CYTOTOXIC EFFECT AND ANTI-OXIDANT ACTIVITY OF BIOASSAY-GUIDED FRACTIONS ISOLATED FROM *Garcinia maingayi*

By

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ABSTRACT

CYTOTOXIC EFFECT AND ANTI-OXIDANT ACTIVITY OF BIOASSAY-GUIDED FRACTIONS ISOLATED FROM *Garcinia maingayi*

Balbita Kaur A/P Manjit Singh

Garcinia maingayi is a medicinal plant that belongs to the family of Clusiaceae or Guttiferae. Traditionally, the leaves are used as anti-pyretic agent and dried fruits are used to treat stomach ache, fever and as an anti-malarial agent. Besides, the stem-bark of *Garcinia maingayi* is used as anti-microbial agents. The ethanolic fraction of stem-bark of *Garcinia maingayi* was evaluated for its anti-oxidant activity using DPPH assay and cytotoxic effect against HeLa and MDA-MB-231 cancer cell lines using MTT assay. From the bioassay-guided fractionation, six major fractions were obtained from the ethanolic fraction of *Garcinia maingayi.* The concentrations used in both assays were ranging from 2 to 5000 µg/mL. Qualitative phytochemical screening revealed the presence of alkaloids, flavonoids, glycosides, phenols, quinones, saponins, tannins and terpenoids based on color changes or precipitation or foams formation. In DPPH assay, fraction 2 showed the highest radical scavenging activity of 94.3±0.05% (EC_{50} 0.02 mg/mL) with references to ascorbic acid which exhibited 94.6±0.01% (EC_{50} 0.01 mg/mL). All the six fractions were proceeded to MTT assay. The highest cytotoxic effect was shown by fraction 6 against HeLa cells with IC_{50} of 1.27 µg/mL. While fraction 3 exhibited IC_{50} of

1.33 µg/mL against MDA-MB-231 cells. Vinblastine sulfate showed lowest IC_{50} of 1.20 and 1.33 µg/mL against HeLa and MDA-MB-231 cells, respectively. Higher radical scavenging activity of all the fractions showed a stronger correlation with higher cytotoxic effect towards tested cancer cell lines. Thus, the secondary metabolites present in the ethanolic fractions may scavenge free radicals that are detrimental to nucleic acids. In conclusion, compounds isolated from ethanolic fraction of *Garcinia maingayi* act as anti-oxidant and anti-cancer agents. Further isolation and purification is useful in the development of drugs to treat various diseases.

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DECLARATION

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

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(BALBITA KAUR A/P MANJIT SINGH)

APPROVAL SHEET

This project report entitled **"CYTOTOXIC EFFECT AND ANTI-OXIDANT ACTIVITY OF BIOASSAY-GUIDED FRACTIONS ISOLATED FROM *Garcinia maingayi*"** was prepared by BALBITA KAUR A/P MANJIT SINGH and submitted as partial fulfilment of the requirements for the degree of Bachelor of Science (Hons) Biomedical Science at Universiti Tunku Abdul Rahman.

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PERMISSION SHEET

It is hereby certified that **BALBITA KAUR A/P MANJIT SINGH (**ID No**: 13ADB07675)** has completed this final year "CYTOTOXIC EFFECT AND ANTI-OXIDANT ACTIVITY OF BIOASSAY-GUIDED FRACTIONS ISOLATED FROM *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,

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(BALBITA KAUR A/P MANJIT SINGH)

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LIST OF ABBREVIATIONS

ABTS	2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonate)
ASR	Age standardized rate
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
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
FBS	Fetal bovine serum
FRAP	Ferric reducing anti-oxidant power
FTC	Ferric thiocyanate
GC-MS	Gas chromatography-mass spectrophotmeter
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer
IC_{50}	Half-maximal inhibitory concentration
LDH	Lactate dehydrogenase
MIC	Minimum inhibitory concentration
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
SRB	Sulphorhodamine B
TBA	Thiobarbituric acid
TEAC	Trolox equivalent anti-oxidant capacity
TLC	Thin layer chromatography
TRAP	Total radical trapping anti-oxidant potential
UV	Ultraviolet
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

Cancer is termed for diseases that are caused by abnormal uncontrollable cell growth causing invasion into nearby tissues and metastasize to other parts from the primary site via the blood circulation and lymphatic system (National Cancer Institute [NCI], 2016). This non-communicable disease has become the prominent cause of mortality and morbidity worldwide in which approximately 8.2 million cancer-related deaths and 14 million new cases are reported in 2012 (World Health Organization [WHO], 2016a). In a recent news update, it was estimated that nearly 1 in 4 of total worldwide deaths caused due to cancer and further increased by 50% to 15 million of new cases in year 2020 (WHO, 2016b).

Besides, in Malaysia, the occurrence of cancer have increased to 37,400 incidence in 2012 with comparison to 32,000 incidence in 2008 and expected to increase to 56,932 cases by 2025 if no action is seriously engaged (The Star Online, 2014). According to International Agency for Research on Cancer (IARC) (2012), the most common type of cancer in both sexes are breast, colorectal, lung, cervical and prostate cancers based on incidence and mortality rates in Malaysia.

There are many risk factors associated with the occurrence of cancer such as exposure to carcinogen, genetic inheritance, environment and lifestyles. The most important risk factor of cancer is the advancing age of host even though the disease can occur at any age (National Cancer Institute [NCI], 2015a). Alteration of certain genes can be caused due to exposure of carcinogen, such as chemicals in tobacco smoke, or ultraviolet lights that may alter the normal function of cells and causing cancer (NCI, 2015b). Moreover, infection due to bacteria (*Helicobacter pylori*), viruses (*Epstein-Barr* and *Human papilloma* viruses) and parasites (*Schistosoma hematobium*) disrupt the growth and proliferation of cells and thus increase the risk of cancer (NCI, 2015c). Besides, behavioral and dietary risks such as high body mass index, low intake of fruits and vegetable, lack of physical activity, consumption of alcohol and tobacco are causing approximately one third of cancer deaths (WHO, 2016a).

Although there are vast number of cancer treatments available, however, yet there are no effective drugs to treat most cancers as mentioned by Cai, et al. (2004). Cancer treatments are very expensive and possess toxic side effects (Cai, et al., 2004; Prasad, et al., 2008). Chemo-drugs travels via the blood circulation, and thus affects the blood-forming cells in bone marrow and hair follicles eventually causing hair loss, cells in the mouth, digestive tract causing gastrointestinal tract discomfort and reproductive system (American Cancer Society, 2016). Cancer cells have also developed drug resistance due to prolonged treatments (Mesquita, et al., 2009). Thus, research on natural products especially medicinal plants is a good attempt as a potential source of drugs to cure cancer and many other diseases.

“An apple a day keeps the doctor away” is a traditional American rhyme and this rhyme does give us an idea that finding healing power in plants is an ancient idea (Cowan, 1999). According to Mahesh and Satish (2008), there are at least 250,000 to 500,000 species of plants on Earth and relatively only a small percentage is investigated phytochemically in pharmacological screening. Hippocrates mentioned that there are estimated 300 to 400 medicinal plants in the late fifth century B.C. and Bible also provided us with medicinal descriptions of approximately 30 healing plants (Cowan, 1999). Besides, Ayurveda tells us about the traditional Indian medicines used from plants as treatment in preventing various tumors (Balachandran and Govindarajan, 2005).

Thus, over the years, medicinal plants has been playing vital roles as a source of anti-cancer and anti-infective agents (Mothana, et al., 2011; Raina, et al., 2014). Based on statistical analysis, approximately 80% of the population uses traditional plants as their medicine in primary health care due to its low toxicity, good therapeutic performances and economic viability as an alternative source to synthetic drugs (Li, et al., 2008; Mathew and Abraham, 2006; Sasidharan, et al., 2011; Surveswaran, et al., 2007).

Natural products span an extremely large and diverse range of chemical compounds from biological sources which include the entire organism (plants, animals, microorganisms) or just derived from a part of an organism (leaves, animal organs) (Sarker, Latif and Gray, 2005). Few example of drugs derived from natural products are paclitaxel from *Taxus brevifolia*, vitamins A and D

from cod liver oil and doxorubicin from *Streptomyces peucetius*. Medicinal plant on the other hand, is defined as medicinal product that contain active ingredients commonly known as secondary metabolites such as, alkaloids, tannins, flavonoids and phenolic compounds. These medicinal properties can be exerted by the plant's leaves, stems, flowers, fruits, seeds, roots, rhizomes and barks where it serves as diagnostic purposes or for prescription as drugs or monitoring of treatments (European Parliament and the Council, 2004; Gandhiraja, et al., 2009; Sarker, Latif and Gray, 2005).

Malaysia is one of the hot spots lie in Asia that has diverse range of plant flora in tropical and subtropical regions (Handa, et al., 2008). Malaysia Nature Society (2016) reported that Malaysia is one of the 12^{th} richest country of biodiversity with approximately 15,000 types of plants. Malaysian plant species have been used traditionally to relieve constipation, headaches and body aches (Saha, et al., 2004). Besides, methanolic extract of Malaysian *Zingiber officinale* showed potential anti-oxidant activity and thus proving higher radical scavenger (Ghasemzadeh, Jaafar and Rahmat, 2010). In addition, *Acalypha indica*, *Cerbera manghas* and *Morinda elliptica* showed significant cytotoxic effect against HeLa cell line with the IC_{50} below 0.02 mg/mL (Ali, et al., 1996).

Thus, this research was conducted to determine the cytotoxic effect and antioxidant activity using *Garcinia maingayi*, a species found in Sabah, Malaysia. Previous studies shown that Garcinia species possess good cytotoxicity against certain human cancer cell lines such as MCF-7 (breast),

DU-145 (prostate) and H460 (non-small cell lung), in which the value of IC_{50} is less than 10 μg/mL (Jabit, et al., 2009). Garcinia species also showed strong antioxidant activity compared with ascorbic acid (Subhashini, Nagarajan and Kavimani, 2011). Previous preliminary studies on the crude extracts of Malaysian species of *Garcinia maingayi* showed a promising biological activity. Thus, the research was proceeded to further purification and isolation.

