CYTOTOXIC EFFECT, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF METHANOLIC BIOASSAY-GUIDED FRACTIONS OF *Garcinia maingayi*

By

POO CHIN LONG

A project report submitted to the Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

in partial fulfillment of the requirements for the degree of

Bachelor of Science (Hons) Biomedical Science

April 2018

ABSTRACT

CYTOTOXIC EFFECT, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF METHANOLIC BIOASSAY-GUIDED FRACTIONS OF *Garcinia maingayi*

Poo Chin Long

Garcinia maingayi or locally known as 'Asam kandis' belongs to the family of Clusiaceae which are native to Malaysia, Thailand and India. Traditionally, Garcinia species has been used to treat skin infections, fever, stomach ache and diuretic. Bioassay-guided fractionation of methanolic fraction of *Garcinia maingayi* were evaluated for antioxidant activity using DPPH assay, antibacterial activity against *Escherichia coli*, *Salmonella enterica* Typhimurium, *Bacillus cereus*, *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* using MIC and MBC assays and cytotoxic effect against COLO 205 cancer cell line using MTT assay. TLC profile revealed the presence of both polar and non-polar compounds in the fractions based on the widest range of R_f values. In DPPH assay, fraction 2 exhibited the highest radical scavenging activity of 93.5±0.003% with EC_{50} value of 21.4 μg/mL. All the fractions showed concentration-dependent antioxidant activity. On the other hand, ascorbic acid exhibited radical scavenging activity of 92.0±0.001% (EC_{50} value 26.5 μg/mL). In antibacterial assays, all the fractions exhibited lowest MIC values of 0.31 mg/mL against *B. cereus* and MRSA 5 except fractions 2, 3 and 4 which showed lowest MIC values of 0.16 mg/mL against MRSA 5.

Fractions 2, 3 and 4 exhibited lowest MIC values of 0.31 mg/mL against MRSA 7 only. All the fractions exhibited bacteriostatic effect against the tested bacteria in MBC assay. In MTT assay, the highest cytotoxic effect was shown by fraction 2 with IC_{50} of 0.70 µg/mL after 72 hours of treatment. Meanwhile, doxorubicin hydrochloride exhibited IC_{50} of 1.40 µg/mL after 48 hours of treatment. The cell viability decreased in the dose-dependent manner. In conclusion, further investigations using pure compounds should be carried out to identify the active constituents and the exact mechanism of action for the reported antioxidant, antibacterial and cytotoxic properties of *Garcinia maingayi* for its potential as pharmaceutical drugs.

ACKNOWLEDGEMENTS

I would like to take this opportunity to extend my sincere gratitude to my family, for always being there for me and giving me the biggest support and encouragement. Without them none of this would have been a success.

Secondly, I would like to thank my supervisor, Ms. Sangeetha Arullappan, for her understanding and precious constructive suggestions and never-ending support which helped me complete this project and from whom I have learnt so much. I am deeply grateful and thankful of her supervision, understanding and motivation in the completion of this project.

Thanks to Faculty of Science, UTAR for providing me a comfortable and sufficient environment and laboratory facilities in completing my project. A huge thanks to all the laboratory officers, Mr. Saravanan, Mr. Tie Shin Wei and Mr. Gee Siew Meng for always being there for us to provide the necessary materials and instruments throughout the project. Thanks to my team mate, Kher Chie Yee and Ooi Kai Xin for always supporting and encouraging me all times during this project.

Last but not least, my greatest appreciation to all my lecturers, course mates and my family and relatives for their patience and support, and those who have directly and indirectly rendered their support, advice and assistance to me and bearing with my nuisance during the completion of this term paper.

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.

_____

(POO CHIN LONG)

APPROVAL SHEET

This project report entitled **"CYTOTOXIC EFFECT, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF METHANOLIC BIOASSAY-GUIDED FRACTIONS OF *Garcinia maingayi*"** was prepared by POO CHIN LONG and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

Approved by:

_____

(Ms. SANGEETHA A/P ARULLAPPAN) Date: _____

Supervisor

Department of Biomedical Science

Faculty of Science

Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

PERMISSION SHEET

It is hereby certified that **POO CHIN LONG (**ID No**: 14ADB03596**) has completed this final year "**CYTOTOXIC EFFECT, ANTIBACTERIAL AND ANTIOXIDANT ACTIVITIES OF METHANOLIC BIOASSAY-GUIDED FRACTIONS OF *Garcinia maingayi***" 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,

_____

(POO CHIN LONG)

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABTS	2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonate)
API	Activator protein-1
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
COLO 205	Colon cancer cell line
COX-2	Cyclooxygenase-2
CSAp	Colon-specific antigen-p
DMSO	Dimethyl sulfoxide
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
EC_{50}	Half-maximal effective concentration
EDTA	Ethylenediaminetetracetic acid
ERK	Extracellular signal-regulated kinase
ESBLs	Extended spectrum β-lactamase
FBS	Foetal bovine serum
FRAP	Ferric reducing antioxidant power
GC-MS	Gas chromatography-mass spectrophotometer
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
IC_{50}	Half-maximal inhibitory concentration
INT	2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium

LDH	Lactate dehydrogenase
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NCBI	National Centre for Biotechnology Information
NCI	National Cancer Institute
NIH	National Institute of Health
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity
PBS	Phosphate buffer saline
R_f	Retention factor
ROS	Reactive oxygen species
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TRAP	Total radical trapping antioxidant potential
UV	Ultraviolet
WHO	World Health Organisation

CHAPTER 1

INTRODUCTION

The usefulness of natural products to humankind can be seen thousand years ago by its undeniable contribution to medicine, agriculture, cosmetics and food sources. Natural products are products of origins which can be derived from living organisms, including animals, plants, insects and microorganisms. However, in most cases the terms natural products refer to pure compounds isolated from plants. Throughout history, medicinal plants have been reported that Hippocrates used approximately 300 different plant species for medicinal purposes (Sarker, Latif and Gray, 2006). The Ebers Papyrus, written in 1550 BC documented a collection of 800 prescriptions and drugs from medicinal plants used for therapy such as caster oil plant, pomegranate, senna, aloe, garlic, onion, coriander and juniper (Dias, et al., 2012; Cseke, et al., 2006; Sarker, Latif and Gray, 2006). Malaysia has a huge tropical rainforest resources and is among the 12 biodiversity-rich countries in the world (Bernama, 2009). Approximately 20,000 plant species are found in Malaysia and 2,000 plant species have been reported to show medicinal values. Therefore, the potential for discovering new and valuable medicinal products is on-going process in Malaysia (Wong, 2009).

Medicinal plants are playing an increasing role in human health due to its substances with therapeutic properties. About 25% of commonly used prescribed drugs are traditionally derived from medicinal plants. According to the World Health Organisation (WHO), 75% of people still rely on plant-based medicir

for primary health care globally (Sarker, Latif and Gray, 2006). Extracts from plants and herbs have been used to treat illness for centuries. For example, morphine is extracted from the seeds of the opium poppy; aspirin is derived from the bark of the willow tree and quinine extracted from the bark of the chinchona tree is used against malaria (Lee and Bishop, 2016).

Plant chemicals can be categorised as primary or secondary metabolites, depending on the essential role in metabolism. Primary metabolites include the lipids, sugars, proteins amino acids, nucleic acids and chlorophyll. On the other hand, secondary metabolites which are also known as phytochemicals are small molecules that are not strictly necessary for the survival of the organism. Secondary metabolites make up all the remaining chemicals form alkaloids to terpenoids and acetogenins to phenolics that represent substances which are not essential in organism's metabolism, development, growth or reproduction (Walton and Brown, 1999). However, some secondary metabolites appeared to have a key role in protecting the plant form environmental pressures, herbivores and microbial infections (Crozier, Clifford and Ashihara, 2006; Iriti and Faoro, 2009). Secondary metabolites possess anti-inflammatory, antioxidant and antimicrobial activities.

Cancer is an abnormal growth of cells or normally called as malignancy. Cancer is the second leading cause of death worldwide and the fourth leading cause of death in Malaysia. The majority of patients are found at late stage of the disease (Desai, 2008). Cancer causes more death than cardiovascular diseases, AIDS, tuberculosis and malaria and reported to be responsible for 8.8 million deaths in

2015. There were 14 million new cases of cancer in 2012 and the number of new cancer cases is expected to rise to 22 million over the next two decades (National Cancer Institute, 2017). According to WHO (2017a), the most common type of cancer is lung cancer which accounts for 1.69 million deaths, followed by liver (788 000 deaths), colorectal (774 000 deaths), stomach (754 000 deaths) and breast (571 000 deaths).

Even though many treatments and therapies are being carried out to prevent this life-threatening disease, however, toxicities or adverse side effects to the cells may occur as a result of these treatments. For example, 5-fluorouracil is known to cause myelotoxicity and cardiotoxicity, whereas doxorubicin caused cardiac toxicity, renal toxicity and myelotoxicity (Desai, 2008). Furthermore, cancer acquired resistance against anticancer drugs by enhancing drug efflux from the cells, induction of drug-detoxifying mechanisms, reduce uptake of drug and alteration in target molecules. In general, failure of tumour cells to respond to chemotherapeutic drug has become a major threat to medicine (Gottesman, 2002). Therefore, researches on medicinal plants for the discovery of new drugs to combat cancers are believed to have less toxic effects and may reduce the adverse side effects (Siddiqui, 1993).

Infectious diseases are caused by pathogenic microorganisms, such as bacteria, viruses, parasites or fungi that can spread directly or indirectly from one person to another (WHO, 2018a). It is one of the leading causes of death worldwide due to its development of resistance to commonly drugs (Young Genomes, 2015). Antimicrobial resistance develops when microorganisms changes upon

continuous exposure of antimicrobial drugs such as antibiotics, antifungals, antivirals, and antihelminthics. These microorganisms are sometimes referred to as “superbugs” and is believed to develop resistance by accumulating mutations in DNA or acquire new gene which allows them to survive contact with antibiotic drugs resulting ineffective to the infections (WHO, 2018b).

Medicinal plants play an important role in drug discovery due to the emergence of antimicrobial resistance bacteria and increases in cancer cases. Medicinal plants have been widely used in primary health care and pharmacological investigations to identify new drugs for the development of new therapeutic agents to treat diseases (Mothana, et al., 2009). The bioactive compounds isolated from medicinal plants are believed to have anticancer and antimicrobial properties with lesser side effects and thus is safer to human health (Joray, et al., 2015).

Garcinia species that are native to Malaysia are studied in this research due to its active metabolites such as xanthones, benzophenones and triterpenoids with numerous biological activities (Jouda, et al., 2016). In previous studies, phytochemical and biological studies of *Garcinia maingayi* are found limited. Thus, this research was conducted to determine the cytotoxic effect, antioxidant and antibacterial activities of fractions isolated from *Garcinia maingayi*.

The objectives of this research are:

1. To isolate compounds from methanolic fraction of *Garcinia maingayi* using gravity column chromatography and thin layer chromatography,
2. To determine the percentage of radical scavenging activity of the isolated fractions using DPPH assay,
3. To evaluate the antibacterial activity of the isolated fractions via minimum inhibition concentration and minimum bactericidal concentration assays,
4. To determine the cytotoxic effect of the isolated fractions against COLO 205 cancer cells using MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 Garcinia Species

2.1.1 Botanical Description

Garcinia species are typically small to medium-sized dioeciously evergreen fruits trees or shrubs, growing up to 25 m in height with a strong trunk tapering to a conical canopy covered with a thick and corky bark (Parthasarathy, Chempakam and Zachariah, 2008). The branches are numerous, decussate and the twigs are distinctly angled (Steven, 2007). The leaves of the trees are usually simple, smooth, leathery, opposite or in whorls of three and ovate or oblong in shape. The length and the width of the leaves are approximately 18 to 23 cm and 9 to 10 cm, respectively. The ventral surface is yellowish green while the dorsal surface of the lamina is dark green in colour. The petiole is thick, robust and approximately 2 to 3 cm long (Idris and Rukayah, 1987).

Garcinia's flowers are terminal, solitary, unisexual on separate trees and borne in tufts or singly in the axils of leaves. The pedicel is straight or curved, light green in colour, 20 mm long and 5 mm thick with four sepals and petals, respectively in red, pink, yellow or white (Te-chato, 2007). The female flowers have a large hypogynous ovary mounted on a receptacle and male flowers have seven or more stamens inserted on a receptacle (Parthasarathy, Chempakam and Zachariah, 2008). The fruits are fleshy berries with smooth fleshy pericarp with high diversity in size and shape. Garcinia fruits have an appetising acidic flavour

(Parthasarathy and Nandakishore, 2014). The plants in this genus are commonly known as kokum, saptrees, mangosteens, garciniasor, and ambiguously ‘‘monkey fruit’’ (Hemshekhar, et al., 2011).

2.1.2 Taxonomical Classification

The genus Garcinia belongs to the Guttiferae family, which comprised of approximately 300 species (Kochummen, 1998). The taxonomical classification of *Garcinia maingayi* is as shown in **Table 2.1**.

Table 2.1: Taxonomical classification of *Garcinia maingayi* (Kochummen, 1998; Hemshekhar, et al., 2011).

Rank	**Taxonomical classification**
Kingdom	Plantae
Phylum	Tracheophyta
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malpighiales
Family	Clusiaceae
Subfamily	Clusioideae
Tribe	Garcinieae
Genus	Garcinia
Species	*Garcinia maingayi*

2.1.3 Habitat and Geographical Distribution

Garcinia is a large genus of polygamous trees or shrubs, distributed in the tropical Asia, Africa and Polynesia (Kumar, Sharma and Chattopadhyay, 2013; Patil and Appaiah, 2015). About 35 species are common in India and are endemic to the evergreen forests of Western Ghats, Gujarat, Andaman and Nicobar Islands and North-Eastern region of India. *G. mangostana*, *G. cambogia*, *G. dulcis* and *G. tinctoria* are widely spread in Asia (Parthasarathy, Chempakam and Zachariah, 2008; Hemshekhar, et al., 2011). Garcinia is found within lowland to montane forest areas in the states of Kedah, Perak, Pahang, Terengganu and Johor. Besides, most of the Garcinia species is distributed in the north of the Malaysian Archipelago, with approximately 28 species in Malaysia, 23 in Thailand, 20 in Indonesia and 19 in the Philippines (Kochummen, 1998; Parthasarathy, Chempakam and Zachariah, 2008).

2.1.4 Traditional and Medicinal Uses

Traditionally, the leaves and fruits are eaten by local people due to its antipyretic properties. In olden days, the dried fruits are used to treat stomach-ache and fever. The extracts of stem bark have been used as antimicrobial agent while yellow dye of *G. opaca* is used to improve blood circulation. Extracts from *G. nervosa* is used to treat skin infections and wounds healing (Jabit, et al., 2009). Garcinia species has been widely used as slimming product due to the presence of hydroxycitric acid that is known to block fat and suppresses the appetite by increasing serotonin levels but its toxicity is still under discussion now (Rasha, et al., 2015).

2.1.5 Chemical Constituents

Detailed chemical studies on *Garcinia maingayi* by Cheng and Cheow (2008) have yielded 1,3,7-trihydroxy-2-3-(3methylbut-2-enyl)-xanthone, benzophenone, isoxanthochymol, benzoic acid derivative 3, 4-dihydroxymethylbenzoate, stigmasterol and sitosterol. Their structures were examined using 1D and 2D nuclear magnetic resonance spectroscopy. Phytochemical investigation of different Garcinia species has resulted in the isolation of complex molecules such as prenylated xanthones, polyisoprenylated benzophenone, flavonoids, lactones and phenolic acids which are known for their antioxidant, cytotoxic, anti-inflammatory, antibacterial, antiviral, antifungal, antiulcer and antiprotozoal activities (Kumar, Sharma and Chattopadhyay, 2013). Xanthone showed antioxidant property by exhibiting a potent radical scavenging activity (Parthasarathy, Chempakam and Zachariah, 2008).

2.2 Pharmacological Studies of Garcinia Species

2.2.1 Antioxidant Activity

Kolaviron isolated from *G. kola* seed extract and garcinol from *G. indica* seed extract appear to act as natural antioxidant (Farombi, et al., 2000). The antiflatoxigenic activities of *G. cowa* and *G. pedunculata* to inhibit the growth of *Aspergillus flavus* might due to the effective anitioxidant property of Garcinia species which could suppress the biosynthesis of aflatoxin (Joseph, et al., 2005). Xanthones were known as strong antioxidant agent due to its uniqueness to maintain intestinal health, neutralising free radicals and promoting a healthy

seasonal respiratory system (Parthasarathy, Chempakam and Zachariah, 2008). Jacob, et al. (2015) found that the *G. gummi-gutta* extract had higher antioxidant property than ascorbic acid. Extracts of *G. gummi-gutta* showed high reducing power with absorbance of 1.372 compared with ascorbic acid with absorbance of 0.252 at 200 µg/mL. Higher absorbance indicates that sample has more antioxidant property. The presence of polyisoprenylated benzophenone and xanthone derivatives makes the plant to have the antioxidant property.

Furthermore, *G. pedunculata* showed highest antioxidant potential with IC_{50} value 47.03±13.48 µg/mL compared to *G. xanthochymus* and *G. morella*. *G. pedunculata* has the highest amount of phenolic content that may correlate to the antioxidant activity of the plants due to its free radical scavenging activity (Gogoi, Tsering and Veer, 2012).

