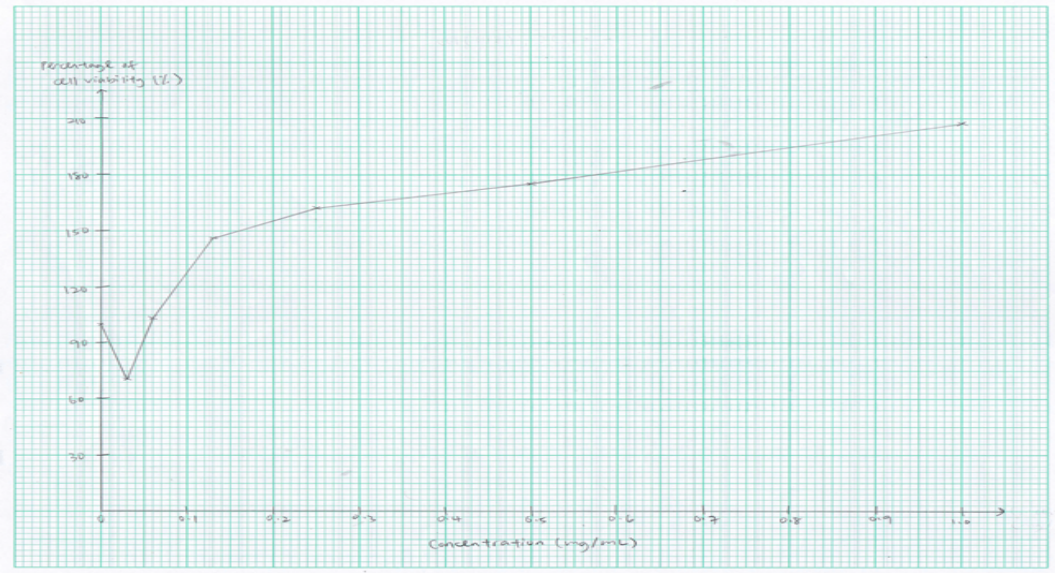

Figure M: The percentage viability of MDA-MB-231 cells against various concentrations of methanol extract of *Calophyllum gracilentum.*

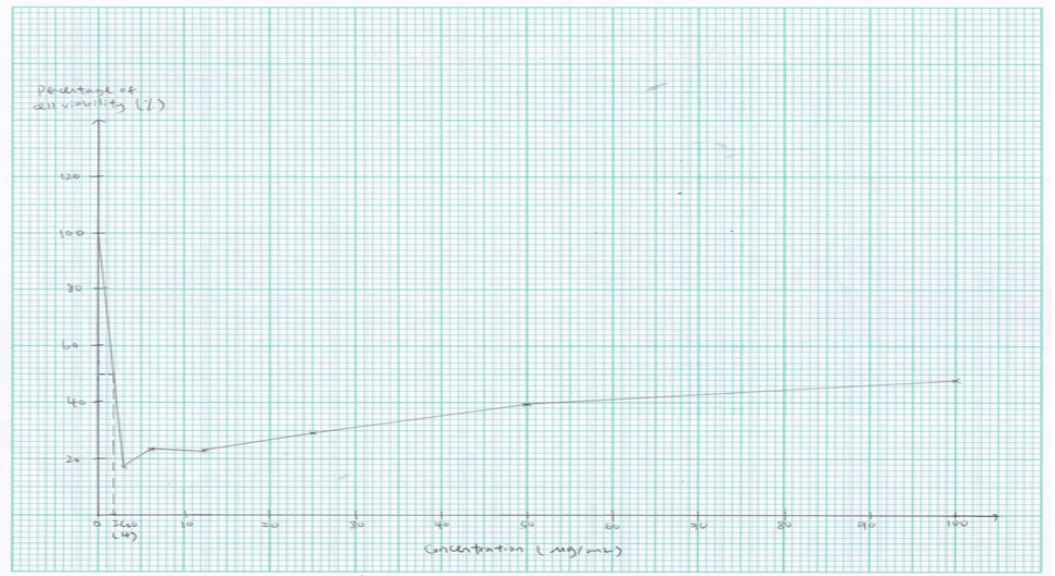

Figure N: The percentage viability of MDA-MB-231 cells against various concentrations of doxorubicin hydrochloride.