Hence, the objectives of this research are:

1) To screen for the presence of bioactive secondary metabolites using qualitative phytochemical tests,
2) To isolate compounds from ethanolic fraction of *Garcinia maingayi* stem-bark using gravity column chromatography and thin layer chromatography,
3) To determine the percentage of radical scavenging activity of the isolated compounds using DPPH assay,
4) To determine the cytotoxic effect of the bioactive compounds against HeLa and MDA-MB-231 cells using MTT assay.

CHAPTER 2

LITERATURE REVIEW

2.1 General Information of Garcinia

2.1.1 Botanical Description

Garcinia species is known as ‘Asam kandis’ or ‘Kandis gajah’ and this polygamous dioecious evergreen fruit tree or shrub is usually small to medium sized as shown in **Figure 2.1** (Asian Plant, n.d.; Chai, 2000; Jabit, et al., 2009; Joseph, et al., 2005). Height of Garcinia species is approximately 12 to 25 m with low horizontal branches, while the leaves are oblong in shape, ranging in length of 18 to 23 cm and width 9 to 10 cm (Idris and Rukayah, 1987; Sethi, 2011). Leaves of Garcinia species are thick with smooth and shiny surfaces. Besides, dorsal surface of the leaves are dark green in color whereas its ventral surface is yellowish green (Idris and Rukayah, 1987). Fruits of Garcinia species are edible with sourly taste in which the fruits have about six to eight seeds (Sethi, 2011; Kumar, Sharma and Chattopadhyay, 2013).

2.1.2 Taxonomical Classification

Garcinia belong to the family of Clusiaceae or Guttiferae (Hemshekhar, et al., 2011). Taxonomical classification of *Garcinia maingayi* is shown in **Table 2.1**.

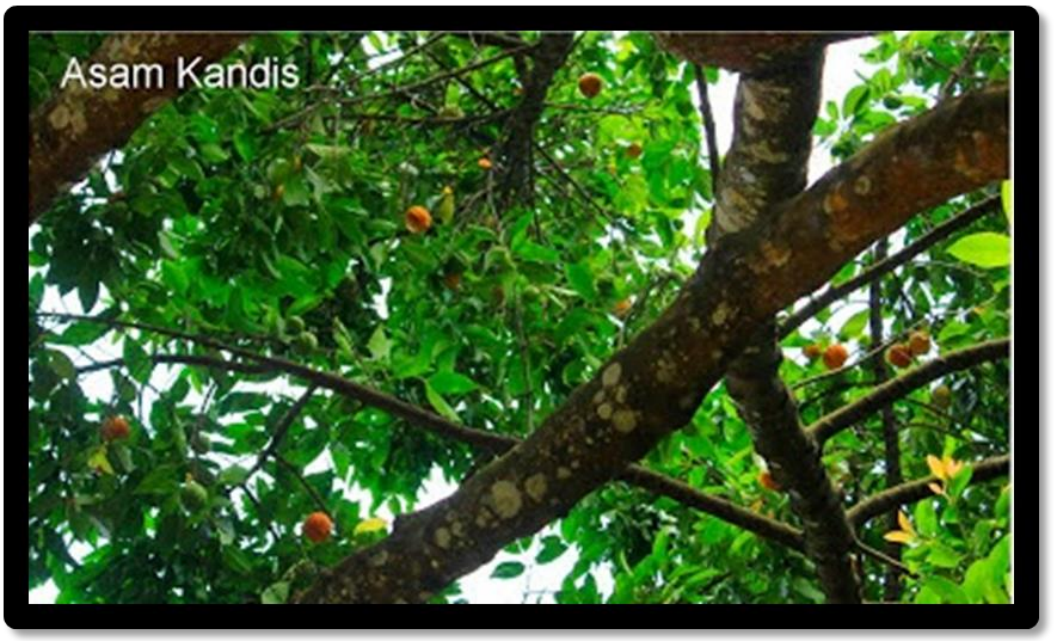

Figure 2.1: Different parts of Garcinia (Asian Plant, n.d.).

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/Guttiferae
Subfamily	Clusioideae
Tribe	Garcinieae
Genus	Garcinia
Species	*Garcinia maingayi*

2.1.3 Habitat and Distribution

Approximately 450 species of genus Garcinia are widely distributed in tropical Asia, South Africa and Polynesia as well as in Australia (Kumar, Sharma and Chattopadhyay, 2013; Patil and Appaiah, 2015). Garcinia species are predominately found in Malaysia specifically in Kedah, Perak, Pahang, Terengganu and Johor whereby its habitat is widely distributed within the montane forest areas to lowlands of rainforest (Kochummen, 1998; Jabit, et al., 2009). However, this species is abundantly present in Sabah and Sarawak (Repin, et al., 2012; Ling and Julia, 2012).

Garcinia species needs maximum sunlight exposure and moisture to ensure well growth of the plant. Hence, Garcinia species is usually found in tropical countries instead of countries with four seasons. Garcinia species also doesn't tolerate any temperature below 4℃ or above 37℃. Besides, Garcinia species thrive better in rich organic soil but able to survive in sandy or clay containing course material (Grant, 2016).

2.1.4 Traditional Uses

Traditionally, leaves of *Garcinia maingayi* is used as an anti-pyretic agent (Jabit, et al., 2009). According to Parthasarathy and Nandakishore (2014) and Chai (2000), dried fruits are usually used for culinary purposes and acts as folk medications to treat stomach ache, fever and used as anti-malaria agent. The stem-bark of Garcinia species can be used as anti-microbial agents, while its latex is used to treat fever (Jabit, et al., 2009).

2.1.5 Chemical Constituents

Detailed chemical studies on *Garcinia maingayi* was done by Cheng and Cheow (2008) using 1D and 2D nuclear magnetic resonance (NMR) spectroscopy. Pure compounds were isolated from *Garcinia maingayi* such as triterpenoids (stigmasterol and sitosterol) as shown in **Figure 2.2**, benzophenone (isoxanthochymol), xanthone (1,3,7-trihydroxy-2-3-(3-methylbut-2-enyl)xanthone) and benzoic acid derivative (3,4-dihydroxy-methylbenzoate) (Cheng and Cheow, 2008). Presence of phenolic compounds shows that *Garcinia maingayi* has anti-oxidant properties with removal of free radical scavengers in cells (Krishnamoorthy, et al., 2014). Xanthone and benzophenone are reported as potent cytotoxic agents toward various human cancer cell lines (Kumar, Sharma and Chattopadhyay, 2013).

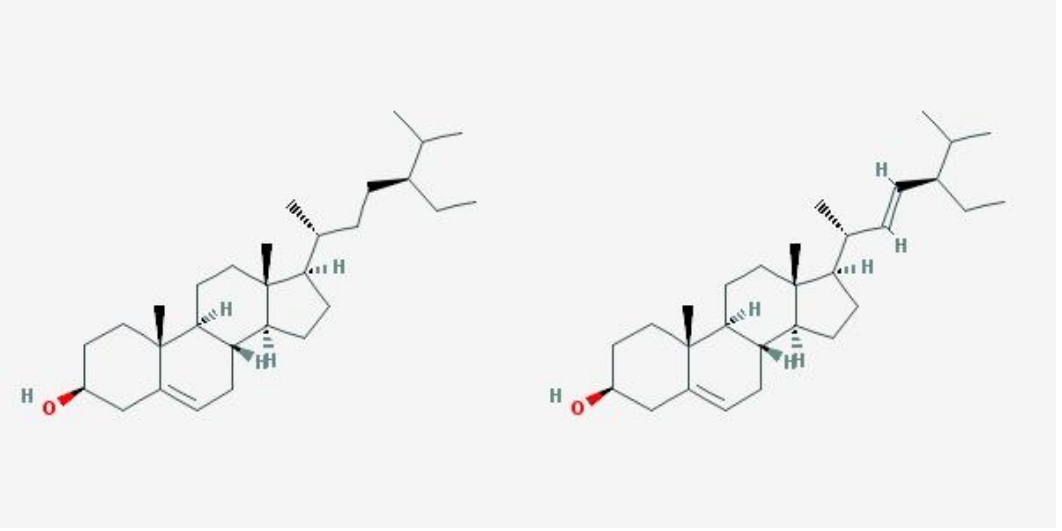

Figure 2.2: Chemical structure of sitosterol (left) and stigmasterol (right) (National Center for Biotechnology Information [NCBI], 2016a; NCBI, 2016b).

2.1.6 Pharmacological Studies of Garcinia Species

2.1.6.1 Anti-oxidant Activity

Aqueous fruits extract of *Garcinia combogia* obtained from maceration technique showed higher radical scavenging activity (Subhashini, Nagarajan and Kavimani, 2011). The aqueous fruits extract recorded IC_{50} value of 36.20 ± 3.04 μg/mL due to the presence of phenolic and flavonoid compounds. Folin-ciocalteau assay showed the values of phenolics and flavonoids content are 75 g pyrocathechol equivalent/mg and 30 g quercetin equivalent/mg in aqueous fruits extract (Subhashini, Nagarajan and Kavimani, 2011).

2.1.6.2 Cytotoxic Effect

In previous studies, few Garcinia species were tested for their cytotoxic effect on breast cancer cells (MCF-7), prostate cancer cells (DU-145) and non-small cell lung cancer cells (H460), in which the IC_{50} values are less than 10 μg/mL. Strong cytotoxic effect was reported due to the presence of xanthones (Jabit, et al., 2009). Stem-bark and leaves extracts of *Garcinia maingayi* showed IC_{50} value of 6 and 10 μg/mL, respectively on MCF-7 cells. Besides, IC_{50} value of 5 and 10 μg/mL were represented by stem-bark extract of *Garcinia nigrolineata* and *Garcinia cantleyana*, respectively on DU-145 cells. Cytotoxic effect on H460 cells were shown by stem extract of *Garcinia nigrolineata* and *Garcinia cantleyana* with IC_{50} value of 3 and 4 μg/mL, respectively. Meanwhile, leaves extract of *Garcinia cantleyana* showed the lowest IC_{50} value of 2 μg/mL, thus showing strongest cytotoxic effect on H460 cells (Jabit, et al., 2009).