2.2.2 Cytotoxic Effect

Jabit, et al. (2009) reported that the extracts from *Garcinia opaca* King (fruit), *Garcinia maingayi* Hook.f. (stem), *Garcinia penangiana* Pierre (leaf) and *Garcinia urophylla* Scortech.ex-King (leaf) showed the most potent and selective cytotoxic effect against breast cancer cells (MCF-7) with IC_{50} ranges from 3 to 8 µg/mL. Stem bark and leaves extracts of *Garcinia maingayi* showed IC_{50} value of 6 and 10 µg/mL, respectively on MCF-7. Besides, the leaf extracts of *Garcinia nigrolineata* and *Garcinia cantleyana* showed strong cytotoxic effect on prostate cancer cells (DU-145) with IC_{50} value of 3 and 4 µg/mL, respectively. Cytotoxic effect on non-small cell lung cancer cells (H460) were

shown by the leaves and stem extracts of *Garcinia cantleyana* and *Garcinia nigrolineata* with IC_{50} value of 2 and 3 µg/mL, respectively (Jabit, et al., 2009).

Furthermore, gambogic acid isolated from the resin of the *G. hurburyi* tree has become a potential anticancer agent due to its ability to induce apoptosis. Besides, Atrovirisidone B isolated from *G. atroviridis* was reported to exhibit significant cytotoxic effect against human breast, prostate and lung cancers (Parthasarathy, Chempakam and Zachariah, 2008). Kumar, Sharma and Chattopadhyay (2013) reported that the polyisoprenylated benzophenones isolated from Garcinia species inhibited growth and induced apoptosis in cancer cells.

2.2.3 Antibacterial Properties

Methanolic stem bark of *Garcinia kola* exhibited highest inhibitory concentration of 0.07, 0.11 and 0.90 mg/mL against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively. The inhibition zones of the extract against these bacteria were ranged from 21.50 to 42.50 mm. The antibacterial activity of the extracts is due to the synergistic action of some bio-reactive substances. Methanol extract has been reported to exhibit highest inhibitory effect when compared with ethanol extract. This could be because of high active compounds or secondary metabolites present in the extract produced with methanol (Ukaoma, et al., 2013). Furthermore, stem bark extract of *Garcinia latissima* Miq showed zone of inhibition of 10.70 and 10.38 mm against *B. subtilis* and *S. aureus*, respectively. This inhibition was reported due to the presence of tannins, saponins and flavonoids (Ambarwati, et al., 2017).

2.3 Cancer

Cancer or neoplasm is a collection of diseases characterised by uncontrolled cell growth and the invasion and spread of cells from primary site to other sites in the body (National Cancer Institute, 2015). A cancer forms when genetic material of the cell is mutated or damaged especially when that mutated gene is responsible for the normal regulation of cell growth. When the genetic control is damaged, the growth and division of cells will become uncontrolled and forming mass of tissue known as tumour (Tannock, et al., 2005).

Cancer is responsible for about 25 to 30% of deaths in developed countries. It is the second cause of death worldwide after cardiovascular disease and the fourth leading cause of death in Malaysia (Desai, 2008). It is estimated that the annual incidence of cancer is 30 000, while the prevalence of cancer was estimated to be approximately 90 000. Lung cancer is the most common killer among malignancies where the majority of patients are found at a late stage of the disease. In a regional cancer registry survey, the most leading cancer among males and females were colorectal and breast cancer respectively (Lim, 2002).

There are many cancer treatments available such as chemotherapy, surgery, radiation, immunotherapy and hormonal therapy. Even though combination of these treatment has been long used, cancer still remains as the one of the leading causes of morbidity and mortality globally. These treatments cause undesired side effects such as anaemia, haemorrhage, nausea, vomiting, diarrhoea and hair

loss (Tannock, et al., 2005). Besides, some chemotherapeutic drugs cause myelotoxicity, cardio toxicity and pulmonary toxicity. Moreover, tumour may develop resistance to treatment after many rounds of chemotherapy and successful shrinkage of tumour. The clinician has to stop the treatment when the side effects prevail over benefits and tumour start to grow again (Desai, 2008). Therefore, many research have been carried out on medicinal plants which acts as potential source of drugs to cure cancer and many other diseases as the secondary metabolites found in the plants might be a safer alternative to synthetic chemotherapeutic drugs (Khan, et al., 2008).

The third most common cancer and the second most common cancer in women worldwide is colon cancer (Arora and Tandon, 2015). There were 64,000 new cases of colon cancer reported in India and out of which 49,000 deaths were seen in 2012 (Globocan Colorectal Cancer Estimated Incidence, 2012). These alarming statistics of colon cancer incidence and mortality has raised serious concerns on the use of radiotherapy and chemotherapy due to their non-specific killing and many undesired side effects (Moongkarndi, Kosem and Kaslungka, 2004). Nevertheless, there were less study of cytotoxic effect of Garcinia species on colon cancer as many studies are focusing on the treatment of breast cancer, lung and prostate cancer cells (Jabit, et al., 2009; Seruji, Khong and Kutoi, 2013). Hence, colon cancer (COLO 205) cell line was used in this research.

2.4 Cancer Cell Line

A cell line is a permanently established cell culture that will proliferate in appropriate fresh medium and space. Cell lines played an important role in studying physiological, pathophysiological and the differentiation processes of specific cells (Ulrich and Pour, 2001). Cancer cell lines grow over time in a favourable artificial environment in a laboratory and used extensively in research to study the normal physiology of cancer cells and the effect of drugs on the cells (National Cancer Institute, 2017). COLO 205 is a human colon carcinoma cell line which was derived from colon and the ascites fluid of a 70-year-old Caucasian male with Dukes' type D, colorectal adenocarcinoma (American Type Culture Collection [ATCC], 2016). The cells are colon-specific antigen-p (CSAp) negative but positive for keratin by immunoperoxidase staining. A 36000 dalton cell surface glycoprotein related to the GA733-2 tumour associated antigen was expressed by COLO 205 cells (ATCC, 2016).

These adherent monolayer cells grow in cuboidal shape at time of isolation but displayed predominantly rounded cells in suspension with cuboidal cells in monolayer. Epithelial-like shaped COLO 205 cells were also observed on cell culture (Public Health England, 2017).

2.5 Infectious Diseases

Infectious diseases are disorders caused by pathogenic microorganisms, such as bacteria, viruses, fungi or parasites that can be transmitted from an infected patient to a susceptible host (Mayo Clinic, 2017). Infectious diseases have a significant impact on the health of the population and are the major cause of

morbidity and mortality worldwide. It has been reported that there was approximately 50,000 deaths caused by infectious disease all around the world every year (World Health Organisation, 2017b). In Malaysia, there have been several outbreaks of infectious diseases including dengue, tuberculosis, Chikungunya, hand, foot and mouth diseases due to multiply resistant organisms (Tee, Takebe and Kamarulzaman, 2009).

Bacterial transmission depends on the ability of bacteria to gain entry and cause damage to the host. Some diseases are easily spread while others require a carrier or intermediate host. Infectious diseases can be transmitted by contact with respiratory secretions from an infected host or even contaminated surfaces. Meanwhile, some are transmitted through ingestion of contaminated food or drink. Vectors such as mosquitoes, fleas and bugs are responsible for the transmission of infectious agent through zoonosis (Mayo Clinic, 2017).

A number of other antimicrobial drugs have been produced to treat infectious diseases since the discovery of the first antibiotic, penicillin in 1928 (American Chemical Society, 2015). These have a huge impact on the human health. Many community-acquired infections have been successfully treated with antibiotics. However, some microorganisms are becoming resistant to the available antibiotics. They are highly adaptable and the over-use or misuse of antibiotics has led to the emergence of resistant bacteria. Bacteria may develop resistance to antimicrobial drugs through a change in the membrane permeability, spontaneous mutations, increased efflux of drug, alteration in the binding sites and others (Lee and Bishop, 2010). Most of the resistant bacteria produce an

enzyme known as extended spectrum β-lactamases (ESBLs) to develop resistance. There are nearly 15 million (25%) of the estimated 57 million deaths worldwide each year are caused by infectious diseases. The emergence of resistant bacteria has increased the number of death. Nevertheless, there have been no significant discoveries of new antibiotics recent years. Therefore, medicinal plant has started to become an alternative that can potentially be effective in the treatment of infectious diseases (Ahmad and Wajid, 2013; Marasini, et al., 2015).

2.6 Bacteria

Bacteria are simple unicellular organisms characterised by a lack of membrane-bound nucleus, mitochondria, endoplasmic reticulum and other defined organelles that can reproduce by asexual division (Murray, Rosenthal and Pfaller, 2015; Lee and Bishop, 2016). Bacteria can be differentiated based on the size, shape, morphology, staining characteristics and cell wall structure. The bacterial cell wall is complex and can be categorised into Gram-positive and Gram-negative. Gram-positive bacteria have a thick layer of peptidoglycan whereas cell wall of Gram-negative bacteria consists of a thin layer of peptidoglycan and an overlying outer membrane (Lee and Bishop, 2016).

2.6.1 *Bacillus cereus*

Bacillus cereus is a Gram-positive aerobic or facultative, motile, anaerobic, spore-forming, rod-shaped bacterium that is almost ubiquitous in the natural environment (Bottone, 2010). It is the major cause of food poisoning and serious and potentially fatal non-gastrointestinal tract infections in human. The bacterium is a spore former and vegetative cell in nature and colonises the human body in a vegetative status. The natural reservoir for *B. cereus* in environment consists of decaying organic matter, vegetables, fomites, fresh and marine water and the intestinal tract of invertebrates, from which soil and food products may become contaminated, leading to colonisation of the human intestine (Bottone, 2010; Savini, 2016). The production of tissue-destructive exoenzymes is closely associated with intestinal or nonintestinal infections. Among these secreted toxins are phospholipases, haemolysins, emesis-inducing toxin, and pore-forming enterotoxins. The enterotoxins secreted by the vegetative cells induce a diarrheal syndrome in the small intestine. Furthermore, *B. cereus* may also cause a number of systemic and local infections such as respiratory infection and nosocomial infection (Bottone, 2010).

2.6.2 *Staphylococcus aureus subsp. aureus*

Staphylococcus aureus is a Gram-positive, grape-like clusters shaped, non-spore-forming bacterium which responsible for many skin and wound infections. Staphylococcus species are normally found in areas of low moisture such as the skin or nose and very tolerant to high salt concentration and dry environments (Lee and Bishop, 2016). The bacteria are always associated with food poisoning such as diarrhoea and vomiting because of their ability to produce enterotoxins.

Staphylococcus aureus poses a serious threat to many patients with wounds infections because they develop resistance quickly to many types of antibiotics especially methicillin. These strains are known as methicillin-resistant *Staphylococcus aureus* (MRSA) which produces a yellow colour on the differential medium, mannitol salt agar. MRSA is often classified their growth on blood agar. Beta-haemolytic Staphylococci produce haemolysin that lyses red blood cells and creating zone of haemolysis on blood agar plates (Foster, 2004; Lee and Bishop, 2010). MRSA can cause a variety of diseases ranging from skin infections, to pneumonia and bloodstream infections. MRSA is endemic in most hospitals and caused about 11% of health care-associated infections in 2011 (Centers for Disease Control and Prevention, 2016). It was reported that the prevalence rate of MRSA infections is the highest in Asia. The prevalence of MRSA in Malaysia has increased from 17% in 1986 to 44.1% in 2007 (Sit, et al., 2017).

2.6.3 *Escherichia coli*

Escherichia coli is a Gram-negative, rod-shaped, facultative anaerobic bacterium which normally present in the intestines of human and animals. Most *E. coli* strains are non-pathogenic and colonise the gastrointestinal tract of humans as a normal flora. However, there are some pathogenic strains which are capable of causing wide variety of illnesses such as diarrhoea, urinary tract infections, respiratory illness, pneumonia and meningitis (Centers for Disease Control and Prevention, 2018; Lim, Yoon and Hovde, 2010). The difference in the pathogenic potential of different strains are due to the specific genes encoded the

virulence factors. Among them, enterohaemorrhagic *E. coli* or Shiga toxin-producing *E. coli* is one type of pathogenic strains that produce Shiga toxins and cause haemorrhagic colitis in humans. *E. coli* is always transmitted through consumption of contaminated food and water and also airborne transmission which has been reported recently as potential route of transmissions (Donnenberg, 2002; Lim, Yoon and Hovde, 2010).

2.6.4 *Salmonella enterica* Typhimurium

The genus Salmonella comprises Gram-negative and non-endospore-forming rods that exists mainly in the mammalian gastrointestinal tract. *Salmonella enterica* Typhimurium is a flagellated facultative anaerobe with approximately 0.7 to 1.5 μm in diameter and 2 to 5 μm in length. There are numerous serovars (serotypes) of Salmonella such as *S. enterica* serovar Typhimurium, Enteritidis, or Choleraesuis (Fabrega and Vila, 2013). *Salmonella enterica* serovar Typhimurium is a primary enteric pathogen and one of the most common causes of food-borne illness such as diarrhoeal diseases. This organism is the major causative agent of nontyphoidal salmonellosis in Malaysia (Ngoi, et al., 2013). *Salmonella enterica* Typhimurium can be transmitted through the ingestion of contaminated food or water. The intestinal tract of animals is the major source of diarrhoea-causing Salmonellae. They are usually found contaminating animal meats and poultry products and cause infection when the food is improperly stored or cooked (Lee and Bishop, 2016). According to Centers for Disease Control and Prevention, approximately 40 000 cases of salmonellosis are

reported each year in the United States leading to the death of 400 persons each year (Fabrega and Vila, 2013).

2.7 Chromatographic Techniques

2.7.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a technique used to separate non-volatile mixtures because it is a simple, cheap and rapid method used to investigate components in a mixture. TLC consists of a thin adsorbent layer of silica, alumina or cellulose (stationary phase) coated onto the surface of a supporting material which can be plastic, aluminium or glass (Sarker, Latif and Gray, 2006). A sample is applied on the baseline of TLC plate and placed into a chamber containing various organic solvents. The sample is separated into its component compounds when the solvent (mobile phase) is allowed to move up the plate by capillary action. The components will travel up the plate at different speeds until it reaches the frontline depending on its affinity for the stationary phase. The speed of migration also depends on the polarity of the eluting solvent. The spot separated would be observed with naked eyes and UV light. The distance that a component moves relative to the solvent front is calculated as retention factor (R_f) values (Coskun, 2016).

In general, low polarity compounds have higher R_f values than higher polarity compounds. The larger an R_f of a compound, the larger the distance it travels on the TLC plate (Massachusetts Institute of Technology, 2012). The adsorptivity of compounds increases with increased polarity which means the more polar the

compound then the stronger it binds to the adsorbent. The stronger a compound is bound to the adsorbent, the slower it moves up the TLC plate. Non-polar compounds move up the plate most rapidly, whereas polar substances travel up the TLC plate slowly or not at all (Busia, 2016; Massachusetts Institute of Technology, 2012).

2.7.2 Gravity Column Chromatography

Gravity column chromatography is an application of column chromatography commonly used for preparative scale separation of components from a crude plant extract based on the gravitational force. Column chromatography is always used for the purification of compounds from a mixture. In practise, a vertical column is packed with a suspension of adsorbent which is usually silica gel. A sample to be purified is placed on the top of the column, and a suitable organic solvent mixture (mobile phase) is allowed to flow down through the column under the force of gravity (Organic Chemistry, 2016). The various components to be separated travel through the column at different rates based on various polarities and interactions ability with stationary phase and mobile phase. The individual compounds are then collected separately as fractions and used for further analysis (Bajpai, Majumder and Park, 2016).

2.8 Bioassays

2.8.1 Antioxidant Assay

DPPH (2, 2-diphenyl-1-picrylhydrazyl) is used in antioxidant assay to assess the free radical scavenging (antioxidant) property of a compound, an extract or other biological sources. It is one of the most widely used methods for screening the antioxidant property of plant extracts. This assay is based on the measurement of scavenging capacity of antioxidants (plant extract) towards the stable free radical DPPH. DPPH is a stable free radical that appears as purple colour with an absorption at 517 nm due to its odd number of electrons. It has an unpaired valence electron at one atom of nitrogen bridge (Sharma and Bhat, 2009). The odd electron of nitrogen atom in DPPH is reduced by receiving a hydrogen atom from antioxidants present in plant extract to the corresponding hydrazine. The purple colour of DPPH will be reduced into yellow colour (Al-Amiery, et al., 2013). Discoloration is measured by using spectrophotometer and the reduction of colour intensity is resembled by the decrease in optical density. The resulting decolourisation is stoichiometric with respect to the number of electrons taken up or captured. The antioxidant activity of the plant extract in DPPH assay can be calculated or determined using EC_{50} value. EC_{50} is defined as the concentration of substrate that causes 50% reduction in the DPPH colour (Kedare and Singh, 2011).

DPPH assay is considered a valid accurate, simple and inexpensive method to evaluate radical scavenging activity of antioxidants because the radical compound is stable and need not be generated. Moreover, it can also be used to quantify antioxidants in complex biological systems, for solid or liquid samples.

The results are more reproducible and comparable to other free radical scavenging methods (Kedare and Singh, 2011).

2.8.2 Cytotoxic Assay

Cell cytotoxicity is the ability of certain chemicals or plant extract to destroy living cells mainly the cancer cells (G-Biosciences, 2012). Healthy living cells or cancer cells can be killed either by apoptosis or necrosis induced by the cytotoxic compounds. Cytotoxic assay is used to determine the effect of certain compounds on the viability of cancer cells grown in culture. Anticancer activity was determined using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) assay for several cancer cell lines (Adan, Kiraz and Baran, 2016).

MTT assay is a sensitive, quantitative and reliable colorimetric assay that measures the cell viability based on the viable cells' ability to reduce the water-soluble yellow MTT dye tetrazolium to insoluble purple formazan dye by the action of mitochondrial dehydrogenase enzyme. The insoluble purple formazan crystals are dissolved by DMSO and the absorbance is measured spectrophotometrically. The colour intensity of the formazan dye yielded by the cell population is proportionate to the number of metabolically active viable cells and inversely proportional to the degree of cytotoxicity (Senthilraja and Kathiresan, 2015). The IC_{50} value can be determined from the dose-response curves. IC_{50} is the concentration of the drugs that will produce a 50% reduction

in the absorbance that measures the effectiveness of a drug in inhibiting certain biochemical or biological function (Jabit, et al., 2009).

2.8.3 Antibacterial Assays

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays are used to test the bacteriostatic and bactericidal activity of a compound. A bactericidal compound is able to kill microorganism whereas bacteriostatic compound inhibits the replication of the microorganism without killing it (Kohanski, Dwyer and Collins, 2010).

The lowest concentration of antimicrobial agents that will inhibit the visible growth of a microorganism is the MIC. It is widely used in the comparative testing of new antimicrobial agents (Andrews, 2002). Dilution methods are always used to determine the MIC of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing where microorganisms are tested for their ability to produce visible growth in microtitration plate wells of broth containing serial dilutions of the antimicrobial agents (Rodr ́guez-Tudela, et al., 2008). The MIC determination on various bacteria in this research were conducted using rapid p-iodonitrotetrazolium chloride (INT) colorimetric assay. INT is a tetrazolium dye that turns purple upon reduction by dehydrogenase activity. Viable bacteria are able to reduce the yellow tetrazolium dye to purple colour formazan. Therefore, MIC was defined as the sample concentration that prevented the colour change of the INT dye and exhibited complete inhibition of

microbial growth which results in colourless solution in the medium (Kuete, et al., 2012).

Minimum bactericidal concentration (MBC) is defined as the lowest concentration of antimicrobial agent that will kill the microorganism after subculture on to antibiotic-free media (Andrews, 2002). MBC is generally determined from MIC in which the sample in MIC wells that shows no bacterial growth is subculture onto the antibiotic-free agar plate. The growth of the bacteria will be observed. The concentration where there is no bacterial growth on the agar plate is the MBC. Therefore, MBC value is the lowest concentration of antimicrobial agent that prevented visible growth on the subculture plate (Goodwin, Blake and Blincow, 1986).

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Plant Material

Stem bark of *Garcinia maingayi* was obtained from Sabah in January 2015 and authenticated by Dr. Lim Chang Kiang, Associate Professor, Department of Chemical Science, Universiti Tunku Abdul Rahman. The stem bark was extracted using various organic solvents via cold extraction method. Only 10.0 g of methanolic fraction was used in this research.

3.1.2 Cancer Cell Line

Human colon cancer cell line (COLO 205) (ATCC® CCL-222TM) was used in this study. The preserved cells were cultured in DMEM and incubated in humidified 5% carbon dioxide incubator.

3.1.3 Bacteria

The glycerol stocks of *Escherichia coli* (ATCC25922), *Salmonella enterica* Typhimurium (ATCC14028), *Bacillus cereus* (ATCC13061), *Staphylococcus aureus subsp. aureus* (ATCC 25923), methicillin-resistant *Staphylococcus aureus subsp. aureus* (ATCC 33591) and methicillin-resistant *Staphylococcus*

aureus subsp. aureus (ATCC 43300) were cultured on Mueller Hinton agar (MHA) and incubated at 37 ℃.

3.1.4 Chemicals and Solvents

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

Table 3.1: List of chemicals and solvents, and their manufacturer or brand.

Chemicals and Solvents	Manufacturer / Brand
Acetone	QRëC™ Grade AR
Ascorbic acid	Gene Chem, Canada
Chloroform	QRëC™ Grade AR
DPPH reagent	Calbiochem®, USA
Dulbecco's Modified Eagle Medium	Nacalai Tesque, Kyoto
Dimethyl sulfoxide (DMSO)	Merck, Germany
Doxorubicin hydrochloride	Fisher Scientific, New Jersey
Ethanol 95% (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Ethyl acetate (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Foetal bovine serum (FBS)	JR Scientific, Inc, USA
Gentamycin sulfate	Bio Basic Inc, Canada
Glycerol	Fisher Scientific (M) Sdn Bhd
Hexane (Industrial grade)	Copens Scientific (M) Sdn.Bhd., Malaysia
INT dye	Bio Basic Inc, Canada
Methanol (Industrial grade)	Irama Canggih Sdn. Bhd., Malaysia
Mueller Hinton agar	HiMedia Laboratories, India
Mueller Hinton broth	Liofilchem, Italy
MTT reagent	Bio Basic Inc, Canada
Penicillin-streptomycin (1U)	Bio Basic Inc, Canada
Phosphate buffered saline (PBS)	MP Biomedicals, France
Potassium chloride	SYSTEM, Malaysia
Potassium dihydrogen phosphate	QRëC™ Grade AR
Silica gel 60	Merck, Germany
Silica gel coated aluminium sheet	Merck, Germany
Sodium chloride	Merck, Germany
Sodium phosphate dibasic anhydrous	Mallinckrodt Baker Inc, Canada
Sodium sulfate anhydrous	John Kollin Corporation, USA
Streptomycin sulfate	Bio Basic Inc
Trypan blue dye (0.4%)	Life Technologies, USA
Trypsin-EDTA (0.25%)	Biowest, USA

3.1.5 Equipment

The equipment used in this research as listed in **Table 3.2**

Table 3.2: List of equipment used, and their manufacturer.

Equipment	Manufacturer
Autoclave	Hvt-50, HICLAVE, USA
Carbon dioxide incubator (5%)	BINDER, Germany
Centrifuge	Kendro, Germany
Electronic balance	Kern, ABJ, Australia
Freezer (-20 ℃)	Snow, Malaysia
Freezer (-80 ℃)	ARDO, Italy
Fume hood	Chemo Resources, Malaysia
Haemocytometer	Marienfeld-Superior, Germany
Incubator (37 ℃)	Memmert, Germany
Inverted contrast phase microscope	Olympus, Malaysia
Laminar flow	Edamix, Malaysia
Microplate reader	TECAN, Australia;
pH meter	Sartorius, Malaysia
Refrigerator (4 ℃)	SAMEMAX, Malaysia
Sonicator	Branson Ultrasonic, USA
Spectrophotometer	Biochrome
Ultraviolet lamp	Spectroline™, USA
Vortex	Bibby Scientific Ltd., UK
Water bath	Memmert, Germany
Weighing balance	Kern and Sohn Gmbh

3.2 Methodology

The workflow of this research is shown in **Figure 3.1**. Stem bark of *Garcinia maingayi* was first extracted using methanol via cold extraction method to obtain methanolic bioassay-fractions. One of the active methanolic fraction was further isolated and analysed using bioassay-guided fractionation.

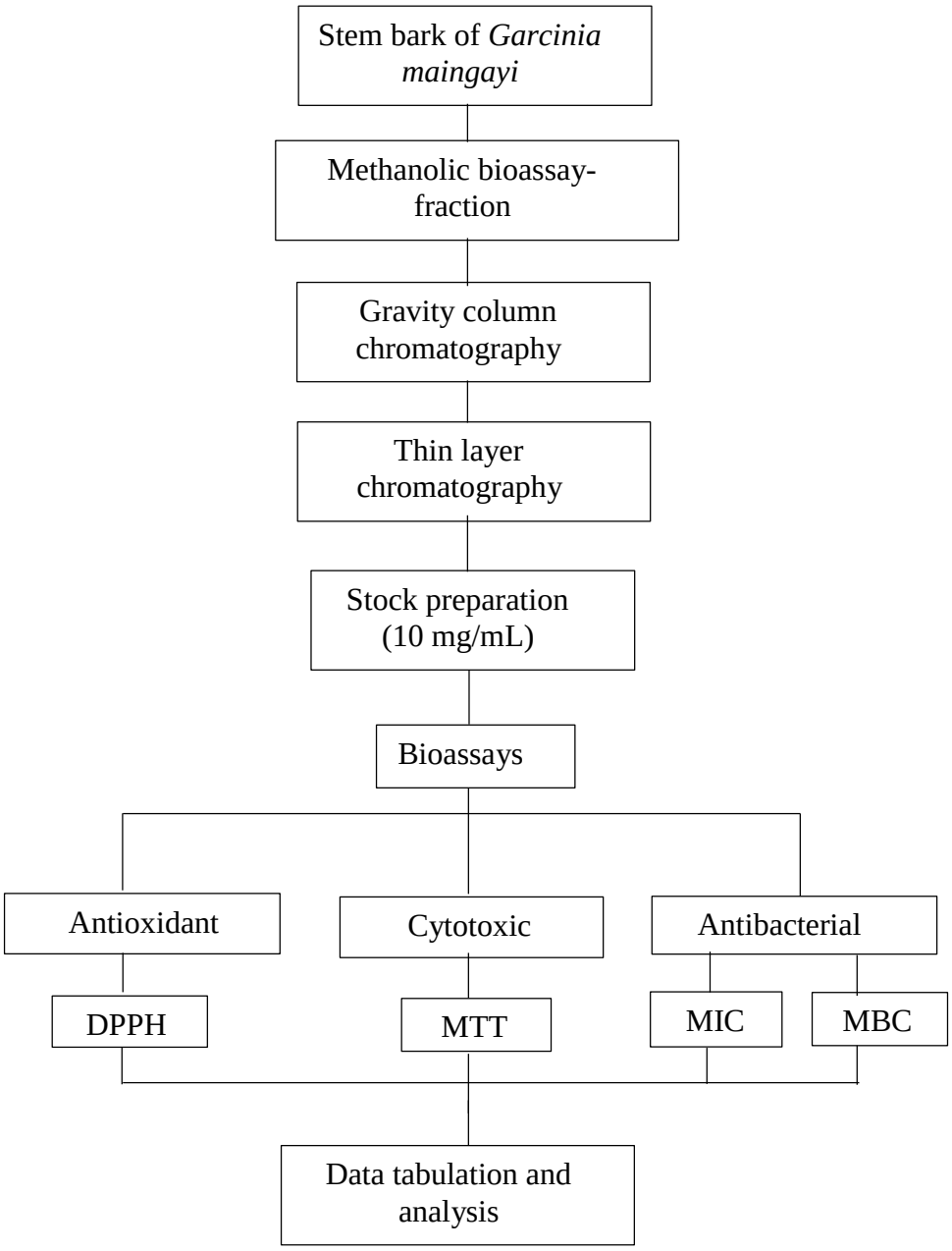

Figure 3.1: A brief workflow of the research using methanolic fraction of *Garcinia maingayi*.

3.3 Gravity Column Chromatography

A glass column sized 4 cm diameter and 100 cm height with sintered layer was packed with silica powder using wet pack method. The column was rinsed with hexane before packing to get rid of any unwanted residues. Silica powder was first mixed with hexane and added into the glass column about third-quarter of the column's height. Hexane was then continuously added into the column to ensure tight packing and at the same time the column was tapped with a rubber hose to remove air bubbles. The methanolic fraction was mixed with silica and air dried. The dried sample was then loaded carefully on top of the packed silica gel in the glass column, followed by two spatulas of sodium sulfate anhydrous layered on sample to absorb water.

Different organic solvent systems with increasing polarities were used in the isolation. The solvent combination ratio used is shown in **Table 3.3**. Mixture of hexane and ethyl acetate was used first based on thin layer chromatography. Approximately 200 mL of eluent was collected in conical flask and concentrated using rotary evaporator (40 ℃). The concentrated fractions were collected in sample vials and the dry weight of each fraction was recorded. Collection and concentration of fractions were repeated until the column become colourless.

Table 3.3: Solvent combination ratio used in gravity column chromatography.

Hexane	Ethyl acetate	Methanol
7	3	-
1	1	-
-	10	-
-	10	0.5
-	10	1.5
-	10	3
-	10	4
-	1	1
-	-	10

Final volume of the mixture was 200 mL.

3.4 Thin Layer Chromatography

Thin layer chromatography (TLC) was used to combine the fractions collected from gravity column chromatography according to the spots. The silica coated aluminium sheet was cut into 10.0 cm long and 5.0 cm width. A baseline of 1.0 cm from bottom and solvent frontline with 1.0 cm from top were drawn on the plate by using a pencil as shown in **Figure 3.2**.

The fractions were first dissolved in chloroform and were spotted on the baseline of the plate using a thin capillary tube. The plate was placed in a beaker saturated with various combinations of organic solvents. The beaker was then covered with aluminium foil to avoid evaporation of organic solvents.

The plate was removed from the beaker once the spots reached the solvent frontline.

All the visible spots were marked immediately using a pencil and the remaining spots were observed under ultraviolet light lamp at short (254 nm) and long (365 nm) wavelengths respectively. Numbers of spots developed and their respective retention factor (R_f) were calculated using the following formula (Massachusetts Institute of Technology, 2012).

$$\text{Retention factor, } R_f = \frac{\text{Distance of spot travelled (cm)}}{\text{Distance of solvent travelled (cm)}}$$

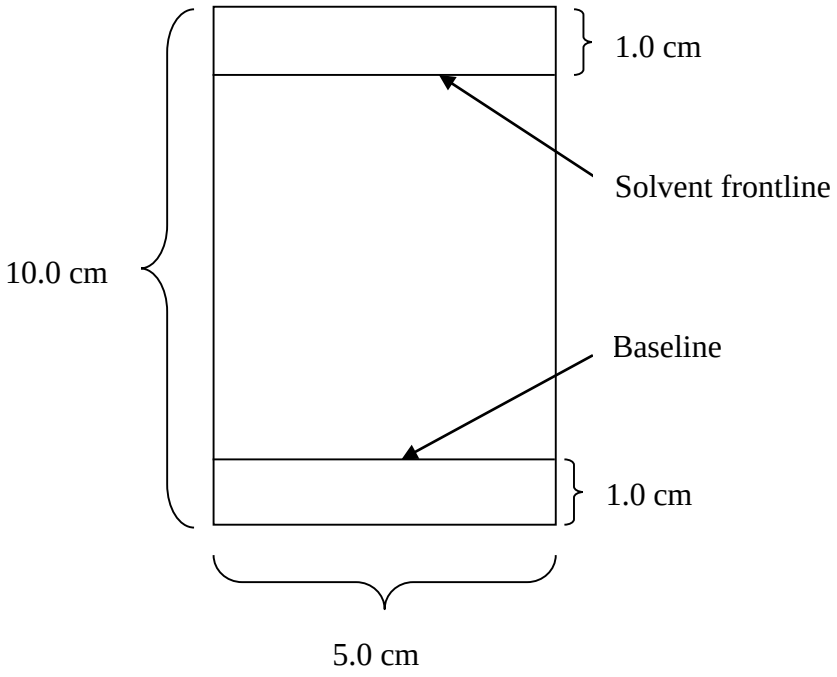

Figure 3.2: Design of thin layer chromatography plate.

3.5 Preparation of Stock Solutions

In DPPH assay, 10 mg of each fraction was added into 1 mL of methanol to obtain concentration of 10 mg/mL and the stock solutions were sonicated for complete solubilisation.

In antibacterial assay, the stock solution was prepared by adding 5 mg of fraction into 1 mL of 100% DMSO to obtain 5 mg/mL. The stock solution was sonicated and further diluted using Mueller Hinton broth.

In MTT assay, stock solution for each fraction was prepared by adding 1 mg of fraction into 1 mL of 100% DMSO to obtain concentration of 1 mg/mL. The stocks were further diluted using basic DMEM by adding 0.1 mL of stock solution into 0.9 mL of DMEM. All the prepared stock solutions were kept at -20 ℃.

3.6 Preparation of Positive and Negative Controls

In DPPH assay, the positive and negative controls were ascorbic acid and methanol, respectively. Ten milligrams of ascorbic acid was dissolved in 1 mL of methanol to obtain concentration of 10 mg/mL. Meanwhile, methanol alone is the negative control.

In antibacterial assay, gentamycin sulfate and streptomycin sulfate were used as positive control, whereas less than 5% DMSO was used as negative control. Both positive controls were prepared by adding 10 mg of the respective

powder into 1 mL of distilled water to obtain 10 mg/mL. The stocks solutions were further diluted using Mueller Hinton broth in a 96-well plate.

In MTT assay, doxorubicin hydrochloride was used as positive control, whereas less than 10% DMSO was used as negative control. The working solutions of 0.1 mg/mL was prepared by diluting 0.01 mL of stocks solution (10 mg/mL) into 0.99 mL of basic DMEM. The negative control was prepared by diluting 100 µL of 100% DMSO with 900 µL of basic DMEM. All the positive and negative controls were kept at -20 ℃.

3.7 Preparation of Reagents and Medium

3.7.1 DPPH Reagent

DPPH reagent (0.02 mM) was prepared by dissolving 1.57 mg of DPPH powder into 200 mL of methanol. The reagent was prepared in dark as DPPH is a light sensitive reagent. The reagent was wrapped with aluminium foil and incubated in dark for 30 minutes and stored in refrigerator at 4 ℃.

3.7.2 MTT Reagent

MTT dye was prepared by adding 50 mg of MTT powder into 10 mL of sterile autoclaved phosphate buffered saline to obtain a concentration of 5 mg/mL. The solution was then vortexed and filtered using 0.22 µm cellulose filter. MTT dye was prepared in dark and covered with aluminium foil since it is sensitive to light. The reagent was stored at -20 ℃.