2.1.6.3 Anti-microbial Activity

Ethanolic stem-bark extract of *Garcinia kola* exhibited lowest inhibitory concentration of 0.03, 0.08 and 0.90 mg/mL against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*, respectively (Ukaoma, et al., 2013). The inhibition zones of the extract against these bacteria were ranging from 20.5 to 40.0 mm (Ukaoma, et al., 2013). The inhibition was mainly due to the presence of high percentage of saponins that act as vital anti-bacterial agent together with synergistic actions of other secondary metabolites such as flavonoids, tannins and alkaloids (Ukaoma, et al., 2013). Furthermore, stem-bark extract of *Garcinia polyantha* showed MIC of 156.25, 78.12, 78.12 and 156.25 µg/mL against *Candida albicans*, *Candida gabrata*, *Microsporum audouinii* and *Trichophyton rubrum*, respectively. These inhibition was reported mainly due to the presence of flavonoids and zanthones (Kuete, et al., 2007).

2.2 Cancer

Cancer is known as non-communicable disease characterized by the abnormal cell proliferation and growth. Cancer has been considered one of the leading cause of mortality and morbidity rate worldwide (Crosta, 2015). Mutation of genes in cell causes abnormal cell division and thus causing the development of cancer. These mutations occurs due to many carcinogens, such as tobacco, exhaust fumes, solar radiation and ultra-violet (UV) light, X-rays that aids and promote in cancer development. These mutated cells undergo unregulated cell proliferation eventually invading and breaching other adjacent cells and

spreading from the primary site of lesion to the entire body via systemic blood circulation and lymphatic system (Crosta, 2015).

According to Lim (2002), cancer has been an on-going growing problem in Malaysia with an annual estimation of 30,000 cases. Based on epidemiology study by Ministry of Health, malignant neoplasms is reported as the third most common cause of death (10.59%) (Malaysian Cancer Statistics, 2006). The most common type of cancer in both sexes in Malaysia are breast, colorectal, lung, cervical and prostate cancers based on incidence and mortality rates (International Agency for Research on Cancer [IARC], 2012). In a recent update, it was estimated that incidence rate of cancer will rise to approximately 56,000 cases in 2025 with the ratio of one out of four Malaysian (National Cancer Society of Malaysia, 2016).

Breast cancer developed due to the malignancy of cell lining in the ducts of breast (PubMed Health, 2016a). Among all the cancers, breast cancer is the main cause of mortality in women based on worldwide statistics (Bustreo and Chestnov, 2015). Breast cancer is also the most common cancer within the Malaysian women population that accounts for 32.1% of incidence rate in 2007 (Kuan, Papapreponis and Hin, 2015). Age standardized rate (ASR) of breast cancer in Malaysia among all the female ethnic groups is 52.8 per 100,000 population (National Cancer Registry, 2003).

Cervical cancer or known as malignant neoplasm of the cervix uteri is caused by the abnormal and uncontrolled proliferation of tissue particularly in cervix (PubMed Health, 2016b). This is the second most common cancer in women that has been reported with 84% of new cases worldwide (World Health Organization [WHO], 2016c). Approximately, 13 human papillomavirus (HPV) out of 100 types causes cancer in which the most common types are HPV-16 and HPV-18 that accounts for 70% of cervical cancer cases (WHO, 2016c). According to National Cancer Registry (2003), cervical cancer is the second most common cancer in Malaysia with ASR of 21.5 per 100,000 population and Malaysia has the highest incidence rate of cervical cancer compared with other Asian and Western countries.

2.3 Cancer Cell Lines

2.3.1 HeLa Cells

HeLa cell line is a human cervical epithelial carcinoma cell line. In the history of animal cell culture, HeLa cells are the first continuous cell line that was isolated and established in 1951 (Masters, 2002; Adey, et al., 2013). HeLa cells were derived from aggressive glandular carcinoma of a 31-year-old woman, Henrietta Lacks (American Type Culture Collection [ATCC], 2014a; Lucey, Nelson-Rees and Hutchins, 2009). This somatic mammalian cells exhibited a uniform morphology with 100% plating efficiency regardless with high or low serum concentrations (Puck and Fisher, 1956). However, with the presence of human sera, these cells are highly stretched, amoeboid shaped with marked motility (Puck, Philip and Cieciura, 1956). So if the same HeLa cells are

cultured in non-human sera condition, these cells would behave differently by packing themselves tightly with columnar and epithelial-like morphology (Puck, Philip and Cieciura, 1956).

2.3.2 MDA-MB-231 Cells

MDA-MB-231 is a human breast epithelial carcinoma cell line which was derived from mammary gland of a 51-year-old Caucasian female with breast adenocarcinoma (ATCC, 2014b). These monolayer cells grow in spindle-shape as well as fibroblast-like shape and adapt to tissue culture conditions due to its polyclonal cell characteristics (Gozgit, et al., 2006; Fillmore and Kuperwasser, 2008). MDA-MB-231 cells are characterized by its significant metastatic pattern that involve the routes of lymph nodes, bone marrow, liver and lung (Müller, et al., 2001). Moreover, these aggressive metastatic cells produce large quantities of lactate due to increase glucose consumption for survival (Gallagher, et al., 2007). MDA-MB-231 cells are known to develop resistance to several anti-cancer agents (Zordoky, et al., 2014).

2.4 Chromatographic Techniques

2.4.1 Thin Layer Chromatography (TLC)

Thin layer chromatography (TLC) or also known as planar chromatography is commonly used laboratory technique because it is the simplest, quick and inexpensive procedure that allow researchers to determine the presence of bioactive compounds (Sasidharan, et al., 2011; Sherma and Fried, 2013).

Researchers are able to choose the best solvent system using TLC based on the best separation of the tested compounds (Sasidharan, et al., 2011).

TLC comprises of stationary and mobile phases, in which the separation depends on the relative affinity of compounds towards those phases (University of Leeds, 2016). Stationary phase in TLC is a thin aluminium plate coated with silica gel, whereas the mobile phase can be an organic solvent (University of Leeds, 2016).

Technically, a little aliquot of tested sample is applied on the baseline and the sample will be placed into a closed, developing chamber containing the organic solvent (Sherma and Fried, 2013). Components in the sample will travel at different rates along the mobile phase through the stationary phase until reaches the frontline. The plate is removed and the spot would be observed and detected in daylight (naked eye) and UV light. Fluorescence quench detection allows the components to be detected under short wavelength (254 nm) and long wavelength (365 nm) due to the presence of the fluorescence indicator impregnated on the TLC plate (Sherma and Fried, 2013).

Retention factor (R_f) is calculated based on the visualized spots, in which the R_f is the ratio of distance travelled by the component from the baseline over the distance travelled by the mobile phase from baseline (Kaur and Arora, 2009). R_f values determine the polarity of the substances present in the extracts.

2.4.2 Gravity Column Chromatography

Gravity column chromatography is a purification technique that isolate desired compounds from a mixture of sample. The compounds are separated based on gravitational forces where the mobile phase moves through the stationary phase based on various polarities (Organic Chemistry, 2016).

Gravity column chromatography comprises of two phases, namely the stationary and mobile phases. The vertical column is packed with silica gel that acts as stationary phase and mobile phase is various organic solvents. The components in the sample possess different interactions between both the phases and those components is eluted with good separation due to different characteristics.

2.5 Bioassays

2.5.1 Anti-oxidant Assay

There are many anti-oxidant assays such as ferric reducing antioxidant power (FRAP), 2,2-azinobis-(3-ethyl-benzothiazoline-6-sulfonate) (ABTS), oxygen radical absorbance capacity (ORAC), thiobarbituric acid (TBA), ferric thiocyanate (FTC) and 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Saha, et al., 2004; Abas, et al., 2006; Katalinic, et al., 2006; Ali, et al., 2008; Krishnaiah, Sarbatly and Nithyanandam, 2011).

However, DPPH assay is the most favored assay due to its nature as DPPH free radical that is commercially available and doesn't need special generation of radicals (Ioannou, et al., 2015). Unlike ABTS assay, free radicals need to be generated by enzymes or chemical reactions. Thus, this proves DPPH assay is more simple and convenient to be used due to its stability and easy colorimetric method besides its better and rapid effectiveness (Arnao, 2000; Krishnaiah, Sarbatly and Nithyanandam, 2011; Mishra, Ojha and Chaudhury, 2012). DPPH assay was established by Brand-Williams and his team in 1995 to measure anti-oxidant content of medicinal plants in different solvents systems such as ethanol and methanol (Cai, et al., 2004; Cheng, Moore and Yu, 2006).

DPPH radical is a free stable radical that gives purple appearance in methanol due to the presence of odd number of electrons while absorbing at 517 nm (Zahin, Aqil and Ahmad, 2009; Mishra, Ojha and Chaudhury, 2012). When DPPH free radical react with anti-oxidant compound (scavenger molecule) present in the plant extracts, the radical would be reduced due to the acceptance of hydrogen atom from the scavenger molecule. The reduced radical causes color change from purple to yellow. The reduction of color intensity is resembled by the decrease in optical density at 517 nm (Mathew and Abraham, 2006). Decolorization is dependent on the amount of electrons captured (Blios, 1958).

Antioxidant activity in DPPH assay is expressed as EC_{50} value that provides the concentration that reduces DPPH free radical by 50% (Basma, et al., 2011).

Based on quantal dose-response, EC_{50} refers to the dose that can be given to the subjects to prevent any side effects such as, arrhythmia, convulsion or even death by the drug (Clarkson, 2012).

2.5.2 Cytotoxic Assay

Cytotoxic assay is based on the mammalian cell lines that are grown actively under optimum condition. Cells undergo continuous mitotic division and treated with tested sample to determine the anti-cancer property of the compound (Houghton, et al., 2007).

There are several measures or assays that can be used to quantify the number of viable cells such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) salt, 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium (XTT) salt or pink aminoxanthine dye in sulphorhodamine B (SRB) assay (Houghton, et al., 2007).

SRB assay may be much simpler and rapid compared to XTT assay that uses salt instead of dye. However, MTT assay is the most common assay used for cytotoxicity screening for comparison and efficient analysis to determine the cell viability.

MTT is a colorimetric assay that quantifies number of viable cells spectrophotometrically at 570 nm (Mahavorasirikul, et al., 2010). The viable cells will cause the reduction of yellow tetrazolium salt into purple formazan

crystals. The reduction is performed by the presence of mitochondrial reductase enzyme which is succinic dehydrogenase (Hansen, Nielsen and Berg, 1989). The amount of formazan crystals formed is directly proportional to the number of viable cells (George, et al., 2010).

The insoluble purple formazan crystals is dissolved by DMSO and the absorbance is measured spectrophotometrically (Moongkarndi, et al., 2004). Thus, the IC_{50} value can be determined from the graph of percentage of cell viability versus concentration of sample (Moongkarndi, et al., 2004). IC_{50} is the half maximal inhibitory concentration that measures the effectiveness of a drug in inhibiting certain biochemical or biological function (Chandra, 2014).

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 proceeded to cold extraction using various organic solvents. Only 10.0 g of ethanolic fraction was used in this research.

3.1.2 Cancer Cell Lines

Human cervical epithelial carcinoma cell line (HeLa) (ATCC® CCL-2™) and human breast epithelial carcinoma cell line (MDA-MB-231) (ATCC® HTB-26™) were used. These cells were cryopreserved in liquid nitrogen vapor tank. The cells were cultured in DMEM medium and was observed using inverted phase contrast microscope.

3.1.3 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 / Solvents	Manufacturer / Brand
Acetone	QRëC™ Grade AR
Ascorbic acid	Gene Chem, Canada
Chloroform	QRëC™ Grade AR
DPPH reagent	Calbiochem®, USA
DMEM	Nacalai Tesque, Kyoto
Ethyl acetate (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Fetal bovine serum (FBS)	JR Scientific, Inc, USA
Hexane (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
Methanol (Industrial grade)	Irama Canggih Sdn. Bhd., Malaysia
MTT reagent	Bio Basic Canada Inc, Canada
Phosphate buffer saline (PBS)	MP Biomedicals, France
Silica gel 60 powder	Merck, Germany
Silica gel coated aluminium sheet	Merck, Germany
Sodium sulfate anhydrous	John Kollin Corporation, USA
Vinblastine sulfate	MP Biomedicals, France
Dimethyl sulfoxide (DMSO)	Merck, Germany
95% Ethanol (Industrial grade)	Copens Scientific (M) Sdn. Bhd., Malaysia
0.4% Trypan blue dye	Life Technologies, USA
0.25% Trypsin-EDTA	Biowest, USA

3.1.4 Equipment

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

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

Equipment	Manufacturer / Brand
Autoclave	Hvt-50, HICLAVE, USA
Carbon dioxide (CO_2) incubator (37 ℃)	BINDER, Germany
Centrifuge (Maximum 12,500 rpm)	Kendro, Germany
Electronic balance	Sartonus, Malaysia
Freezer (-20 ℃)	Snow, Malaysia
Freezer (-80 ℃)	ARDO, Italy
Fume hood	Chemo Resources, Malaysia
Hemocytometer	Marienfeld-Superior, Germany
Incubator (37 ℃)	Memmert, Germany
Inverted contrast phase microscope	Olympus, Malaysia
Laminar flow	Edamix, Malaysia
Microplate reader	TECAN, Australia; OMEGA, Germany
Refrigerator (4 ℃)	SAMEMAX, Malaysia
Rotary evaporator	R-200 Buchi, Swirtzerland
Sonicator	Branson Ultrasonic, USA
Ultraviolet lamp	Spectroline™, USA
Vortex mixer	Bibby Scientific Ltd., UK
Water bath	Memmert, Germany

3.2 Methodology

Figure 3.1 shows a brief workflow of isolation and purification of ethanolic fraction of *Garcinia maingayi*.

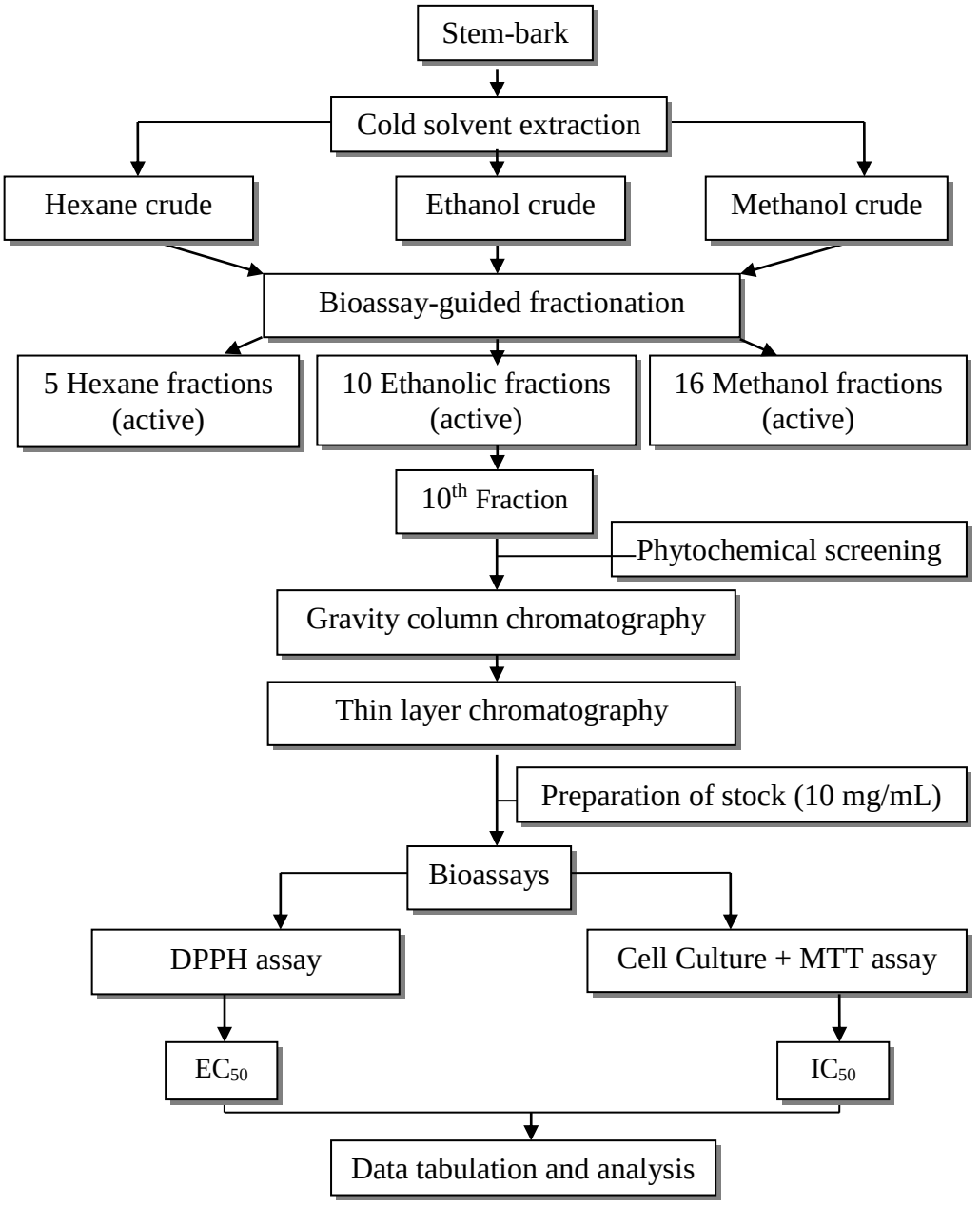

Figure 3.1: A brief summary of bioassay-guided fractionation of ethanolic stem-bark extract of *Garcinia maingayi*.

3.3 Phytochemical Screening

3.3.1 Test for Alkaloids

Five drops of Wagner's reagent that consist of 1.27 g of iodine and 2 g of potassium iodide in 100 mL in water was added in the test tube containing ethanolic fraction of *Garcinia maingayi*. Presence of alkaloids were indicated when a brownish-red precipitation was observed at the bottom of test tube (Mojab, et al., 2003).

3.3.2 Test for Flavonoids

In a test tube containing 2 mL of ethanolic fraction, five drops of 20% sodium hydroxide was added and within few seconds intense yellow coloration was formed. Few drops of diluted hydrochloric acid (0.1M) was then added and the yellow coloration changed colorless indicating the presence of flavonoids (Ugochukwu, Uche and Ifeanyi, 2013).

3.3.3 Test for Glycosides

Yellow precipitation was formed indicating presence of glycosides when five drops of 1% bromine water was added to a test tube containing 1 mL of the ethanolic fraction of *Garcinia maingayi* (Bhandary, et al., 2012).

3.3.4 Test for Phenols

In a test tube, five drops of aqueous 5% of ferric chloride was added to 2 mL of ethanolic fraction and a black color was observed showing the presence of phenols (Gowri and Vasantha, 2010).

3.3.5 Test for Quinones

A yellow precipitation was observed and indicating the presence of quinones upon the addition of five drops of concentrated hydrochloric acid (1M) into 1 mL of ethanolic fraction in a test tube (Ugochukwu, Uche and Ifeanyi, 2013).

3.3.6 Test for Saponins

Six millilitres of distilled water was mixed with 2 mL of ethanolic fraction in a test tube. The tube was closed tightly with rubber stopper and was shaken vigorously. Persistent formation of foam at the top layer of the test tube indicates the presence of saponins (Ayoola, et al., 2008).

3.3.7 Test for Tannins

In a test tube containing 1 mL of ethanolic fraction, five drops of 1% gelatin were added and a white precipitate was observed thus indicating the presence of tannins (Bhandary, et al., 2012).

3.3.8 Test for Terpenoids

Approximately 2 mL of ethanolic fraction was added into a test tube, followed by addition of 1 mL of chloroform and five drops of concentrated sulphuric acid (1M). Presence of terpenoids were confirmed when an immediate formation of reddish brown precipitate observed at the bottom of the tube (Edeoga, Okwu and Mbaebie, 2005).

3.4 Bioassay-guided Fractionation

3.4.1 Sample Preparation for Isolation

Approximately 10.0 g of ethanolic fraction of *Garcinia maingayi* was dissolved in 20 mL of chloroform. The fraction was sonicated and was then further added with 30.0 g of silica powder. The mixture were stirred thoroughly using mortar and pestle to obtain completely dried and fine powder-like form of sample.