3.7.3 Phosphate Buffered Saline

Phosphate buffered saline (PBS) was prepared by adding 1.60 g of sodium chloride, 0.04 g of potassium chloride, 0.29 g of disodium phosphate and 0.05 g of monopotassium phosphate into a Schott bottle containing 200 mL of distilled water. The pH of PBS was adjusted to 7.4 and autoclaved at 121 ℃ for 15 to 20 minutes. The sterile PBS was sealed with parafilm and kept at 4 ℃.

3.7.4 Iodotetrozolium Chloride Dye

Iodotetrozolium chloride **(**INT) dye at concentration of 0.4 mg/mL was prepared by adding 16 mg of INT powder in 40 mL of distilled water. INT dye was then filtered using 0.20 μm cellulose membrane filter. The preparation of INT was performed in dark and the tube was wrapped with aluminum foil due to its light sensitivity. The INT dye was kept at -20 ℃.

3.7.5 Complete Growth Medium

Complete medium was prepared aseptically by adding 20 mL of 10% FBS into 180 mL of basic DMEM medium. The prepared complete medium was incubated overnight at CO_2 incubator for quality control purposes. The medium was then sealed with parafilm and stored in the 4 ℃ refrigerator.

3.7.6 Mueller Hinton Agar and Mueller Hinton Broth

Mueller Hinton agar (MHA) was prepared by adding 15.2 g of agar powder into 400 mL of distilled water, whereas Mueller Hinton broth (MHB) was prepared by dissolving 8.4 g of broth powder into 400 mL of distilled water. Both MHA and MHB were autoclaved at 121 ℃ for 15 minutes. The cold MHB was then sealed with parafilm and stored at room temperature. Meanwhile, the agar was poured aseptically into sterile petri dishes and was allowed to solidify. The plates were sealed with parafilm to avoid contamination and kept at room temperature.

3.8 Cell Culture

3.8.1 Culture and Subculture of Cell Line

COLO 205 cell line was taken out from liquid nitrogen tank and was thawed immediately in water bath at 37 ℃. Approximately 1 mL of cells were pipetted into a 25 cm^3 culture flask containing 4 mL of complete medium. The flask was then observed under inverted phase contrast light microscope and incubated in humidified 5% CO_2 incubator at 37 ℃.

Subculture was performed once the cells reached 70 to 80% confluency. The old medium was discarded and the cells were rinsed twice with 5 mL of PBS. Approximately 2 mL of 0.25% trypsin-EDTA solution was added into the flask and incubated in CO_2 incubator for 5 to 10 minutes for detachment of cells. The flask was observed under inverted light microscope to ensure complete detachment of cells and 3 mL of complete medium was added into the flask.

The cells were transferred into a centrifuge tube, sealed with parafilm and centrifuged at 1000 rpm for 6 minutes. The supernatant was discarded and the pellet was resuspended with 1 mL of complete medium. The mixture was then transferred into a new 25 cm^3 flask containing 4 mL of complete medium and incubated in 5% CO_2 humidified incubator at 37 ℃.

3.8.2 Culture and Subculture of Bacteria

Glycerol stocks of *Escherichia coli*, *Salmonella enterica* Typhimurium, *Bacillus cereus, Staphylococcus aureus subsp. aureus* and two strains of methicillin resistant *Staphylococcus aureus* were thawed and inoculated onto MHA plate aseptically by using inoculating loop. The plates were sealed with parafilm and incubated at 37^{o}C incubator for 24 hours. The bacteria colonies were then transferred from the plates into 30 mL of fresh MHB and incubated in shaking incubator at 37^{o}C with speed of 200 rpm. The bacterial suspension was subcultured by adding 10 mL of bacterial suspension into 20 mL of new broth.

3.9 Cell Counting

3.9.1 Cancer Cell Line

Cell counting of cancer cells were performed prior to MTT assay using haemocytometer. Approximately 100 µL of cell suspension was mixed with 100 µL of 0.4% trypan blue in a microcentrifuge tube. The cells were incubated for 3 minutes and 20 µL of the mixture were loaded onto haemocytometer through capillary action. The cells were then observed under an inverted light microscope at 100x magnification. Dead cells were stained blue whereas viable cells were unstained and only the viable cells were counted using a cell counter. The concentration of cells was calculated using the following formula (Katsares, et al., 2015).

Concentration (cells/mL) = the average of viable cells in four grids $\times$ dilution factor $\times 10^4 \times$ volume of original cell suspension

3.9.2 Bacteria

In antibacterial assay, 10^6 CFU/mL of the bacteria was seeded into the 96-well plate. The bacterial suspension was transferred to a cuvette and the absorbance was measured at 600 nm using spectrophotometer. The concentration of bacteria was adjusted by adding bacteria suspension or MH broth to obtain absorbance ranging from 0.08 to 0.10 which is equivalent to 0.5 McFarland standard (Donay, et al., 2007).

3.10 Bioassays

3.10.1 DPPH Assay

Round bottom 96-well plate was used in DPPH assay and the design is shown in **Figure 3.3**. Approximately 50 µL of methanol was added to all the wells. In the first row of columns 3 to 12, 50 µL of ascorbic acid and various fractions were added respectively and serially diluted with methanol to obtain concentrations ranging from 0.04 to 5.00 mg/mL. Meanwhile, approximately 50 µL of methanol only was added into columns 1 to 2 as negative control. About 20 µL of DPPH reagent was added into each well and the plate was wrapped with aluminium foil. The plate was incubated in dark for 30 minutes. The absorbance was read by using a microplate reader at 517 nm. The percentage of radical scavenging activity was calculated using the following formula (Basma, et al., 2011). The percentage of radical scavenging activity against concentration of sample was plotted to determine EC_{50} value.

$$\text{Percentage of radical scavenging activity} = (A_{NC} - A_S / A_{NC}) \times 100\%$$

Where,

A_S refers to average absorbance of the sample

A_{NC} refers to the average absorbance of the negative control

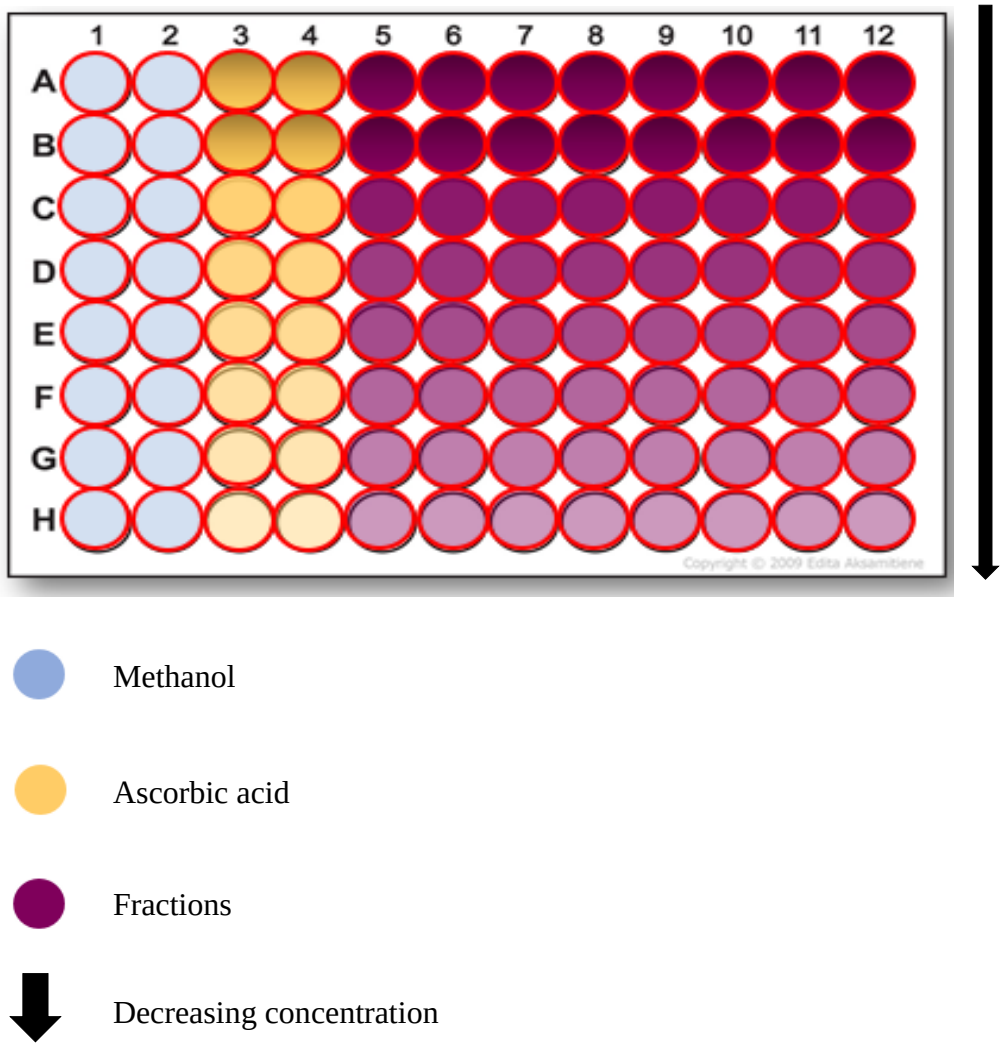

Figure 3.3: Design of 96-well plate used in DPPH assay.

3.10.2 MTT Assay

MTT assay was performed on a flat bottom 96-well plate and the design used is shown in **Figure 3.4.** A concentration of 1 x 10^4 cells/mL were seeded on each well except sterility control that contain only basic medium. The plate was then incubated in 5% CO_2 incubator at 37°C for 24 hours and the attachment of cells were observed under an inverted microscope. Various fractions were added into the first row of columns 5 to 12 and serially diluted to obtain concentrations

ranging from 0.39 to 50.00 µg/mL. Subsequently, approximately 100 µL of 10% DMSO was added into columns 1 to 2 while doxorubicin hydrochloride was added into columns 3 to 4. Both DMSO and doxorubicin hydrochloride were further serially diluted. After addition of all the samples, the plate was incubated for 24, 48 and 72 hours in 5% CO_2 incubator.

After incubation, the mixture in each well was removed, followed by addition of 10 µL of MTT dye into each well. The plate was sealed with parafilm and covered with aluminium foil. The 96-well plate was further incubated for 4 hours. Ten microliters of 100% DMSO was added into all the wells and incubated for 15 to 20 minutes to dissolve the insoluble purple crystal formazan. The absorbance was measured at 570 nm using microplate reader. The percentage of cell viability was calculated using the following formula (Fatemeh and Khosro, 2013). A graph of the percentage of the cell viability versus concentration of sample was plotted and IC_{50} values were determined from the graph.

$$\text{Percentage of cell viability} = (A_{Sample} / A_{NC}) \times 100\%$$

Where,

A_{Sample} refers to the average absorbance of the various samples or doxorubicin

A_{NC} refers to average absorbance of negative control

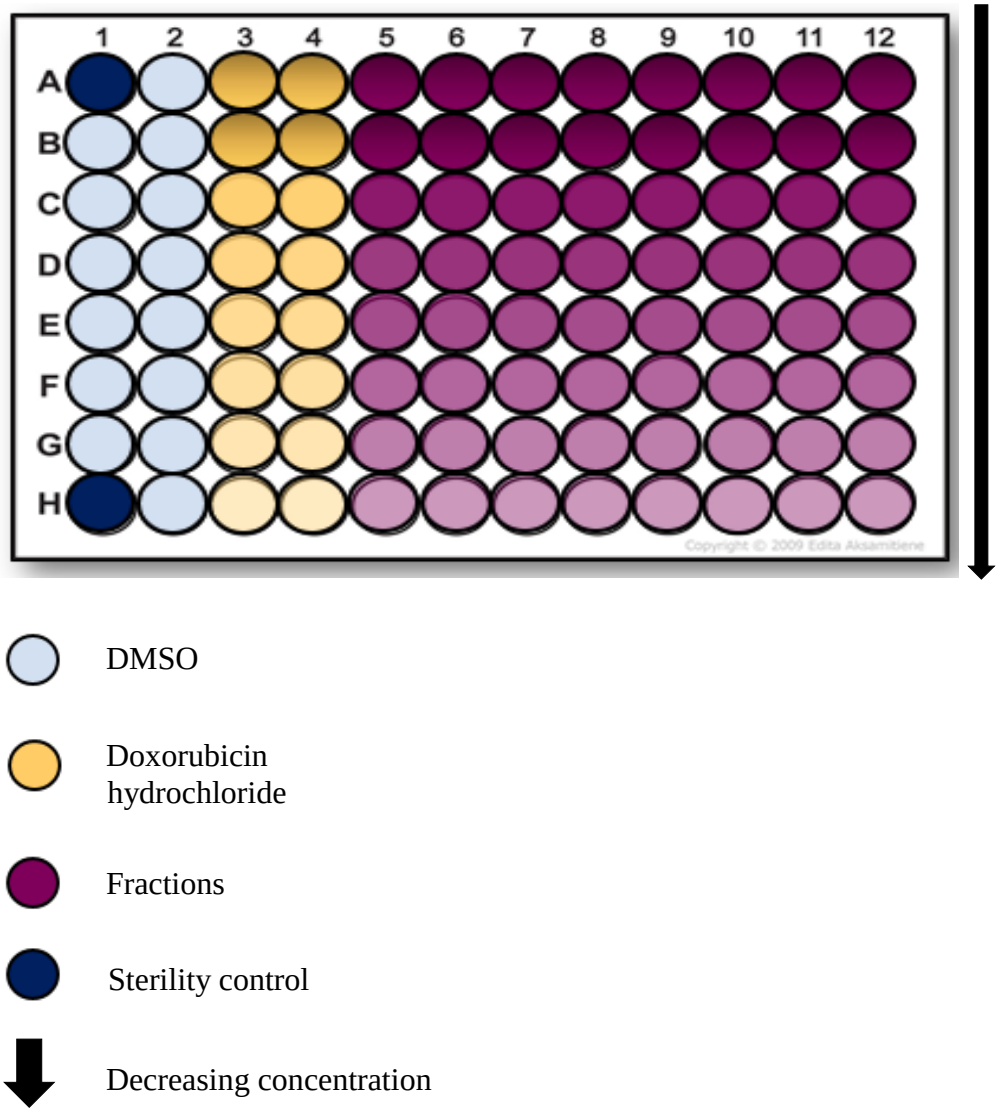

Figure 3.4: Design of 96-well plate used in MTT assay.

3.10.3 Minimum Inhibitory Concentration Assay

MIC assay was carried out using round bottom 96-well plate and the design used is shown in **Figure 3.5.** Approximately 50 µL of MH broth was added to each well. Columns 1 to 2 and 3 to 4 were added with 50 µL of 5% DMSO and gentamicin sulfate or streptomycin sulfate, respectively, while 50 µL of fractions were added into the first row of the columns 5 to 12. All the samples were serially

diluted with MH broth. Subsequently, each well of the plate was added with $1x10^6$ CFU/mL of the bacteria and the plate was incubated at 37°C for 18 to 24 hours. After incubation, 20 µL of INT dye was added into all the wells and incubated for 30 minutes. The plates were covered with aluminium foil due to its light sensitivity. The sample that remained yellow or no changes showed inhibition and indicating lowest MIC value.

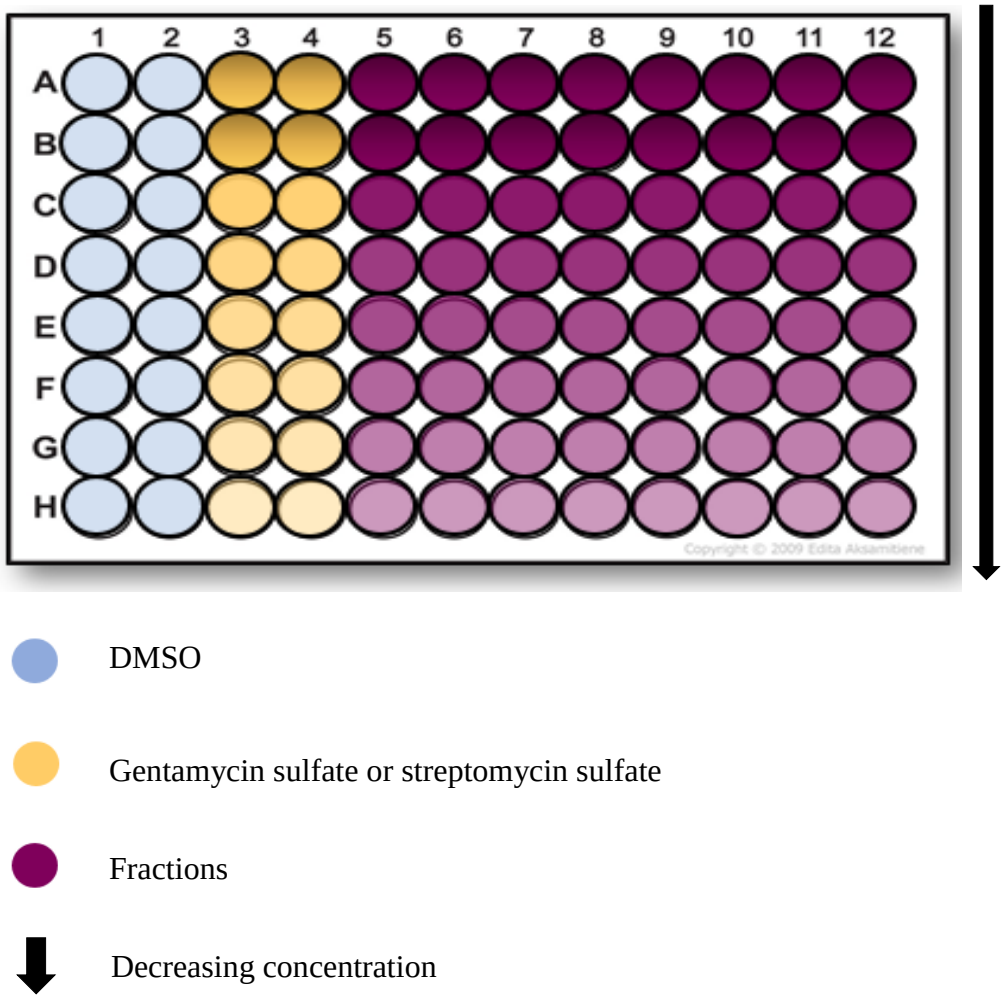

Figure 3.5: Design of 96-well plate used in MIC assay.