3.4.2 Gravity Column Chromatography

A glass column sized 4 cm diameter and 100 cm height as well as with sintered layer at the bottom was packed with silica gel 60 powder using wet pack method. Prior to packing, the column was rinsed with hexane to get rid of any contaminants. Silica powder was mixed with hexane and added into the glass column about 11 to 12 cm of its height. Hexane was continuously added into the column to ensure tight packing and in certain interval, the column was tapped with a rubber hose to remove air bubbles which can be a hindrance

during elution. Approximately three spatulas of sodium sulfate anhydrous were added on the top layer of the packed column to absorb any water.

The powdered sample was loaded carefully on top of the packed silica gel in the glass column. Elution was performed with various organic solvents in an increasing polarity. Hexane with the lowest polarity was used first for elution, followed by ethyl acetate, methanol and finally ethanol. **Table 3.3** shows the ratio of volume of organic solvents used during the elution. A few 250 mL conical flasks were used to collect approximately 200 mL of eluent from the column and were concentrated using rotary evaporator. Collection and concentration of fractions were repeated until the eluents become colorless. The fractions were then dried in drying oven at 37 ℃. The dry weight of each fraction was obtained and recorded.

Table 3.3: Solvent combination used in gravity column chromatography.

Hexane (mL)	**Ethyl acetate (mL)**	**Methanol (mL)**	**Ethanol (mL)**	**Final volume (mL)**
200	200	-	-	400
100	300	-	-	400
-	400	-	-	400
-	300	100	-	400
-	200	200	-	400
-	-	-	400	400

3.4.3 Thin Layer Chromatography (TLC)

Fractions that were collected from gravity column chromatography were combined according to similar spots observed on TLC plate. The silica coated aluminium sheet of TLC was cut with width of 5.0 cm and length of 10.0 cm. 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 dried fractions were dissolved in chloroform and were spotted on the baseline of the plate using a thin capillary tube. The plate was placed in an enclosed chamber saturated with various types of organic solvents, commonly hexane, ethyl acetate and methanol. Once the spot reached the solvent frontline, the plate was removed.

The visible spots that were observed by the naked eyes were marked immediately using a pencil and the rest of the spots were visualized 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 (Kaur and Arora, 2009).

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

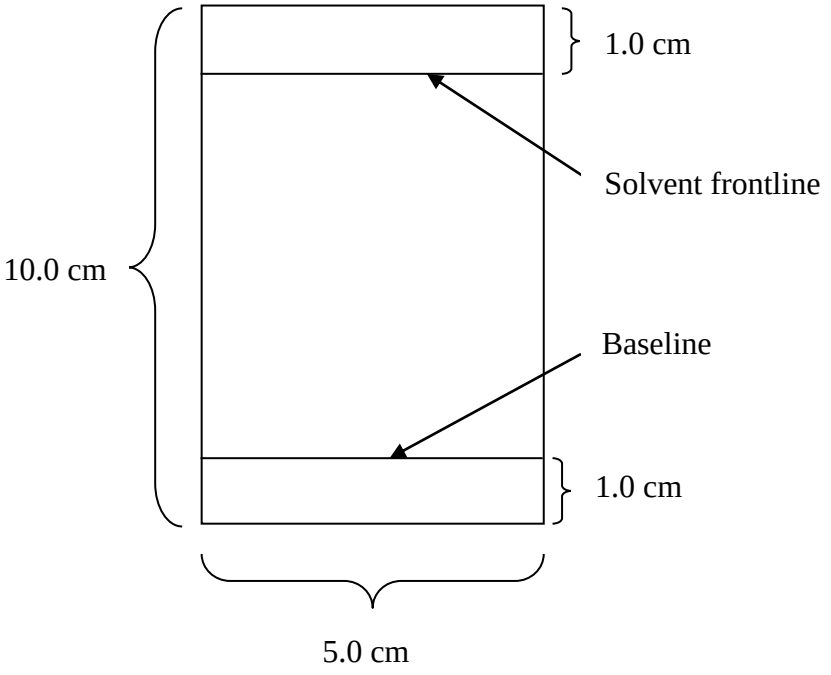

Figure 3.2: Design of thin layer chromatography (TLC) plate.

3.5 Preparation of Chemicals, Medium and Fractions

3.5.1 Stock and Working Solutions

In DPPH assay, stock solution for each fraction was prepared by adding 10 mg of respective fraction into 1 mL of methanol to obtained concentration of 10 mg/mL. The stock solutions were mixed thoroughly using vortex and sonicated further for complete solubilization. A volume of 50 µL of stock solution of each fraction was added into 96-well plate containing 50 µL of methanol and was serially diluted to obtain concentrations ranging from 0.039 to 5.000 mg/mL.

In MTT assay, stock solution for each fraction was prepared by adding 10 mg of fraction into 1 mL of 100% dimethyl sulfoxide (DMSO). The prepared stock solution was mixed thoroughly using vortex and sonicated. The stocks were further diluted by using basic medium to obtain 0.1 mg/mL. Working solutions were prepared at concentrations ranging from 2.0 to 10.0 µg/mL.

All the stock and working solutions for both DPPH and MTT assays were kept in refrigerator at 4 ℃.

3.5.2 Positive and Negative Controls

Ascorbic acid was used as positive control in DPPH assay. Stock solution of 10 mg/mL was prepared by adding 10 mg of ascorbic acid powder into 1 mL of methanol. The prepared stock solution was mixed thoroughly using vortex. A volume of 50 µL of stock solution of ascorbic acid was added into 96-well plate containing 50 µL of methanol and was serially diluted to obtain concentrations ranging from 0.039 to 5.000 mg/mL. Meanwhile, negative control was only methanol and was added lastly to the respective wells to prevent evaporation.

In MTT assay, vinblastine sulfate was used as positive control. The stock solution was prepared by adding 10 mg of powder into 1 mL of 100% DMSO to obtain concentration of 10 mg/mL. The prepared stock solutions were mixed thoroughly using vortex and was further diluted using basic medium to obtain 0.1 mg/mL. The working solutions for assay were prepared at concentrations

ranging from 2.0 to 10.0 µg/mL. Negative control used in MTT assay was DMSO (less than 1%).

All the positive and negative controls for both assays were kept at 4 ℃.

3.5.3 DPPH and MTT Reagents

DPPH reagent at 2 mg/mL was prepared by dissolving 20 mg of DPPH powder in a sample vial containing 10 mL of methanol. The mixture were incubated in dark for 30 minutes to allow complete solubilization. DPPH is a light sensitive reagent and thus the sample vial was wrapped with aluminium foil and sealed with parafilm. The reagent was stored in refrigerator at 4 ℃.

As for MTT reagent, 150 mg of MTT powder was dissolved in 30 mL of sterile autoclaved PBS to obtain concentration of 5 mg/mL. The solution was then filtered using cellulose filter membrane with diameter of 0.22 µm into a test tube. Since MTT reagent is light sensitive, the tube was wrapped with aluminium foil and sealed with parafilm. The reagent was then stored at 4 ℃ refrigerator.

3.5.4 Complete Medium

Complete DMEM medium was prepared by adding 50 mL of 10% heat-inactivated FBS into 450 mL of basic DMEM. The medium was sealed with parafilm and then stored in refrigerator at 4 ℃.

3.5.5 Phosphate Buffer Saline (PBS)

Five PBS tablets were dissolved in 500 mL of ultrapure water in a sterile Schott bottle. The PBS solution was autoclaved at 121℃ at 15 Ibs for 15 minutes. The solution was allowed to cool down at room temperature, sealed with parafilm and kept at 4℃ for further uses.

3.6 Culture and Subculture of Cell Lines

HeLa and MDA-MB-231 cancer cell lines were taken out from liquid nitrogen tank and were thawed immediately within one minute in water bath at 37℃. For each cancer cell line, approximately 1 mL of cells were pipetted into separate 25 cm^3 tissue culture flask that contain 4 mL of pre-warmed complete medium. The flasks were then observed under microscope and placed in humidified 5% CO_2 incubator at 37℃. Regular observation on the cultured cells using inverted phase contrast light microscope was performed to ensure no contamination.

Once the cells reached 80 to 90% confluency, subculture was performed. The old medium was discarded and the attached cells were rinsed twice with 5 mL of PBS. Approximately, 3 mL of 0.25% trypsin-EDTA solution was added into the flask and was then incubated for 5 to 10 minutes. To ensure complete detachment of cells, the flask was viewed under microscope and 4 mL of complete medium was added into the flask to stop trypsinzation.

The medium was then transferred into 15 mL centrifuge tube and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the pellet was re-suspended with 1 mL of complete medium and the mixture was then transferred into two new 25 cm^3 flasks that contain 4 mL of complete medium. All the flasks were labelled and placed in 5% CO_2 humidified incubator at 37 ℃.

3.7 Cell Counting

Prior to MTT assay, cancer cells were counted using hemocytometer where the cells were stained with 0.4% trypan blue dye. Approximately, 50 µL of cell suspension was mixed with 50 µL of the dye in an eppendorf tube. The mixture was then mixed aseptically and incubated at room temperature for two to three minutes in laminar flow.

Ten microliters of cells were pipetted onto hemocytometer through capillary action. The cells were then counted under 100x magnification using inverted light microscope and only the viable cells were counted using a cell counter. The average number of viable cells in the four main counting grids and the concentration of cells were calculated, respectively as shown in **Appendix A**.

3.8 Bioassays

3.8.1 DPPH Assay

Layout of 96-well plate (U-bottom) used in DPPH assay is as shown in **Figure 3.3**. Approximately, 50 µL of methanol was added into all the wells except for wells in columns 11 and 12. Ascorbic acid at concentration of 10 mg/mL was added into columns 1 and 2 whereas fractions were added into columns 3 to 10. These samples were serially diluted to obtain concentrations ranging from 0.039 to 5.000 mg/mL. In columns 11 and 12, 100 µL of methanol were added. Ten microliters of DPPH reagent (2 mg/mL) was added into all the wells and incubated in dark for 30 to 45 minutes at room temperature. Absorbance of each well was then measured using microplate reader at 517 nm.

The percentage of radical scavenging activity was calculated based on following formula (Basma, et al., 2011). The EC_{50} values were determined from the graph of percentage radical scavenging activity against concentration of fractions.

Percentage of radical scavenging activity = $(A_0 - A_1) \times 100\% / A_0$

Where,

A_0 refers to average absorbance of negative control

A_1 refers to average absorbance of fraction sample

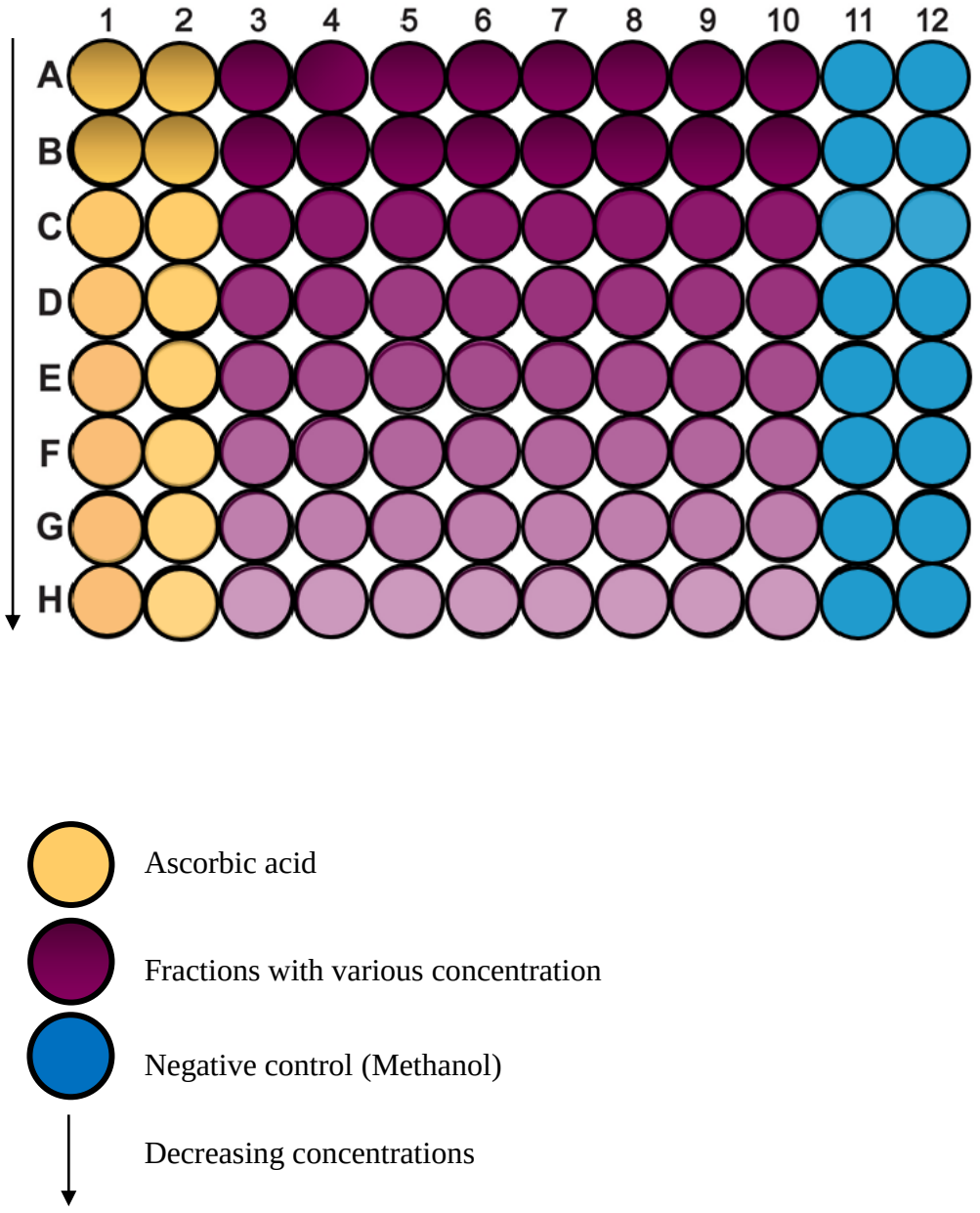

Figure 3.3: Layout of 96-well plate used in DPPH assay.

3.8.2 MTT Assay

A flat-bottom 96-well plate was used in MTT assay and 100 µL of cells were seeded into each well at concentrations ranging from 1 x 10^4 to 1 x 10^5 cells per well. The plate was then incubated for 24 hours in humidified 5% CO_2 incubator at 37 ℃.

After incubation, the cells were observed under an inverted microscope to ensure for its attachment. A volume of 50 µL at various concentration of each fraction and vinblastine sulfate were added into rows A to E and row G, while negative control and sterility controls were added into rows F and H, respectively. **Figure 3.4** shows the design used in MTT assay.

After 72 hours incubation, 20 µL of MTT solution was added into all the wells. The plate was then wrapped with aluminium foil and further incubated at 37 ℃ for four hours. Approximately, 70 µL of the samples in each well was removed and 100 µL of 100% DMSO was added to dissolve the purple formazon crystals. Absorbance of each well was then measured at 570 nm using a microplate reader.

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

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

Where,

A_{Sample} refers to the average absorbance of the various fractions or vinblastine sulfate

A_{NC} refers to average absorbance of negative control

3.9 Data Analysis

DPPH and MTT assays were repeated four and five times, respectively. The data obtained was expressed as mean ± standard deviation which was then tabulated and analyzed using Microsoft Office Excel 2013.

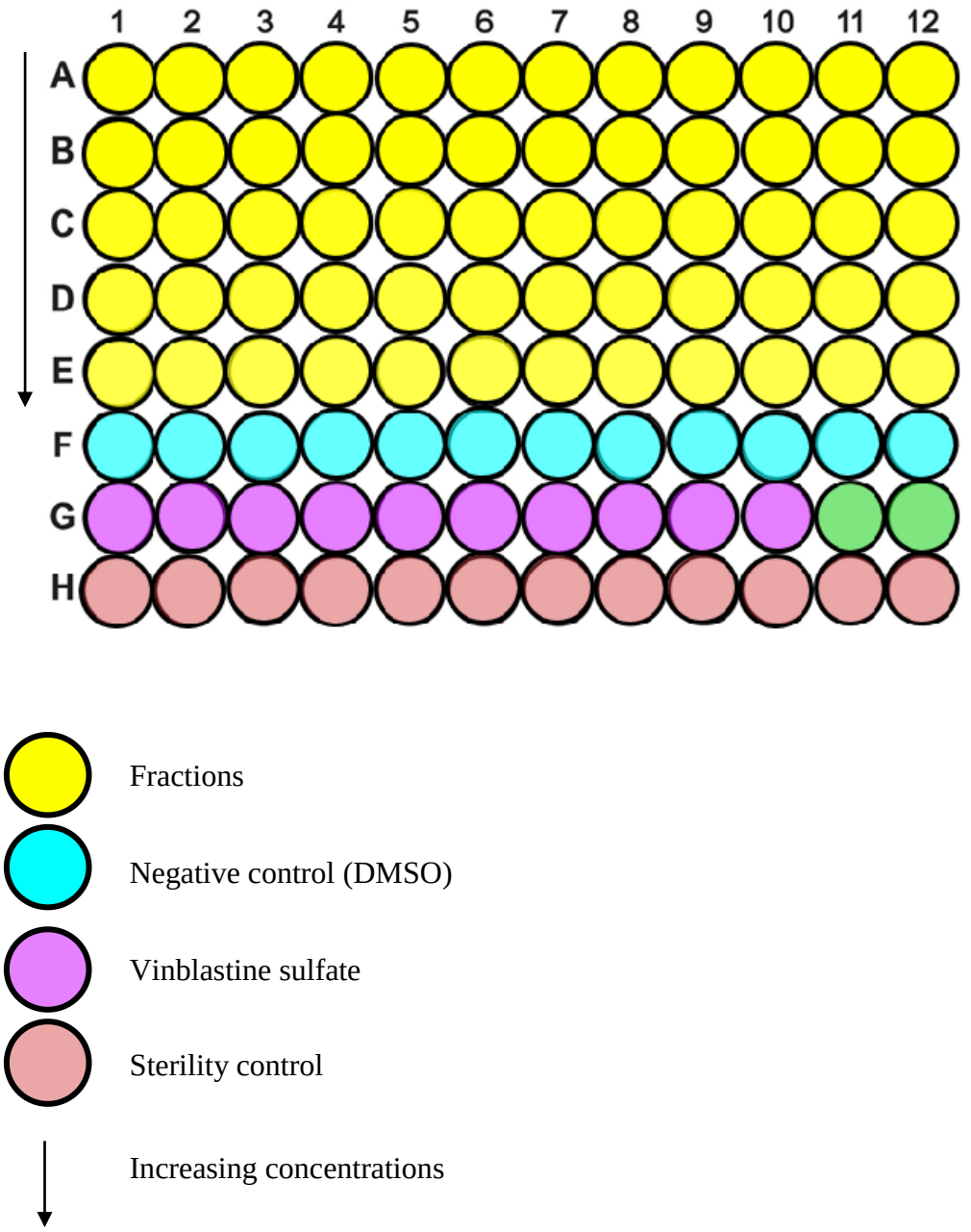

Figure 3.4: Layout of 96-well plate used in MTT assay.

CHAPTER 5

DISCUSSION

5.1 Percentage Yield of Fractions

Extraction yield is defined as the amount of extract recovered in mass as compared with the initial amount of whole plant(Murugan and Parimelazhagan, 2014). The yields obtained indicating the solvent's efficiency to isolate certain components from the original material (Murugan and Parimelazhagan, 2014). Extraction of plant is vital in isolation of bioactive components from medicinal plants. In order to increase the yield of extraction, stem-barks of *Garcinia maingayi* were grounded into small particles, and it also eventually increases the surface area allowing good mixing with the solvent (Sarker, Latif and Gray, 2006; Azmir, et al., 2013). At the end of isolation, the yield of bioactive compounds were obtained in higher amount that is vital in natural product research (Murugan and Parimelazhagan, 2014).