3.10.4 Minimum Bactericidal Concentration Assay

MBC assay was performed on Mueller Hinton agar. The samples with the lowest MIC value were streaked on the agar plate by using inoculating loop. The agar plates were sealed with parafilm and incubated at 37^{o}C incubator for 24 hours. The absence of colonies formed in agar indicates the bactericidal ability of the test samples. The lowest concentration of sample that showed no colonies formed and killed the bacteria was categorised as the minimum bactericidal concentration (MBC) (Omara, Zawrah and Samy, 2017).

3.11 Data Analysis

DPPH assay was repeated thrice, while MIC, MBC and MTT assays were repeated twice. The results obtained from these assays were calculated by using Microsoft Office Excel 2007.

CHAPTER 5

DISCUSSION

5.1 Bioassay-guided Fractionation

Various polarity solvents such as hexane, ethyl acetate and methanol were optimised in both gravity column chromatography and thin layer chromatography to isolate and separate polar and non-polar compounds present in the fractions of *Garcinia maingayi*. A gradual gradient method was applied in the isolation and separation of compounds in which the fractionation was initiated by using non-polar solvent (hexane), followed by solvents with increasing polarity, namely ethyl acetate and methanol. Lower polarity was used because all the compounds will be eluted out leading to clustering of compounds if polar solvent is used at first. Besides, the separation of compounds is inefficient due to cracking of the packed column (Columbia University, 2007). Thus, a gradual gradient method will ideally elute the compounds with different polarity one at a time.

Proper solvent system was selected based on the chemical properties of the compounds present in the fractions. Hexane and ethyl acetate mixture at 7:3 ratio and ethyl acetate and methanol mixture at 10:0.5 ratio were selected as the mobile phase in TLC after a few trials and error methods. These solvent systems produced many separated spots indicating the presence of both polar and non-polar compounds present in the fractions of *Garcinia maingayi*.

The yield of the fractions is depending on the extraction method and solvents used in the isolation of compounds from plants (Dhanani, et al., 2017). Based on **Figure 4.2**, fraction 7 showed the highest extraction yield and was isolated using mixture of ethyl acetate and methanol indicating this fraction contain mostly polar metabolites. On the other hand, fraction 1 showed the lowest extraction yield and was isolated using hexane and ethyl acetate indicating more non-polar metabolites are present in this fraction. This shows that the extraction yield increases with increasing polarity of the solvent used in extraction (Do, et al., 2014). However, quantitative phytochemical screening was not performed in this study as the yield of each fraction is too low which is less than 1 g.

Based on **Table 4.1**, the high resolution of spots and many spots produced on TLC plate deduced that the isolated fractions contain polar and non-polar compounds. The compounds with different polarities contribute to the physiological and pharmacological activities of the fractions. However, fraction 1 contain more polar compounds because of its widest range of R_f values and was isolated using mixture of hexane and ethyl acetate. The reason for the successful separation of compounds is due to the type of solvent used in the chromatography. Different polarities of solvents were used in and these solvents are capable to form non-covalent bonds with functional groups of secondary metabolites. The solvents used were selected based on their different polarity ranges. Non-polar substances will dissolve in non-polar solvents while polar substances will dissolve in polar solvents (Kagan and Flythe, 2014). In this case, the mixture of non-polar solvent (hexane) and intermediate polar solvent (ethyl acetate) produced the widest range of R_f values.

On the other hand, fraction 3 gave the narrowest range of R_f value in a solvent system of hexane and ethyl acetate. Ethyl acetate is an intermediate solvent that can separate both polar and non-polar compounds, thus intermediate compounds moved slowly resulting in smallest R_f values (Sarker, Latif and Gray, 2006). Meanwhile, fraction 6 showed narrower range of R_f value in a solvent system of ethyl acetate and methanol at 10:0.5 ratio. This is because the overall polarity of the solvent mixture is much polar compared to previous system. Furthermore, methanol is a polar solvent which can hydrogen bonding with other molecules due to the presence of hydroxyl group that has high electronegative oxygen atom (Master Organic Chemistry, 2018). Thus, most of the compounds separated are polar compounds which has a strong affinity for a polar adsorbent like silica which consists of surface silanol groups with exposed hydroxyl group and thus move slowly and not well separated (Sarker, Latif and Gray, 2006).

5.2 Bioassays

5.2.1 DPPH Assay

The role of free radical reactions and other reactive oxygen species (ROS) in disease pathology is well established and known to be involved in the pathogenesis of many acute and chronic diseases such as asthma, diabetes, aging, immunosuppression, neurodegeneration, cancers and atherosclerosis (Saeed, Khan and Shabbir, 2012). The major contributing factor is the oxidative stress when there is an imbalance between ROS and antioxidants. An antioxidant can be defined as any substance that delays or prevents oxidative damage to a target molecule due to its ability to trap free radicals (Mahdi-Pour, et al., 2012). Besides

the ability of scavenge free radicals, antioxidants can also reduce the valence electrons of an element from a higher level to a lower valence level due to its ability to donate its electrons (Mahayasih, Elya and Hanafi, 2018). It is believed that free radicals cause cell damage through covalent binding and lipid peroxidation with subsequent tissue injury. Recent studies have shown that the antioxidants present in medicinal plants such as flavonoids, phenolics and tannins possess free radical scavenging properties and are effective to prevent the destructive processes caused by oxidative stress. Medicinal plants with high level of antioxidant phytochemicals constituents have been used as an effective therapeutic approach for many diseases (Tjahjani, et al., 2014).

In this study, the radical scavenging activity was measured as a decrease in the absorbance of DPPH. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. Radical scavenging potential was expressed as percentage of radical scavenging activity (Policegoudra, et al., 2012). Concentration-dependent manner activity were shown by the fractions, in which the antioxidant activity increased as the concentration of the fraction increased. This may due to the different amount of bioactive compounds present at various concentration. The bioactive compounds may be diluted at lowest concentration. The highest radical scavenging activity could be attributed to the presence of high amount of the compounds at higher concentrations (Lehàr, et al., 2009).

Based on the results, all the isolated fractions showed higher percentage of free radical scavenging activity except fraction 7 with moderate activity. Fractions 2 and 3 showed the highest scavenging activity with 93.5±0.003% at 0.63 mg/mL

and 0.16 mg/mL, respectively comparable to ascorbic acid with 92.0±0.001% at 0.16 mg/mL. High antioxidant activity possessed by fractions 2 and 3 might be due to higher amount of bioactive phytochemicals present in the fraction. Besides, the mixture of several scavenging compounds in the fractions could act in a synergetic manner to enhance the antiradical activity (Sylvie, et al., 2014). In the contrary, the lowest antioxidant activity displayed by fraction 7 could be due to compounds in the fraction which is likely to work antagonistically. Fraction 7 consists of combination of many similar fractions using TLC, thus the antagonism of many phytochemicals producing opposing effect causing them to act differently (Mahayasih, Elya and Hanafi, 2018). Besides, antagonistic relationship among alkaloids and saponins in fractions could also attributable to low antioxidant activity as fraction 7 was isolated using solvent combination of ethyl acetate and methanol, thus this fraction could consist of both intermediate and polar compounds, such as alkaloids, flavonoids, saponins and terpenoids (Milugo, et al., 2013).

Based on the results, the isolated fractions exhibited high free radical scavenging activity. According to Zarena and Sankar (2009), antioxidant activity of various types of plant extracts is actually depends on the solvent used in extraction. This might be due to different extraction capacity of each solvent to extract different types of bioactive components from certain plant (Tjahjani, et al., 2014). For example, polar compounds such as glycosides of many flavonoids and phenolic acids generally extracted using polar solvents such as water and alcohols. Non-polar solvents are mainly used for isolation of flavonoids and carotenoids. It has been found that methanol is the best solvent for extraction of many active

constituents such as polyphenols, lactones, phenones, quassinoids, flavones, saponins and some terpenoids (Policegoudra, et al., 2012). Mahayasih, Elya and Hanafi (2018) reported that the methanol extract of *G. lateriflora* exhibited the highest radical scavenging activity when compared with hexane and ethyl acetate extracts due to the highest phenolic content. The methanol extract is also known to contain flavonoids, alkaloids, anthraquinones, tannins and saponins (Zarena and Sankar, 2009). Meanwhile, the methanol extract from bark of *G. nervosa* demonstrated higher scavenging activity of 98.73% by DPPH radical scavenging assay (Seruji, Khong and Kutoi, 2013).

In the present study, the data obtained showed that the fractions are potential free radical scavengers. The antioxidants are believed to react with free radical by donating hydrogen from polar bioactive compounds and break the free radical chain of oxidation. As a result, a stable end product which does not initiate or propagate further oxidation was formed (Zarena and Sankar, 2009). As mentioned earlier, medicinal plants contain a broad range of bioactive compounds such as terpenoids, carotenoids, phenolics, anthocyanins and flavonoids. The antioxidant activity may be attributed to the presence of these compounds in the fractions. In most of the studies, phenolic compounds such as tannins, xanthones and flavonoids are always associated with strong antioxidant activities (Kosem, Han and Moongkarnd, 2007).

Phenolic compounds are one of the largest and widely occurring category of phytochemicals and are considered as physiological and morphological importance in plants. The antioxidant activity of phenolic compounds is due to

their rich in hydroxyl groups which allow them to donate hydrogen atoms or electron, scavenge free radicals and chelate metal cations. The antioxidant activity of phenolic acids is depending on the degree of hydroxylation. For instance, trihydroxylated gallic acid shows a high antioxidant activity due to high degree of hydroxylation. Besides, the antioxidant activity of flavonoid is due to the relative structural complexity of the flavonoid molecules. The position of hydroxyl groups and degree of hydroxylation contributes to higher activity as it confers higher stability to the aroxyl radical by electron delocalisation or it might act as the preferred binding site for trace metals (Balasundram, Sundram and Samman, 2006).

Tannin can also act as an antioxidant because it can act as lipoxygenase inhibitor and thus stabilizing lipid fraction (Mahayasih, Elya and Hanafi, 2018). Studies on *Garcinia rigida*, *Garcinia mangostana* and *Cratoxylum cochinchinense* demonstrated various biological activities such as antioxidant, anti-inflammatory, antibacterial, antifungal, cytotoxic and anti-HIV. These plants are source of xanthone derivatives that possess one or more hydroxyl groups in the aromatic scaffold which contributes to its antioxidant activities (Francik, et al., 2016). Besides, xanthones can either donate or accept electrons in order to deactivate or scavenge free radicals via single electron transfer mechanism (Mart ńez, Hernandez-Marin and Galano, 2012).

The concentration of sample producing 50% scavenging of the DPPH radical (EC_{50} value) was determined to conclude the significant radical scavenging activity for further analysis. Lower EC_{50} values indicate a stronger antioxidant

potential (Ali Hassan, et al., 2013). Based on the result, fraction 2 showed the highest scavenged activity with EC_{50} value of 21.4 µg/mL as compared to other fractions and ascorbic acid. This study revealed that all the fractions and ascorbic acid except fraction 7 have prominent antioxidant activity but fraction 7 revealed a very poor antioxidant activity with EC_{50} value of 108.0 µg/mL. The result is in agreement with the previous study in which the methanol stem bark extract of *Garcinia benthamiana* showed high antiradical activity with low EC_{50} value of 79.1 µg/mL (See, et al., 2017). The presence of reactive metabolites such as polyphenols, flavonoids and xanthones are mainly found in these fractions and could be attributable to the observed high antioxidant properties of these fractions (Mahdi-Pour, et al., 2012).

In this study, the isolated fractions were compared with ascorbic acid due to its natural antioxidant potential. Ascorbic acid scavenges peroxyl radical, inhibit cytotoxicity induced by oxidants, help to reduce or prevent the formation of OH-deoxyguanosinereduce and hydrogen peroxide-induced lipid peroxidation (Yen, Duh and Tsai, 2002). Besides, it also interacts with the plasma membrane due to its ability to donate electrons to the alpha-tocopheroxyl radical and converts into ascorbate radical (May, 1999).

5.2.2 MTT Assay

In this study, COLO 205 cancer cell line was incubated for 24, 48 and 72 hours. The viability of COLO 205 cells after different incubation period was determined by MTT assay. This is because previous study has shown that the viability of COLO 205 cells decreased by the treatment with α-mangostin of *G. mangostana* in both concentration and time-dependent fashions (Watanapokasin, et al., 2011). Most of the studies used 72 hours of treatment because the amount of signal generated in MTT assay is dependent on many parameters including the length of the incubation period, concentration of MTT, the number of viable cells and their metabolic activity. Longer incubation time will increase the sensitivity and lower the IC_{50} values (Riss, et al., 2013). In addition, the structural complexity of secondary metabolites also attributed is also one of the reasons of longer incubation of treatment. The structure of phytochemicals is very large so it requires more time to penetrate efficiently through the cell membrane of cancer cells to obtain more reliable results (Bednarek, 2005). This can be observed in fractions 2 and 3 which showed lower IC_{50} values at 72 hours treatment compared to 24 and 48 hours.

The IC_{50} values were determined from concentration response curve. The IC_{50} values represented the concentration that reduced the mean absorbance at 570 nm to 50% that measures the effectiveness of a drug in inhibiting certain biological function (Seruji, Khong and Kutoi, 2013). All the isolated fractions exhibited cytotoxicity in dose-dependent manner. Significant difference was observed at selected treatment concentrations when compared to doxorubicin hydrochloride. From the results, doxorubicin hydrochloride showed strongest

cytotoxic activity with lowest IC_{50} value of 1.40 µg/mL at 48 hours. According to US NCI plant screening program, crude extract is considered cytotoxic when IC_{50} value is lower than 30 µg/mL, whereas fraction is lower than 10 µg/mL. The lower the IC_{50} value, the more effective the anticancer potential of the pure compound or crude extract (Hamidon, et al., 2016). Since IC_{50} for all the fractions were lower than 10 µg/mL, it can be concluded that these fractions pose stronger cytotoxic activity and can be considered as a potential cytotoxic agent.

Fractions that displayed higher antioxidant activity were used in the study as therapeutic antioxidants may prevent early events in tumour development (Liou and Storz, 2010). All the fractions gave positive response to COLO 205 cells especially for fraction 2 that showed higher cytotoxicity with lowest IC_{50} values of 0.70 µg/mL after 72 hours. According to Yang, et al. (2009), specific phytochemicals might act additively, synergistically, and/or antagonistically with other compounds to display cytotoxic activity. The presence of active compounds and also the synergistic interaction between the phytochemicals might be responsible for the high cytotoxic activity against COLO 205 cells in this study.

On the other hand, fractions 4 and 5 showed slightly lower cytotoxicity compared to the other fractions. This difference in cytotoxic activity could be due to the solubility of biologically active ingredients (Arora and Tandon, 2015). Higher number of spots present in fractions 4 and 5 indicated the presence of many metabolites with different polarities. These variety of compounds and impurities in fractions might interact with each other and hide the potential

cytotoxic activity of other metabolites. The interaction among compounds may reduce the anticancer activity due to the antagonism effect exhibited by the polar bioactive compounds in the fraction (Hamidon, et al., 2016). Basri, et al. (2014) reported that the methanol extract of *Canarium odontophyllum* stem bark displayed low cytotoxicity against human HCT116 colon cancer cell line due to the variety of phytochemicals acting in antagonism to reduce the cytotoxic effect. This shows that different phytochemicals can show opposing effect to produce antagonism.

According to Arast, et al. (2017), the active compounds with potential antitumour properties are non-polar in nature. Potential anticancer bioactive compounds in non-polar extracts is due to the nature of cell membrane that is amphiphilic and thus anticancer agent can penetrate the cell membrane easily. This statement correlates with the present study, in which fractions 2 and 3 exhibited higher cytotoxic effect and lower IC_{50} value against COLO 205 cells than fractions 4 and 5. This is because fractions 4 and 5 contain more polar metabolites which were isolated using more polar solvent system.

Besides, the higher cytotoxic effect of the fractions could be attributed to the presence of active compounds such as tannins, xanthones, flavones, isoflavones and saponins. It has been proven that some bioactive compounds from Garcinia species exhibited a wide range of biological and pharmacological activities such as antioxidant and cytotoxicity (Seruji, Khong and Kutoi, 2013). Saponins are natural glycosides with triterpenoids or spirostane aglycones that possess various pharmacological properties. Saponins exhibit *in vitro* and *in vivo* anticancer

effect, such as antiproliferation, antimetastasis, antiangiogenesis and antimultidrug resistance. It has shown that effectiveness of cytotoxic effect in various cancer cell lines are by inhibiting cell growth, inducing apoptosis, inhibiting invasion and migration (Xu, et al., 2016).