Different solvents were used during extraction of targeted compounds due to the formation of non-covalent bonds with functional groups of secondary metabolite and thus solubilizing and extracting out these molecules. For an example, polar solvents such as methanol or ethanol was used to isolate hydrophilic compounds, whereas non-polar solvent like hexane can be used for extraction of hydrophobic compounds (Sasidharan, et al., 2011). In this research, fraction 6 showed the highest extraction yield and was isolated using ethanol only indicating *G. maingayi* fraction contain mostly polar secondary

metabolites. Hence, based on phytochemical analysis, *G. maingayi* fraction may contain alkaloids, saponins, terpenoids, tannins and glycosides which are secondary metabolites with high polarity (Tara, et al., 2010). This is because ethanol is a polar solvent due to the presence of hydroxyl group that has high electronegative oxygen atom which eventually allows hydrogen bonding with other molecules thus attracting polar compounds (Schiller, 2010).

5.2 Bioassay-guided Fractionation

5.2.1 Thin Layer Chromatography (TLC)

Various polarity solvents were optimized for mobile phase such as hexane, ethyl acetate, methanol and ethanol. Hexane usually acts as the diluent of non-polar constituents, whereas the other organic solvents are involve in selectivity of bioactive compound (Sherma and Fried, 2013). Aluminium plate coated with silica gel acted as the stationary phase.

Polar compounds have stronger affinity towards the stationary phase, thus compounds will move slower and giving smaller R_f values. On the other hand, non-polar compounds will move faster compared to polar compounds resulting in wider R_f values. Based on **Table 4.3**, fraction 3 showed the widest range of R_f values and was isolated using mixture of hexane and ethyl acetate. Hexane isolates non-polar compounds such as saponins, whereas ethyl acetate isolates intermediate polar compound such as flavonoids. Thus this combination produced the widest range of R_f due to the respective affinity towards the stationary phase. On the other hand, fraction 6 gave the narrowest range of R_f

value and was isolated with only ethanol. Ethanol is a polar solvent that isolates only polar compounds such as alkaloids and glycosides, thus these polar compounds moved slowly resulting in smallest R_f values.

5.2.2 Gravity Column Chromatography

In this research, the solvent extraction of plant material relies on 'solid-liquid' extraction, in which the fractions were placed in contact with the solvent. The solvent will then diffuse into the plant cells, eventually solubilizing the metabolites and finally extracting out of the plant cells (Sarker, Latif and Gray, 2006).

In gravity column chromatography, silica gel 60 powder was used to pack the column and acted as the stationary phase. Meanwhile, various type of organic solvents were used as the mobile phase. Silica gel powder was used because it is made up of spherical particle that allows for an optimal resolution efficiency resulting in better separation of secondary metabolites (Sigma-Aldrich, 2016). Silica gel powder acts as the polar adsorbent in gravity column chromatography will interact and bind to the polar compounds by forming intermolecular hydrogen bond with its available hydroxyl group. Thus, bioactive compounds in fractions with different functional group eventually causing variation in the strength of intermolecular forces, so these compounds are eluted at different elution rates (Amrita.edu, 2011).

A gradual gradient method was applied in the isolation of compounds, where fractions were eluted out by using various solvent system with different polarity. Therefore, chromatography was initiated by using hexane, a non-polar solvent and followed by solvents with increasing polarity, namely ethyl acetate, methanol and ethanol. This is because if polar solvent is used at first it might cause elution of all the compounds at once without separation. Besides, it may cause cracking of the packed column that eventually causes inefficient separation of compounds (Columbia University, 2007). Thus, a gradual gradient method will ideally elute the compounds with different polarity one at a time. Hexane eluted compounds that are least polar and weakly adsorbed to the polar stationary phase. So, these compounds will have the shortest retention time compared to polar compounds (Betacourt and Gottlieb, 2014). While, polar compounds were eluted lastly throughout the chromatography process because these compounds are retained by the polar stationary phase (Swambe Chemicals, 2015).

Prior to elution various colored bands were observed travelling down the column and the elution was only completed when the eluent becomes colorless. The collected fractions were then concentrated by using rotary evaporator under reduced pressure ensuring the stability of the compounds collected (Lee, et al., 2003; Organic Chemistry, 2016). Polar secondary metabolites such as alkaloids, flavonoids and tannins appeared as dark spots against a bright green background at short wavelength UV light (254 nm). The non-polar compounds such as carotenes and chlorophylls appeared as yellow spots (Sarker, Latif and Gray, 2006).

5.3 Bioassays

5.3.1 DPPH Assay

Reactive oxygen species (ROS) are by products of cellular metabolism especially during physiological and biochemical processes (Cai, et al., 2004). Superoxide radical ($O_2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$) and peroxyl radical ($ROO^{\cdot}$) are types of ROS produced during normal metabolic processes (Wong, et al., 2006). Balance of anti-oxidation and oxidation processes are important in maintaining a healthy biological system (Katalinic, et al., 2006). Antioxidants are used to prevent oxidative reactions.

In general, there are commercial synthetic antioxidants such as, butylatedhydroxyanisole and butylatedhydroxytoluene. However, these synthetic antioxidants are termed as 'generally recognized as safe' because they have the potential to be carcinogenic (Cheung, Cheung and Ooi, 2003). According to Matkowski (2008), secondary metabolites of plants do possess antioxidant activity with good therapeutic performance and low toxicity.

Based on **Table 4.4**, fraction 2 exhibited the highest radical scavenging activity similar to ascorbic acid, thus proving to be equivalent to ascorbic acid activity. Furthermore, all the fractions exhibited higher radical scavenging activity. These fractions were isolated using combination of hexane, ethyl acetate, methanol and ethanol. So, non-polar, intermediates polar and polar compounds such as alkaloids, flavonoids, glycosides, phenols, quinones, tannins and terpenoids are present and responsible for the antioxidant activity. Besides, presence of these secondary metabolites have been revealed in phytochemical

screening of ethanolic fraction of stem-barks of *Garcinia maingayi* as shown in **Table 4.1**.

According to the results, concentration-dependent manner activity were shown by these fractions, in which the lowest concentration exhibited lowest radical scavenging activity, whereas higher percentage of radical scavenging activity was exhibited at highest concentration. This may due to the different amount of bioactive compounds present at various concentration. At lowest concentration, the bioactive compounds may be diluted. Presence of high amount of the compounds at higher concentrations contributed to the highest radical scavenging activity (Lehàr, et al., 2009).

However, a slight decrease in the scavenging activity was observed in fractions 4, 5 and 6. One of the possible reason is due to the high absorbance value which was caused by the high intensity colors of the fractions itself. This can be proved by the Beer-Lambert's law in which it shows linear relationship between absorbance and concentration of absorbing species. Thus, the higher the amount of molecules in the sample, the higher the absorbance (Clark, 2007). Dark-brownish fractions 4, 5 and 6 were isolated using solvent combination of ethyl acetate, methanol and ethanol, thus these fractions consists of huge amount of both intermediate and polar compounds, such as alkaloids, flavonoids, saponins and terpenoids. Besides, it could be due to antagonistic relationship among alkaloids and saponins in fractions (Milugo, et al., 2013). Antagonism refers to the interaction between two or more drug

compounds that have opposite effects thus reducing the effectiveness of the drug (AIDSinfo, 2016).

As shown in **Table 4.4**, ascorbic acid exhibited the highest percentage of radical scavenging activity as compared to fractions. Ascorbic acid or also known as vitamin C is an odorless white to pale yellow crystalline powder and soluble in water, thus acting as a good candidate of antioxidant agent in human (National Center for Biotechnology Information [NCBI], 2016c). Ascorbic acid helps to prevent cell damages that are caused by ROS by acting as reducing agent, free radical scavenger as well as a detoxifying agent in humans (National Cancer Institute Thesaurus, 2016). Furthermore, since ascorbic acid is a pure compound, therefore it possesses the highest scavenging activity as compared to fractions. Pure compound has only one substance without any other mixtures of element, thus absences of any other impurities couldn't start other reactions that might interfere with the reaction that is being studies (Davies, Austin and Partridge, 1991; Crawford, 2003). Ascorbic acid interacts with plasma membrane by donating electrons to the α-tocopheroxyl radical, and thus converts into ascorbate radical (May, 1999). Ascorbate radical in turn directly neutralize hydroxyl, alkoxyl and lipid peroxyl radicals, thus forming water, alcohol and lipid hydroperoxides, respectively as shown in **Figure 5.1** (Lu, Lin and Chen, 2010).

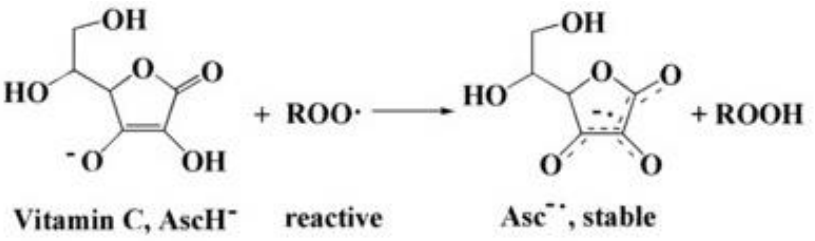

Figure 5.1: Ascorbate radical neutralize the reactive radical (Lu, Lin and Chen, 2010).

Effective concentration (EC_{50}) is a concentration of a compound that is required to obtain a 50% of free radical scavenging activity (Chen, Bertin and Froldi, 2013). EC_{50} values of compound is inversely proportional to the percentage of scavenging activity, in which compound that exhibit higher percentage of scavenging activity shows lower EC_{50}. However, in this research, the EC_{50} values for ascorbic acid and fractions were the same. This is because, all the fractions exhibited equivalent scavenging activity when compared with ascorbic acid due to high presences of bioactive compounds. Hence, fractions isolated from *Garcinia maingayi* possesses good and strong antioxidant activity similar with the antioxidant activity of ascorbic acid.