Abu Bakar, et al. (2015) reported that the inhibition of liver cancer cell line could be explained by the presence of phenolic acids, carotenoids, anthocyanins, tannins and xanthones. The anticancer properties of phenolic acids are mainly due to its ability to scavenge free radicals, regulate gene expression, modulate DNA damage repair, cell proliferation, apoptosis and invasion. Apart from that, phenolic acids have been reported to inhibit transcription factors linked to inflammation, pro-inflammatory cytokines, COX-2, inducible nitric oxide synthase and lipoxygenases (Rosa, et al., 2016). On the other hand, tannins are capable of inducing caspase-3-dependent apoptosis in cancer cells through extracellular signal-regulated kinase and P38 mitogen-activated protein kinase pathway blockage, inhibiting transcription factors activation such as activator protein-1 and growth factor-mediated pathways suppression (Dai and Mumper, 2010).

Other than that, the cell viability of most of the fractions decreased when the concentration of the fractions is increased. This study is in agreement with previous study where the viability of MCF-7 breast adenocarcinoma decreased at high concentration of *G. xanthochymus* stem bark extract (Hamidon, et al., 2016). However, higher cell viability was observed for fractions 2 and 3 when the concentration of fractions increased after 72 hours. Possible reason could be

the antagonism of multiple phytoconstituents present in the fractions which cause them to act differently. Antagonistic interactions of phytochemicals in the fractions would affect the efficacy of fractions resulting in the decrease of biological effects rather than enhancing the effects (Milugo, et al., 2013). At lower concentration, lesser phytoconstituents are present in the fractions to cause antagonistic effect whereas higher number of phytochemicals present at higher concentration would act as antagonist in the fractions and reduce the cytotoxic effect of fractions, thus leading to an increase in the percentage of cell viability (Odhiambo, et al., 2009).

Doxorubicin hydrochloride or commonly known as adriamycin is an anthracycline drug used in the treatment of several cancers including lung, breast, gastric, ovarian, sarcoma, and paediatric cancers. Doxorubicin acts on the cancer cells by intercalating into DNA, disrupt the topoisomerase-II-mediated DNA repair and generates free radicals resulting in DNA damage and cell death (Thorn, et al., 2011). Abu Bakar, et al. (2015) stated that doxorubicin exhibited highest cytotoxic activity with IC_{50} value of 2.0 µg/mL. Similarly, doxorubicin in the present study also showed the strongest anticancer property with lowest IC_{50} value of 1.40 µg/mL after 48 hours of treatment.

5.2.3 MIC and MBC Assays

Based on **Table 4.6**, all the fractions were active against all the six bacteria with MIC more than 0.31 mg/mL. *Bacillus cereus* was more susceptible to the fractions compared to other bacteria as it was inhibited at MIC of 0.31 mg/mL. However, the most susceptible strain was found to be MRSA 5 (ATCC 33591) as a lower concentration of fraction is enough to inhibit its growth. The above findings suggest the presence of antibacterial compounds in *Garcinia maingayi*. According to Rios and Recio (2005), extract or fraction is considered to possess strong antibacterial activity when the MIC is less than 100 μg/mL, whereas moderate activity is shown when MIC is less than 625 μg/mL and weak activity when MIC of fraction is more than 625 μg/mL against the corresponding pathogens. Since the MIC obtained in the present study are more than 100 μg/mL, the methanol fractions of *Garcinia maingayi* are said to possess moderate antibacterial activity against selected Gram-positive and Gram-negative bacteria.

Among the six bacteria tested, only *B.cereus*, MRSA 5 (ATCC 33591) and MRSA 7 (ATCC 43300) were inhibited by the isolated fractions. However, other bacteria may have inhibitory if the concentration used is more than 0.31 mg/mL. The result is in accordance with the previous studies which reported that most plant extracts are more active against Gram-positive bacteria which have only one outer peptidoglycan layer and is easier to penetrate than Gram-negative ones, which contain outer membrane with a lipopolysaccharide layer that makes the cell wall impermeable to certain antibiotics and antibacterial compounds (Wintola and Afolayan, 2015). However, the growth of *S. aureus* in this present study was not inhibited by the fractions. This result is also contradictory to

previous study which reported that *S. aureus* is susceptible to the methanol extract of stem bark from *Garcinia latissima* Miq (Ambarwati, et al., 2017). This may be due to the antagonistic effect of the mixture of natural compounds found in the fractions that might reduce the antibacterial activity of the fractions (Toroglu, 2007). The fractions may possibly contain a low concentration of antibacterial compounds and can be related to the absence of bactericidal activity (Wintola and Afolayan, 2015).

MBC values showed that all the fractions exhibited only bacteriostatic effect. Although some of the bacteria tested can be inhibited by the fractions but they are not able to be killed by the fractions. This indicates the low efficacy of antibacterial activity of the fractions. This is because the antibacterial activity of fractions was not only due to one main active compounds but to the combined action of additional other compounds. The phytochemicals present in fractions 2, 3 and 4 might interact and give synergistic effect to enhance the effect of metabolites that increase the overall efficacy of antibacterial activity and thus able to inhibit MRSA 7 (Ambarwati, et al., 2017). This explains why fractions 2, 3 and 4 exhibited higher bacteriostatic effects against MRSA 5 and MRSA 7. In addition, there are no previous reports on chemical components and biological activities from *Garcinia maingayi.* The first report on the antibacterial activity of *Garcinia maingayi* showed that the extracts tested demonstrated only moderate or weak activity against certain tested bacteria. Thus, further investigations on the mode of action of this plant are required (Lin, 2005).

The preliminary phytochemical analysis of fractions from stem bark of Garcinia species revealed the presence of alkaloids, flavonoids, tannins, phenols, polyphenols and saponins which could be responsible for the observed antimicrobial activity (Ambarwati, et al., 2017). The secondary metabolites found in plant might be capable of increasing plasma membrane permeability and ion leakage from the cells and inhibiting several cellular processes to inhibit bacterial growth (Khan, et al., 2009).

Flavonoids are commonly found in fruit, vegetables, nuts, seeds, stems, flowers, wine, tea and honey. It has been reported that flavonoids act through inhibiting DNA gyrase and β-hydroxyacyl carrier protein dehydratase activities as well as by inhibition of cytoplasmic membrane function (Paiva, et al., 2010). Flavonoids also are able to complex with extracellular and soluble proteins and to complex with bacterial cell walls (Mujeeb, Bajpai and Pathak, 2014).

Terpenoids have been suggested that these metabolites exhibit antibacterial activity by promoting bacterial lysis and membrane disruption (Souza, et al., 2010). Terpenoids act on the membrane proteins and induce uncontrolled efflux of ions and metabolites by increasing the permeability of the membranes, which lead to cell leakage, resulting in apoptotic cell death (Khan, et al., 2009).

Saponins are naturally occurring glycosylated phytoanticipins that are found mainly in plant species. Saponins are believed to confer protection against various pathogens in plants. Antibacterial property of saponin is due to its ability to cause leakage of proteins and certain enzymes from the cell (Shihabudeen,

Priscilla and Thirumurugan, 2010). Besides, coumarins are able to cause reduction in cell respiration to act against Gram-positive bacteria (Paiva, et al., 2010).

Gentamycin sulfate is a broad-spectrum aminoglycoside antibiotic that is bactericidal and active against Gram-negative bacteria and some Gram-positive bacteria except Streptococci. Aminoglycosides work by binding to the bacterial 30S ribosomal subunit and 16S rRNA, causing inhibition of bacterial protein synthesis (DrugBank, 2005). In addition, streptomycin sulfate is also a type of aminoglycoside antibiotic and works the same like gentamycin as a protein synthesis inhibitor. Streptomycin inhibited Gram-positive and Gram-negative bacteria and *Mycobacterium tuberculosis*. In this study, bacteria were killed by the gentamycin sulfate or streptomycin sulfate at a lowest concentration due to its active pure compounds in nature that act selectively to bacteria (Schmitz, et al., 1999).

5.3 Limitations of Study

The limitations of this study are more focused on the methodology of the experiment. In the present study, the isolated fractions were dissolve in DMSO and some fractions were found difficult to dissolve. Therefore, these fractions were sonicated for a longer time to ensure complete solubilisation. However, heat will be generated from the sonicating reaction and might disturb some of the secondary metabolites found inside the fractions.

In addition, improper pipetting techniques as well as uneven cell seeding into the 96-well plates gave rise to inaccuracy and inconsistent absorbance readings for the assays. Hence, the assays were repeated for at least twice and the average of the absorbance reading was calculated to obtain a reliable and significant result.

5.4 Future Studies

Future studies are required to investigate more details about the bioactive compounds present in *Garcinia maingayi*. The isolated individual compounds can be further purified and analysed using high performance liquid chromatography (HPLC) and gas chromatography linked with mass spectrophotometry (GC-MS). Besides, the structure of the isolated bioactive compounds can be elucidated by analysis of spectroscopic data using nuclear magnetic resonance (Sasidharan, et al., 2011; Teke, et al., 2011).

Furthermore, other bioassays can be conducted to further evaluate the antioxidant activity of the fractions because a single assay is very difficult to conclude the potential activity of the isolated fractions. Therefore, antioxidant assays such as ferric reducing antioxidant power assay, ABTS radical scavenging activity, oxygen radical absorbance capacity, total radical trapping antioxidant potential, nitric oxide scavenging activity and trolox equivalent antioxidant capacity assays can be carried out to further evaluate the free radical scavenging activity of the fractions (Wintola and Afolayan, 2015).

In addition, *in vitro* assays such as protease viability marker, DNA fragmentation, neutral red and ATP assays can be conducted to evaluate the cytotoxicity of the fractions towards cancer cells (Riss, et al., 2013). Apart from that, cell cycle analysis, apoptosis analysis using Annexin V-FITC apoptosis detection kit, caspases activity and alkaline comet assay can be performed to investigate the anticancer potential of the fractions. The morphological changes of COLO 205 cells upon treatment with fractions should be observed too in the future study (Abu Bakar, et al., 2015). Besides, the antibacterial activity of the fractions should be tested on more clinical and multidrug strains.

In the present study, only one cancer cell line (COLO 205) was used, hence, in the future, more cell lines, preferably both monolayer and suspension cell lines should be tested to have a better assessment on the anticancer effect. Normal cell lines also should be tested to ensure isolated compounds cause no or minimal side effects on normal healthy cells besides killing the cancer cells.

In order to evaluate the efficacy and validate the *in vitro* findings, studies of the isolated fractions should be carried out using animal models such as mice and rodents. Further analysis on mechanism of action, therapeutic index and dosage efficacy of isolated active compounds can be done to provide the researchers better evidence of the activity exerted by the plant (Monte, et al., 2014).

CHAPTER 6

CONCLUSIONS

Bioassay-guided fractionation of methanolic fraction of *Garcinia maingayi* yielded seven semi-purified fractions. TLC profile showed that fraction 1 exhibited the widest range of R_f values, whereas fraction 3 exhibited the narrowest range of R_f values. The results of isolation demonstrated that fraction 1 produced highest yield whereas fraction 7 produced lowest yield.

In DPPH assay, fraction 2 demonstrated highest radical scavenging activity of $93.5 \pm 0.003\%$ with EC_{50} value of 21.4 µg/mL, while fraction 7 exhibited the lowest radical scavenging activity of $38.1 \pm 0.004\%$ with EC_{50} value of 108.0 µg/mL.

The results of MTT assay revealed that fraction 2 can be a promising cytotoxic agent against COLO 205 cells with IC_{50} of 0.70 µg/mL after 72 hours of treatment, whereas doxorubicin hydrochloride exhibited IC_{50} value of 1.40 µg/mL. In antibacterial assays, all the fractions exhibited lowest MIC values of 0.31 mg/mL against *B. cereus* and MRSA 5 but only fractions 2, 3 and 4 were active against MRSA 7 with lowest MIC value of 0.31 mg/mL. All the fractions exhibited bacteriostatic effect against the tested bacteria in MBC assay.

In conclusion, fractions isolated from *Garcinia maingayi* are potential antioxidant, cytotoxic and antibacterial agents.

REFERENCES

Abu Bakar, M. F., Ahmad, N.E., Suleiman, M., Rahmat, A., Isha, A., 2015. *Garcinia dulcis* fruit extract induced cytotoxicity and apoptosis in HepG2 liver cancer cell line. *BioMed Research International,* 2015(1), pp. 6-8.

Adan, A., Kiraz, Y. and Baran, Y., 2016. Cell proliferation and cytotoxicity assays. *Current Pharmaceutical Biotechnology,* 17(14), p. 1213.

Ahmad, M. and Wajid, M., 2013. Plants as potential source of antimicrobial agents. *Journal of Alternative and Complementary of Medicine*, 2(3), pp.18-25.

Al-Amiery, A. A., Kadhum, A. A. H., Obayes, H. R. and Mohamad, A. B., 2013. Synthesis and antioxidant activities of novel 5-chlorocurcumin, complemented by semiempirical calculations. *Bioinorganic Chemistry and Applications*, 2013(1), pp. 4-5.

Ali Hassan, S. H., Fry, J. R. and Abu Bakar, M. F., 2013. Phytochemicals content, antioxidant activity and acetylcholinesterase inhibition properties of indigenous *Garcinia parvifolia* fruit. *BioMed Research International*, 2013(1), pp. 5-6.

Ambarwati, N. S. S., Elya, B., Malik, A. and Hanafi, M., 2017. Evaluation of antimicrobial activities of *Garcinia latissima* Miq. stem bark extract. *Journal of Young Pharmacists*, 9(1), p. 57.

American Chemical Society, 2015. *Discovery and development of penicillin.* [online] Available at: <https://www.acs.org/content/acs/en/education/whatischemistry/landmarks/flemingpenicillin.html> [Accessed 25 March 2018].

American Type Culture Collection, 2016. *COLO 205 (ATCC® CCL-222™).* [online] Available at: <https://www.atcc.org/Products/All/CCL-222.aspx#generalinformation> [Accessed 9 February 2018].

Andrews, J. M., 2002. Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy,* 49(6), p. 1049.

Arast, Y., Razi, N.S., Seydi, E., Naserzadeh, P., Nazemi, M. and Pourahmad, J., 2017. Selective toxicity of non polar bioactive compounds of Persian gulf sea squirt *Phallusia nigra* on skin mitochondria isolated from rat model of melanoma. *Asian Pacific Journal of Cancer Prevention*, 18(3), p.811.

Arora, S. and Tandon, S., 2015. Mitochondrial pathway mediated apoptosis and cell cycle arrest triggered by aqueous extract of wheatgrass in colon cancer colo-205 cells. *Journal of Plant Biochemistry and Biotechnology*, 25(1), pp. 56-63.

Bajpai, V., Majumder, R. and Park, J., 2016. Isolation and purification of plant secondary metabolites using column-chromatographic technique. *Bangladesh Journal of Pharmacology*, 11(1), pp. 846-847.

Balasundram, N., Sundram, K. and Samman, S., 2006. Phenolic compounds in plants and agri-industrialby-products: Antioxidant activity, occurrence, and potential uses. *Food Chemistry*, 99(1), pp. 192-194.

Basma, A. A., Zakaria, Z., Latha, L. Y. and Sasidharan, S., 2011. Antioxidant activity and phytochemical screening of the methanol extracts of *Euphorbia hirta* L. *Asian Pacific Journal of Tropical Medicine*, 4(5), pp. 386-390.

Basri, D., Mohd, M., Meng, C., Latif, E. and Huyop, F., 2014. Cytotoxic activity of stem bark extracts from *Canarium odontophyllum* Miq (Dabai) against human colorectal carcinoma HCT 116 cell line. *American Journal of Plant Sciences*, 5(26), pp. 6-7.

Bednarek, P., 2005. Structural complexity, differential response to infection, and tissue specificity of indolic and phenylpropanoid secondary metabolism in Arabidopsis roots. *Plant Physiology*, 138(2), p. 1058.

Bernama, 2009. *Malaysia one of 12 mega biodiversity countries in the world*. [online] Available at: <https://bigcatrescue.org/malaysia-one-of-12-mega-biodiversity-countries-in-the-world/> [Accessed 22 March 2018].

Bottone, E. J., 2010. *Bacillus cereus*, a volatile human pathogen. *Clinical Microbiology Reviews*, 23(2), pp. 382–398.

Busia, K., 2016. *Fundamentals of herbal mdicine: major plant families, analytical methods, materia medica*. 2nd ed. Birkbeck: Xlibris Corporation.

Centers for Disease Control and Prevention, 2018. *Escherichia coli*. [online] Available at: https://www.cdc.gov/ecoli/index.html [Accessed 7 Februrary 2018].

Centers for Disease Control and Prevention, 2016. *Methicillin-resistant Staphylococcus aureus (MRSA).* [online] Available at: https://www.cdc.gov/mrsa/community/index.html [Accessed 7 Februrary 2018].

Cheng, G.L.E. and Cheow, Y.L., 2008. Secondary metabolites from two Garcinia species and their biological activities. *Asian Journal of Chemistry*, 20(1), pp. 343-351.

Columbia University, 2007. *Chromatography.* [online] Available at: <http://www.columbia.edu/cu/chemistry/ugrad/hssp/EXP_8.html> [Accessed 5 March 2018].

Coskun, O., 2016. Separation techniques: chromatography. *Northern Clinics of Istanbul*, 3(2), pp. 156–160.

Crozier, A., Clifford, M. N. and Ashihara, H., 2006. *Plant secondary metabolites: occurrence, structure and role in the human diet.* Victoria: Blackwell Publishing.

Cseke, L. J., Kaufman, P. B., Warber, S. L. and Duke, J. A., 2006. *Natural products from plants.* 2nd ed. Boca Raton: Taylor and Francis Group.

Dai, J. and Mumper, R. J., 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, 15(10), pp. 7320-7323.

Desai, A., 2008. Medicinal plants and cancer chemoprevention. *Current Drug Metabolism*, 9(7), pp. 581-591.

Dhanani, T., Shah, S., Gajbhiye, N. and Kumar, S., 2017. Effect of extraction methods on yield, phytochemical constituents and antioxidant activity of *Withania somnifera*. *Arabian Journal of Chemistry*, 10(1), pp. 1197-1198.

Dias, D.A., Urban, S. and Roessner, U., 2012. A historical overview of natural products in drug discovery. *Metabolites*, 2(2), pp.303-336.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S. and Ju, Y.H., 2014. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. *Journal of Food and Drug Analysis*, 22(3), pp.296-302.

Donay, J. L., Fernandes, P., Lagrange, P. H. and Herrmann, J. L., 2007. Evaluation of the inoculation procedure using a 0.25 McFarland standard for the BD phoenix automated microbiology system. *Journal of Clinical Microbiology*, 45(12), pp. 4088-4089.

Donnenberg, M.S., 2002. *Escherichia coli: virulence mechanisms of a versatile pathogen*. 1st ed. Maryland: Elsevier.

DrugBank, 2005. Gentamycin. [online] Available at: <https://www.drugbank.ca/drugs/DB00798> [Accessed 20 March 2018].

Fabrega, A. and Vila, J., 2013. *Salmonella enterica* Serovar Typhimurium skills to succeed in the host: virulence and regulation. *Clinical Microbiology Reviews*, 26(2), pp. 308-341.

Farombi, E.O., Tahnteng, J.G., Agboola, A.O., Nwankwo, J.O. and Emerole, G.O., 2000. Chemoprevention of 2-acetylaminofluorene-induced hepatotoxicity and lipid peroxidation in rats by kolaviron-A *Garcinia kola* seed extract. *Food and Chemical Toxicology*. 38(6), pp. 535–541.

Fatemeh, K. and Khosro, P., 2013. *In vitro* cytotoxic activity of aqueous root extract of *Althea kurdica* against endothelial human bone marrow cells (line k562) and human lymphocytes. *Bulletin Environment, Pharmacology and Life Sciences*, 2(6), pp.23-29

Foster, T. J., 2004. The *Staphylococcus aureus* "superbug". *The Journal of Clinical Investigation,* 114(12), pp. 1693-1694.

Francik, R., Szkaradek, N., Zelaszczyk, D. and Marona, H., 2016. Antioxidant activity of xanthone derivatives. *Journal of Drug Biochemistry*, 73(6), p. 1508.

G-Biosciences, 2012. *What is cell cytotoxicity and how to measure it?*. [online] Available at: <https://info.gbiosciences.com/blog/bid/164400/what-is-cell-cytotoxicity-and-how-to-measure-it> [Accessed 25 March 2018].

Globocan Colorectal Cancer Estimated Incidence, 2012. *Mortality and prevalence worldwide in 2012*. [online] Available at: <http://globocan.iarc.fr/Pages/fact_sheets_cancer.aspx> [Accessed 25 March 2018].

Gogoi, B. J., Tsering, . J. and Veer, V., 2012. Antioxidant potential of Garcinia species from Sonitpur district, Assam, Northeast India. *International Journal of Pharmaceutical Sciences and Research,* 205(1), pp. 3472-3475.

Goodwin, C. S., Blake, P. and Blincow, E., 1986. The minimum inhibitory and bactericidal concentrations of antibiotics and anti-ulcer agents against *Campylobacter pyloridis*. *Journal of Antimicrobial Chemotherapy*, 17(3), p. 310.

Gottesman, M. M., 2002. Mechanisms of cancer drug resistance. *Annual Review of Medicine,* 53(1), pp. 615-627.

Hamidon, H., Taher, M., Jaffri, J.M., Zakaria, T.M.T., Sulaiman, W. MAW., Susanti, D., Ichwan, S. JA. and Zakaria, Z.A., 2016. Cytotoxic and anti-inflammatory activities of *Garcinia xanthochymus* extracts on cell lines. *Makara Journal of Health Research,* 20(1), pp. 11-17.

Hemshekhar, M., Sunitha, K., Santhosh, M.S., Devaraja, S., Kemparaju, K., Vishwanath, B.S., Niranjana, S.R. and Girish, K.S., 2011. An overview on genus Garcinia: phytochemical and therapeutical aspects. *Phytochemical Review,* 10, pp. 325-351.

Idris, S. and Rukayah, A., 1987. Description of the male mangosteen (*Garcinia mangostana L.*) discovered in Peninsular Malaysia. *MARDI Research Bulletin,* 15(1), pp. 63-66.

Iriti, M. and Faoro, F., 2009. Chemical diversity and defence metabolism: How plants cope with pathogens and ozone pollution. *International Journal of Molecular Sciences,* 10(8), pp. 3372-3373.

Jabit, M. L., Wahyuni, F.S., Khalid, R., Israf, D.A., Shaari, K., Lajis, N.H. and Stanslas, J., 2009. Cytotoxic and nitric oxide inhibitory activities of methanol extracts of Garcinia species. *Pharmaceutical Biology,* 47(11), pp. 1024-1026.

Jacob, K.M.P., Ali, M.A., Vishnu, H., Shylaja, G., Mythili, S. and Sathiavelu, A., 2015. Evaluation of antibacterial and antioxidant activity of *Garcinia gummi-gutta*. *International Journal of Drug Development and Research,* 7(3), pp. 057-059.

Joray, M. B., Trucco, L.D., Gonz áez, M.L., Napal, G.N.D., Palacios, S.M., Bocco, J.L. and Carpinella, M.C., 2015. Antibacterial and cytotoxic activity of compounds isolated from *Flourensia oolepis*. *Evidence-Based Complementary and Alternative Medicine,* 2015(2015), pp. 1-11.

Joseph, G. S., Jayaprakasha, G.K., Selvi, A.T., Jena, B.S. and Sakariah, K.K., 2005. Antiaflatoxigenic and antioxidant activities of Garcinia extracts. *International Journal of Food Microbiology,* 101(2), pp. 153-160.

Jouda, J. B., Mbazoa, C.D., Sarkar, P., Bag, P.K. and Wandji, J., 2016. Anticancer and antibacterial secondary metabolites from the endophytic fungus Penicillium sp. CAM64 against multi-drug resistant Gram-negative bacteria. *African Health Sciences,* 16(3), p. 734.

Kagan, I. A. and Flythe, M. D., 2014. Thin-layer chromatographic (TLC) separations and bioassays of plant extracts to identify antimicrobial compounds. *Journal of Visualized Experiments*, 1(85), pp. 1-8.

Katsares, V., Petsa, A., Felesakis, A., Paparidis, Z., Nikolaidou, E., Gargani, S., Karvounidou, I., Ardelean, K.A., Grigoriadis, N. and Grigoriadis, J., 2015. A rapid and accurate method for the stem cell viability evaluation: the case of the thawed umbilical cord blood. *Laboratory Medicine,* 40(9), pp.557-560.

Kedare, S. B. and Singh, R. P., 2011. Genesis and development of DPPH method of antioxidant assay. *Journal of Food Science and Technology,* 48(4), pp. 412-422.

Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A.A., Ali, S.M., Siddiqui, M. and Khan, A.U., 2009. Antimicrobial activity of five herbal extracts against multi drug resistant (MDR) strains of bacteria and fungus of clinical origin. *Molecules,* 14(2), pp. 586-597.

Khan, M.J., Drochner, W., Steingass, H. and Islam, K.M.S., 2008. Nutritive evaluation of some tree leaves from Bangladesh for feeding ruminant animals. *Indian Journal of Animal Sciences*, 78 (11), pp. 1273-1277.

Kochummen, K.M., 1998. *Garcinia maingayi*. [online] Available at: <http://www.iucnredlist.org/details/37830/0> [Accessed 19 September 2017].

Kohanski, M.A., Dwyer, D.J. and Collins, J.J., 2010. How antibiotics kill bacteria: from targets to networks. *Nature Reviews Microbiology*, 8(6), p.423.

Kosem, N., Han, Y.-H. and Moongkarnd, P., 2007. Antioxidant and cytoprotective activities of methanolic extract from *Garcinia mangostana* hulls. *Science Asia,* 33(1), p. 283.

Kuete, V. Betrandteponno, R., Mbaveng, A.T., Tapondjou, L.A., Meyer, J.J.M., Barboni, L. and Lall, N., 2012. Antibacterial activities of the extracts, fractions and compounds from *Dioscorea bulbifera*. *BMC Complementary and Alternative Medicine,* 12(1), p. 228.

Kumar, S., Sharma, S. and Chattopadhyay, S. K., 2013. The potential health benefit of polyisoprenylated benzophenones. *Fitoterapia,* 89(10), pp. 86-125.

Lee, G. and Bishop, P., 2016. *Microbiology and infection control for health professionals.* 6th ed. Melbourne: Pearson.

Lee, G. and Bishop, P., 2010. *Microbiology and infection control for health professionals.* 4th ed. Sydney: Pearson .

Lehàr, J., Krueger, A.S., Avery, W., Heilbut, A.M., Johansen, L.M., Price, E.R., Rickles, R.J., Short, G.F., Staunton, J.E., Jin, X., Lee, M.S., Zimmermann, G.R. and Borisy, A.A., 2009. Synergistic drug combinations improve therapeutic selectivity. *Nature Biotechnology*, 27(7), pp. 659-666.

Lim, J.Y., Yoon, J.W. and Hovde, C.J., 2010. A brief overview of *Escherichia coli* O157: H7 and its plasmid O157. *Journal of microbiology and biotechnology*, 20(1), p.5.

Lim, G. C., 2002. Overview of cancer in Malaysia. *Japanese Journal of Clinical Oncology,* 32(1), pp. 37-42.

Lin, C. Y., 2005. Chemical constituents and biological activities from *Garcinia Maingayi* and *Garcinia Parvifolia*. Doctoral dissertation. Universiti Putra Malaysia.

Liou, G. and Storz, P., 2010. Reactive oxygen species in cancer. *Free Radical Research*, 44(5), pp. 1-3.

Mahayasih, P. G. M. W., Elya, B. and Hanafi, M., 2018. Fractionation and antioxidant activity potency of the extract of *Garcinia lateriflora* Blume *var. javanica* Boerl leaf. *American Institute of Physics*, 1933(1), pp. 2-4.

Mahdi-Pour, B., Jothy, S.L., Latha, L.Y., Chen, Y. and Sasidharan, S., 2012. Antioxidant activity of methanol extracts of different parts of *Lantana camara*. *Asian Pacific Journal of Tropical Biomedicine*, 2(12), pp. 964-965.

Marasini, B.P., Baral, P., Aryal, P., Ghimire, K.R., Neupane, S., Dahal, N., Singh, A., Ghimire, L. and Shrestha, K., 2015. Evaluation of antibacterial activity of some traditionally used medicinal plants against human pathogenic bacteria. *BioMed Research International*, 2015(1), pp. 1-6.

Martínez, A., Hernández-Marin, E. and Galano, A., 2012. Xanthones as antioxidants: a theoretical study on the thermodynamics and kinetics of the single electron transfer mechanism. *Food and Function*, 3(4), pp.442-450.

Massachusetts Institute of Technology, 2012. *Thin layer chromatography*. [online] Available at: <https://ocw.mit.edu/courses/chemistry/5-301-chemistry-laboratory-techniques-january-iap-2012/labs/MIT5_301IAP12_TLC_Handout.pdf > [Accessed 21 September 2017].

Master Organic Chemistry, 2018. *All about solvents.* [online] Available at: <https://www.masterorganicchemistry.com/2012/04/27/polar-protic-polar-aprotic-nonpolar-all-about-solvents/> [Accessed 8 March 2018].

May, J. M., 1999. Is ascorbic acid an antioxidant for the plasma membrane?. *The Federation of American Societies for Experimental Biology Journal*, 13(9), pp. 995-1006.

Mayo Clinic, 2017. *Infectious diseases.* [online] Available at: <https://www.mayoclinic.org/diseases-conditions/infectious-diseases/symptoms-causes/syc-20351173>
[Accessed 20 December 2017].

Milugo, T. K., Omosa, L.K., Ochanda, J.O., Owuor, B.O., Wamunyokoli, F.A., Oyugi, J.O. and Ochieng, J.W., 2013. Antagonistic effect of alkaloids and saponins on bioactivity in the quinine tree (*Rauvolfia caffra* sond.): further evidence to support biotechnology in traditional medicinal plants. *BMC Complementary and Alternative Medicine,* 13(1), p. 285.

Monte, J., Abreu, A. C., Borges, A., Simões, L. C., and Simões, M., 2014. Antimicrobial activity of selected phytochemicals against *Escherichia coli* and *Staphylococcus aureus* and their biofilms. *Pathogens,* 3(2), pp. 480-481.

Moongkarndi, O., Kosem, N. and Kaslungka, S., 2004. Antiproliferation,antioxidation and induction of apoptosis by *Garcinia Mangostona* (Mangosteen) on Skbr3 human breast cancer cell line. *Journal of Ethnopharmacology*, 90(1), pp. 161-166.

Mothana, R. A., Lindequist, U., Gruenert, R. and Bednarski, P. J., 2009. Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine,* 9(1), pp. 1-11.

Mujeeb, F., Bajpai, P. and Pathak, N., 2014. Phytochemical evaluation, antimicrobial activity, and determination of bioactive components from leaves of *Aegle marmelos*. *BioMed Research International.,* 2014(1), p. 6.

Murray, P.R., Rosenthal, K.S. and Pfaller, M.A., 2015. *Medical microbiology*. 8th ed. Maryland: Elsevier Health Sciences.

National Cancer Institute, 2017. *Cancer cell line.* [online] Available at: <https://www.cancer.gov/publications/dictionaries/cancer-terms/def/cancer-cell-line> [Accessed 9 February 2018].

National Cancer Institute, 2015. *Statistics at a glance: The burden of cancer worldwide.* [online] Available at: <https://www.cancer.gov/about-cancer/understanding/statistics> [Accessed 19 September 2017].

Ngoi, S. T., Lindstedt, B.-A., Watanabe, H. and Thong, K. L., 2013. Molecular characterization of *Salmonella enterica* serovar Typhimurium isolated from human, food, and animal sources in Malaysia. *Japanese Journal of Infectious Diseases,* 66(3), pp. 180-188.

Odhiambo, J., Siboe, G., Lukhoba, C. and Dossaji, S., 2009. Antifungal activity of crude extracts of selected medicinal plants used in combinations in Lake Victoria Basin, Kenya. *Plant Products Research Journal,* 13(1), pp. 35-43.

Omara, S.T., Zawrah, M.F. and Samy, A.A., 2017. Minimum bactericidal concentration of chemically synthesized silver nanoparticles against pathogenic Salmonella and Shigella strains isolated from layer poultry farms. *Journal of Applied Pharmaceutical Science,* 7(8), pp.214-221.

Organic Chemistry, 2016. *Column chromatography.* [online] Available at: <http://orgchemboulder.com/Technique/Procedures/Columnchrom/Procedure.shtml>
[Accessed 10 October 2017].

Paiva, P. M. G., Gomes, F.S., Napoleão, T.H., Sá, R.A., Correia, M.T.S. and Coelho, L.C.B.B., 2010. Antimicrobial activity of secondary metabolites and lectins from plants. In: A. Méndez-Villas, ed. *Current Research, Technology and Education Topics in Applied Microbiology and Microbial Biotechnology*. Pernambuco: Formatex, p. 399.

Parthasarathy, U. and Nandakishore, O. P., 2014. Morphological characterisation of some important Indian Garcinia species. *Dataset Papers in Science,* 2014(1), pp. 3-4.

Parthasarathy, V. A., Chempakam, B. and Zachariah, T. J., 2008. *Chemistry of spices.* Wallingford, UK: Cabi.

Patil, M.M. and Appaiah, K.A., 2015. Garcinia: Bioactive compounds and health benefits. *Introduction to Functional Food Science*, pp.110-125.

Policegoudra, R. S., Saikia, S., Das, J., Chattopadhyay, P., Singh, L., and Veer, V., 2012. Phenolic Content, antioxidant activity, antibacterial activity and phytochemical composition of *Garcinia lancifolia*. *Indian Journal of Pharmaceutical Sciences,* 74(3), pp. 268-271.

Public Health England, 2017. *COLO 205.* [online] Available at: <https://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=87061208&collection=ecacc_gc#>
[Accessed 9 February 2018].

Rasha, H.M., Salha, A., Thanai, A. and Zahar, A., 2015. The biological importance of *Garcinia cambogia*: A review. *Journal of Nutrition and Food Sciences,* 5(4), pp. 1-2.

Rios, J. L. and Recio, M. C., 2005. Medicinal plants and antimicrobial activity. *Journal of Ethnopharmacology,* 100(2005), pp. 81-82.

Riss, T. L., Moravec, R.A., Niles, A.L., Duellman, S., Benink, Helen, A., Worzella, T. J. and Minor, L., 2013. Cell viability assays. In: G. S. Sittampalam , N. P. Coussens, K. Brimacombe and A. Grossman, eds. *Assay Guidance Manual.* Bethesda: Eli Lilly and Company and the National Center for Advancing Translational Sciences, pp. 355-357.

Rodriguez-Tudela, J.L., Arendrup, M.C., Arikan, S., Barchiesi, F., Bille, J., Chryssanthou, E., Cuenca-Estrella, M., Dannaoui, E., Denning, D.W., Donnelly, J.P. and Fegeler, W., 2008. European Committee for Antimicrobial Susceptibility Testing (EUCAST) definitive document 9.1: Method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. *Clinical Microbiology Infection,* 14(1), pp.982-984.

Rosa, L. Silva, N.J.S., Soares, N.C.P., Monteiro, M.C. and Teodoro, A.J., 2016. Anticancer properties of phenolic acids in colon cancer a review. *Journal of Nutrition and Food Sciences,* 6(2), p. 468.

Saeed, N., Khan, M. R. and Shabbir, M., 2012. Antioxidant activity, total phenolic and total flavonoid contents of whole plant extracts *Torilis leptophylla* L. *BMC Complementary and Alternative Medicine,* 12(1), p. 221.

Sarker, S. D., Latif, Z. and Gray, A. I., 2006. *Natural products isolation.* 2nd ed. New Jersey: Human Press Inc.

Sasidharan, S. Chen, Y., Saravanan, D., Sundram, K.M. and Latha, L.Y., 2011. Extraction, isolation and characterization of bioactive compounds from plants' extracts. *African Journal of Traditional, Complementary, and Alternative Medicines,* 8(1), pp. 1-5.

Savini, V., 2016. *The diverse faces of Bacillus cereus.* 1st ed. Boston: Academic Press, pp. 1-13.

Schmitz, F.-J., Fluit , A.C., Gondolf, M., Beyrau, R., Lindenlauf, E., Verhoef, J., Heinz, H.P. and Jones, M.E., 1999. The prevalence of aminoglycoside resistance and corresponding resistance genes in clinical isolates of Staphylococci from 19 European hospitals. *Journal of Antimicrobial Chemotherapy*, 43(2), pp. 253-255.

See, I., Ee, G.C.L., Mah, S.Hui., Jong, V.Y. M. and Teh, S.S., 2017. Effect of solvents on phytochemical concentrations and antioxidant activity of *Garcinia benthamiana* stem bark extracts. *Journal of Herbs, Spices and Medicinal Plants*, 23(2), p. 6.

Senthilraja, P. and Kathiresan, K., 2015. *In vitro* cytotoxicity MTT assay in vero, HepG2 and MCF-7 cell lines study of Marine Yeast. *Journal of Applied Pharmaceutical Science*, 5(3), pp. 080-084.

Seruji, N. M. U., Khong, H. Y. and Kutoi, C. J., 2013. Antioxidant, anti-inflammatory and cytotoxic activities of *Garcinia nervosa* (Clusiaceae). *Journal of Chemistry*, 2013(2013), p.4.

Sharma, O. P. and Bhat, T. K., 2009. DPPH antioxidant assay revisited. *Food Chemistry*, 113(4), p. 1202.

Shihabudeen, H. M. S., Priscilla, D. H. and Thirumurugan, K., 2010. Antimicrobial activity and phytochemical. *International Journal of Pharma Sciences and Research*, 1(10), p. 432.

Siddiqui, H.H., 1993. Safety of herbal drugs - an overview. *Drugs News and Views*, 1(2), pp. 7-10.

Sit, P., Teh, C., Idris, N., Sam, I., Syed Omar, S., Sulaiman, H., Thong, K., Kamarulzaman, A. and Ponnampalavanar, S., 2017. Prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) infection and the molecular characteristics of MRSA bacteraemia over a two-year period in a tertiary teaching hospital in Malaysia. *BMC Infectious Diseases*, 17(1). p. 274.

Souza, A. B., Martins, C. H.G., Souza, M.G.M., Furtado, N.A.J.C., Heleno, V. C.G. and Rocha, E.M.P., 2010. Antimicrobial activity of terpenoids from *Copaifera langsdorffii* Desf. against cariogenic bacteria. *Phytotherapy Research*, 25(2011), p. 219.

Stevens, P.F., 2007. Clusiaceae-Guttiferae. In: Kubitzki K. (eds) Flowering Plants • Eudicots. *The families and genera of vascular plants*. St. Louis: Springer, pp. 48-50.

Sylvie, D. D., Anatole, P. C., Cabral, B. P. and Veronique, P. B., 2014. Comparison of *in vitro* antioxidant properties of extracts from three plants used for medical purpose in Cameroon: *Acalypha racemosa*, *Garcinia lucida* and *Hymenocardia lyrata*. *Asian Pacific Journal of Tropical Biomedicine*, 4(1), p. S631.

Tannock, I., Hill, R., Bristow, R. and Harrington, L., 2005. *The basic science of oncology*. 5th ed. New York: McGraw Hill.

Te-chato, S., 2007. Floral and fruit morphology of some species in. *Warasan Wichakan Songkhlanakharin*, 29(2), pp. 247-248.

Tee, K. K., Takebe, Y. and Kamarulzaman, A., 2009. Emerging and re-emerging diseases in Malaysia. *International Journal of Infectious Diseases*, 13(3), pp. 307-318.

Teke, G. N., Lunga, P.K., Wabo, H.K., Kuiate, J.R. and Vilarem, G., 2011. Antimicrobial and antioxidant properties of methanol extract, fractions and compounds from the stem bark of *Entada abyssinica* Stend ex A. Satabie. *BMC Complementary and Alternative Medicine*, 11(1), p. 57.

Thorn, C. F., Oshiro, C., Marsh, S., Hernandez-Boussard, T., McLeod, H., Klein, T.E. and Altman, R.B., 2011. Doxorubicin pathways: pharmacodynamics and adverse effects. *Pharmacogenetics and Genomics*, 21(7), pp. 440-441.

Tjahjani, S., Widowati, W., Khiong, K., Suhendra, A. and Tjokropranoto, R., 2014. Antioxidant properties of *Garcinia Mangostana* L (Mangosteen) Rind. *Procedia Chemistry*, 13(1), pp. 200-202.

Toroglu, S., 2007. *In vitro* antimicrobial activity and antagonistic effect of. *Journal of Environmental Biology*, 28(3), pp. 555-556.

Ukaoma, A. A., Ukaoma, V. O., Okechukwu, R.I. and Iwuagwu, M., 2013. Phytochemcial screening and antibacterial properties of *Garcinia kola*. *The Journal of Phytopharmacology*, 2(3), pp. 34-38.

Ulrich, A. B. and Pour, P. M., 2001. Cell lines. *Encyclopedia of Genetics*, 1(73), pp. 310-311.

Walton, N. and Brown, D., 1999. *Chemicals: perspectives on plant secondary products from plants.* London: Imperial College Press.

Watanapokasin, R., Jarinthanan, F., Nakamura, Y., Sawasjirakij, N., Jaratrungtawee, A. and Suksamrarn, S., 2011. Effects of α-mangostin on apoptosis induction of human colon cancer. *World Journal of Gastroenterology*, 17(16), pp. 2093-2095.

Wintola, O. and Afolayan, A., 2015. The antibacterial, phytochemicals and antioxidants evaluation of the root extracts of *Hydnora africana* Thunb. used as antidysenteric in Eastern Cape Province, South Africa. *BMC Complementary and Alternative Medicine,* 15(1), p. 307.

Wong, K.M., 2009. *Herbs of Malaysia: an introduction to the medicinal, culinary, aromatic and cosmetic use of herbs.* Malaysia: Marshall Cavendish Editions.

World Health Organisation, 2018a. *Infectious disease.* [online] Available at: < http://www.who.int/topics/infectious_diseases/en/> [Accessed 16 December 2017].

World Health Organisation, 2018b. *Antimicrobial resistance*. [online] Available at: <http://www.who.int/mediacentre/factsheets/fs194/en/> [Accessed 16 December 2017].

World Health Organisation, 2017a. *Cancer*. [online] Available at: < http://www.who.int/mediacentre/factsheets/fs297/en/> [Accessed 15 December 2017].

World Health Organisation, 2017b. *World Health Report 1996: Infectious diseases.* [online] Available at: <http://www.who.int/whr/1996/media_centre/press_release/en/> [Accessed 20 December 2017].

Xu, X. H., Li, T., Fong, C., Chen, X., Chen, X.J., Wang, Y.T., Huang, M. and Lu, J.J., 2016. Saponins from chinese medicines as anticancer agents. *Molecules*, 21(12), p. 1326.

Yang, J., Liu, R. H. and Halim, L., 2009. Antioxidant and antiproliferative activities of common edible nut seeds. *LWT - Food Science and Technology*, 42(1), pp. 1-3.

Yen, G.-C., Duh, P.-D. and Tsai, H.-L., 2002. Antioxidant and pro-oxidant properties of ascorbic acid and gallic acid. *Food Chemistry,* 79(1), p. 307.

Yoshioka, K., Kataoka, T., Hayashi, T., Hasegawa, M., Ishi, Y. and Hibasami, H., 2000. Induction of apoptosis by gallic acid in human stomach cancer KATO III and colon adenocarcinoma COLO 205 cell lines. *Oncology Reports*, 7(6), pp. 1221-1224.

Young Genomes, 2015. *What are infectious diseases?*. [online] Available at: https://www.yourgenome.org/facts/what-are-infectious-diseases [Accessed 5 January 2018].

Zarena, A. S. and Sankar, K. U., 2009. A study of antioxidant properties from garcinia mangostana l. pericarp extract. *Acta Scientiarum Polonorum Technologia Alimentaria,* 8(1), p. 27.

APPENDIX A

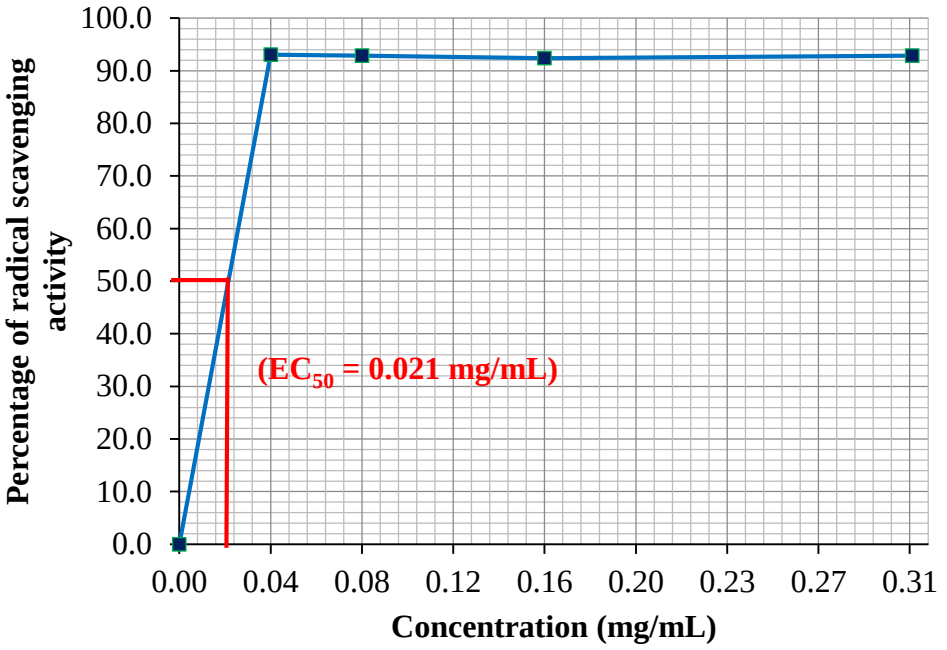

Figure A: The percentage radical scavenging activity of fraction 2 at various concentrations.

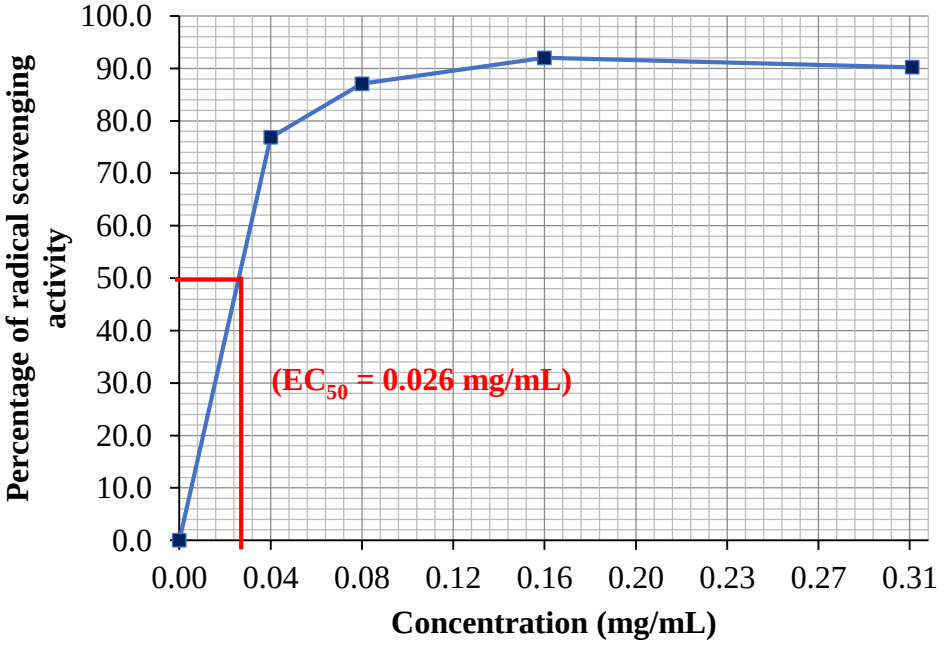

Figure B: The percentage radical scavenging activity of ascorbic acid at various concentrations.

APPENDIX B

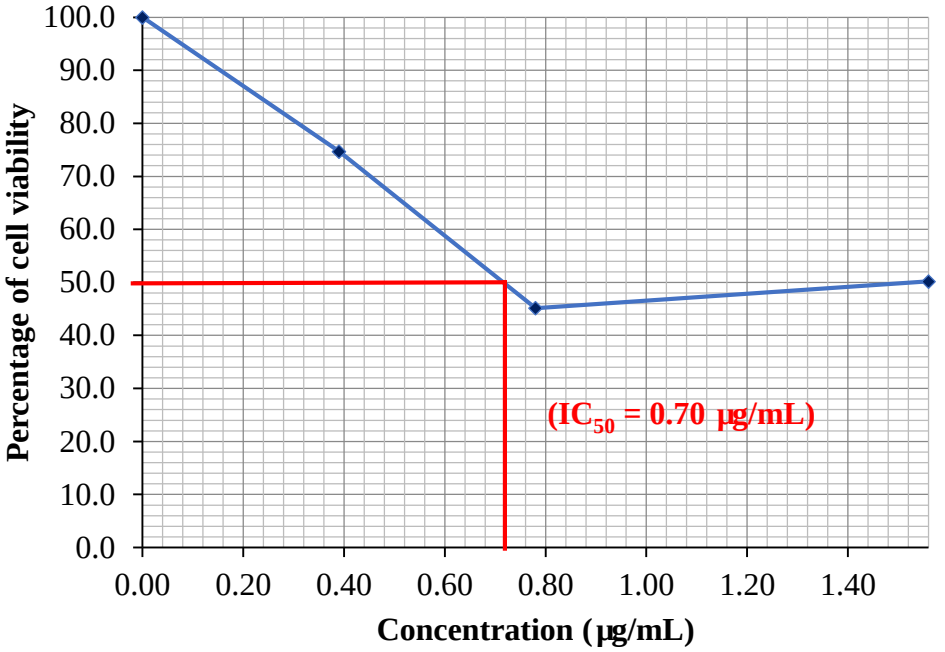

Figure C: The percentage of cell viability of fraction 2 at various concentrations.

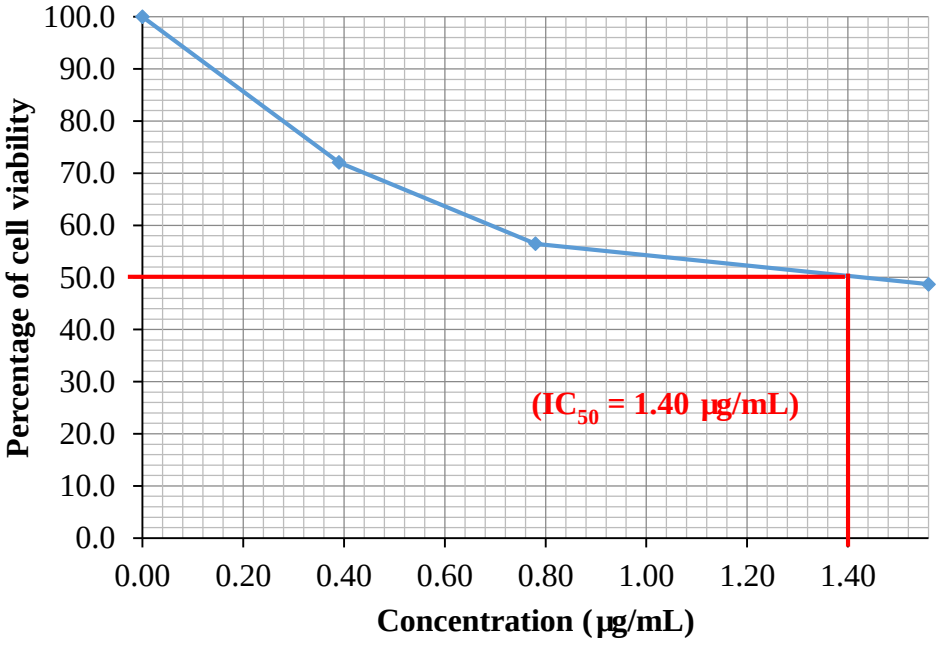

Figure D: The percentage of cell viability of doxorubicin hydrochloride at various concentrations.