There is strong correlation between antioxidant activity and cytotoxic effect of *Garcinia maingayi* fraction. High level of ROS in human body promotes the development as well as progression of tumor (Liou and Storz, 2010). This is because ROS causes oxidative damage to host cellular DNA leading to gene mutation causing activation of oncogenes that may be the initiation step of carcinogenesis (Waris and Ahsan, 2006). Therefore, compounds with good

antioxidant property, will be able to exert anti-proliferative effect towards cancer cells. Hence, all the fractions isolated from *Garcinia maingayi* that exhibited strong antioxidant activity with low EC_{50} values showed higher cytotoxic effect.

5.3.2 MTT Assay

Cancer may arises from oxidative stress due to ROS or any DNA damage. Hence, in order to prevent the onset or progression of cancer without affecting the normal healthy cells, flavonoids and polyphenolic compounds from plant extracts plays an important role as they possess biological effect at nontoxic concentration in humans (Ren, et al., 2003). Furthermore, phenolic compounds such as flavonoids and xanthones have been thoroughly studied for their anti-cancer, anti-angiogenic and pro-apoptotic effects (Carocho and Ferreira, 2013).

Based on comparison of cytotoxic effect of fractions against HeLa and MDA-MB-231 cells, all the six fractions of ethanolic stem-bark extract of *Garcinia maingayi* exhibited the highest cytotoxicity against MDA-MB-231 cells. Presence of high amount of flavonoids could be the possible reason, as studies have shown mixture of flavonoids inhibited metastasis by inhibiting mRNA expression of GDP dissociation inhibitor (D4-GDI) and cell division cycle 42 (Cdc42) (Jiang, et al., 2015). Besides, MDA-MB-231 cells have spindle epithelial-like shape which causes them to undergo down-regulation of the tight junction proteins such as, occludin, claudin-1, claudin-5, JAM-1 and JAM-2, thus making them more permeable to macromolecules (Martin, et al.,

2013). Hence, permeability of MDA-MB-231 cells allows the entry of bioactive secondary metabolite to exert their cytotoxicity. Apart from this, presence of large amount of bioactive compounds in concentration dependent manner, may target various biosynthetic pathways thus suppressing cell adhesion as well as migration (Jiang, et al., 2015). Inhibitory effect of bioactive compounds on cell adhesion protein can be related to the PI3K signaling pathway, subsequently reducing adhesiveness of cancer cells (Jiang, et al., 2015).

HeLa cells showed slightly lesser cytotoxicity as compared to MDA-MB-231 cells against the fractions of *Garcinia maingayi*. This incidence can be due to the presence of 76 to 80 heavily mutated chromosomes instead of normal 46 chromosomes. This deviation of chromosome abnormality is caused by the DNA incorporation of human papilloma virus (HPV) into the host genome causing itself to grow unusually fast as compared to other cancer cells (Carpio, 2014).

In this research, all the cancer cell lines were incubated for 72 hours of treatment. Viewing from toxicology perspective, this incubation period was selected because permeability of membrane played a vital role in ensuring the efficacy of drug in absorption, metabolism, distribution and elimination (Katzung and Trevor, 2015). Diffusion rate of molecules depends on its size as well as its strength of hydrophobicity. Hence, phospholipid bilayer is way much less permeable to polar compounds whereas hydrophobic compounds readily across the bilayer (Fulton, 2016). This statements correlates with the

research, in which bioactive compounds comes with large functional group such as, aromatic rings, carboxyl groups and hydroxyl groups that requires longer time period to penetrate into the cells.

In HeLa cells, lowest cell viability was shown by fraction 6 that was solely extracted using ethanol. Ethanol isolates polar secondary metabolites, such as xanthones as shown in **Table 4.6**. Xanthones are proven as natural occurring anti-cancer agent in higher plant family, namely in Guttiferae (Carocho and Ferreira, 2013). In MDA-MB-231 cells, the lowest cell viability was exhibited by fraction 3 extracted using solvent combination of hexane and ethyl acetate as shown in **Table 4.8**. Possible reasoning could be that xanthones derived from Garcinia species may down regulate the expression of Mcl-1 protein causing changes in mitochondrial membrane eventually induces apoptosis in cancer cells (Wang, Sanderson and Zhang, 2011; Su, et al., 2011).

However, the cytotoxic effect exerted also could be possibly due to the presence of semi-polar compounds such as flavonoids that could be isolated in ethyl acetate. Based on previous studies, flavonoids blocks nuclear factor-kappa B eventually inhibit the activation of matrix metalloproteinases and vascular endothelial growth factor (VEGF) expression. Down regulated expression of VEGF in cancer cells causes inhibition of angiogenesis of the cells thus inhibiting the progression of tumor (Batra and Sharma, 2013). Besides, flavonoids also able to inhibit ornithine decarboxylase and thus decreasing the amount of polyamine biosynthesis. Eventually, DNA and

protein synthesis of cancer cells would be inhibited too due to the inhibition of polyamine biosynthesis (Katyal, Bhardwaj and Khajuria, 2014).

All the fractions showed approximately equivalent and higher percentage of cell viability showed IC_{50} values less than 10 µg/mL against HeLa and MDA-MB-231 cells, proving strong activity of ethanolic stem-bark fraction of *Garcinia maingayi* (Jabit, et al., 2009).

Vinblastine sulfate was used as a positive control in MTT assay. Vinblastine sulfate is classified as a 'plant alkaloid' with anti-neoplastic activity (Chemocare, 2002). This chemotherapy drug is used to treat breast, testicular cancers, kaposi sarcoma and Hodgkin and non-Hodgkin lymphoma (NCI, 2011). Cell growth of cancer cells are arrested at metaphase when vinblastine sulfate binds to the micro-tubular protein of mitotic spindle eventually preventing polymerization of spindle fibers (Hospital UK, 2016).

5.4 Limitations of Study

Slurry method was used during the research, whereby the homogenous mixture of solid stationary phase and non-polar solvent were added into the column. Even though, this method gives the best separation, however, packing the column with homogenous mixture is a difficult technique to master since it can cause the formation air bubbles and cracks in the column leading to poor separation.

During the preparation of stock solution for both the bioassays, the isolated fractions were unable to fully dissolve in 100% DMSO. Thus, prepared samples were sonicated longer to ensure the samples are completely dissolved. Hence, longer time was required to complete the sonication process.

Improper pipetting techniques as well as uneven cell seeding into the 96-well plates gave rise to fluctuation and inconsistency of absorbance readings for both assays. Hence, to obtain a reliable and significant result, DPPH and MTT assays were repeated for four and five times, respectively.

5.5 Future Studies

The isolated compounds can be further purified and analyzed using high performance liquid chromatography (HPLC) and gas chromatography linked with mass spectrophotometry (GC-MS). Besides, the structure of the isolated bioactive compounds should be elucidated using nuclear magnetic resonance (NMR).

In this research, only two cancer cell lines were used for the assessment of cytotoxic effect. Hence, in future, ethanolic fraction of *Garcinia maingayi* can be tested on other cell lines, preferably both adherent and suspension cell lines. Normal cell lines also should be evaluated to ensure isolated compounds cause no or minimal side effects on normal healthy cells besides killing the cancer cells.

There are various bioassays available to evaluate antioxidant activity isolated compounds, which can be divided into hydrogen atom transfer reaction assays and electron transfer reaction assays. Assays such as oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant potential (TRAP) and β-carotene bleaching assay, Trolox equivalent antioxidant capacity (TEAC), total phenolic assay and hydroxyl radical scavenging can be used (Katzung and Trevor, 2015). Besides, to evaluate the 'basal' cytotoxicity activity of the tested compounds towards cancer cells, few other *in vitro* assays can be conducted, such as ATP assay, LDH leakage assay, neutral red assay as well as DNA fragmentation assay.

In future the *in vitro* studies of ethanolic fraction of stem-bark on anti-bacterial, anti-malarial, anti-viral activities are needed to be conducted as reported in a number of literature review. Besides, to evaluate the efficacy and validate the *in vitro* findings, studies using animal models such as mice and other rodents should be carried out. Analysis on mechanism of action, dosage efficacy, therapeutic index and toxicity of isolated active compounds can be done as well as provide the researcher a better and promising evidences on the activities posed by the plant.

CHAPTER 6

CONCLUSIONS

Phytochemical screening of ethanolic fraction of *Garcinia maingayi* showed the presence of alkaloids, flavonoids, glycosides, phenols, quinones, saponins, tannins and terpenoids. Bioassay-guided fractionation has isolated six major fractions using gravity column chromatography and thin layer chromatography. The highest yield of 22.7% was obtained by fraction 6, whereas lowest yield of 2.5% was produced by fraction 4. In DPPH assay, fraction 2 and ascorbic acid exhibited higher scavenging activity of 94.3 and 94.6%, respectively in which the EC_{50} values for both were of 0.02 mg/mL. However, all the fractions also exhibited higher radical scavenging activity and hence these fractions were then further tested on their cytotoxicity effect against HeLa and MDA-MB-231 cell lines.

In MTT assay, fraction 6 showed the highest cytotoxic effect against HeLa cells with IC_{50} value of 1.27 µg/mL, whereas vinblastine sulfate showed IC_{50} value of 1.20 µg/mL. Meanwhile, fraction 3 showed the highest cytotoxic effect against MDA-MB-231 cells with similar IC_{50} value of 1.33 µg/mL as compared to vinblastine sulfate. However, good cytotoxicity activity were exhibited by all the fractions against HeLa and MDA-MB-231 cells. The cytotoxic and anti-oxidant results showed strong activity and thus can be further analyzed for its bioactivity.

As a conclusion, compounds isolated from stem-bark of *Garcinia maingayi* possess potential anti-oxidant and anti-cancer agents. However, further purification and isolation are required to obtain pure bioactive compounds as therapeutic agents in drug development.

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APPENDIX A

Cell Counting

Concentration of cell suspension = A x B x 10^4 (Grigoryev, 2014).

Where,

A refers to average number of viable cells in four different grids

B refers to dilution factor of trypan blue dye

10^4 refers to volume of chamber = 1 x 1 x 0.1 mm = 0.1 mm^3 = 10^4

Percentage of Yield

The percentage of yield was calculated using the formula as shown below (Ahmad, et al., 2009).

Percentage yield of fraction = (W_1 / W_2) x 100%

Where,

W_1 refers to the dry weight of fraction in gram

W_2 refers to the weight of ethanolic extract in gram

APPENDIX B

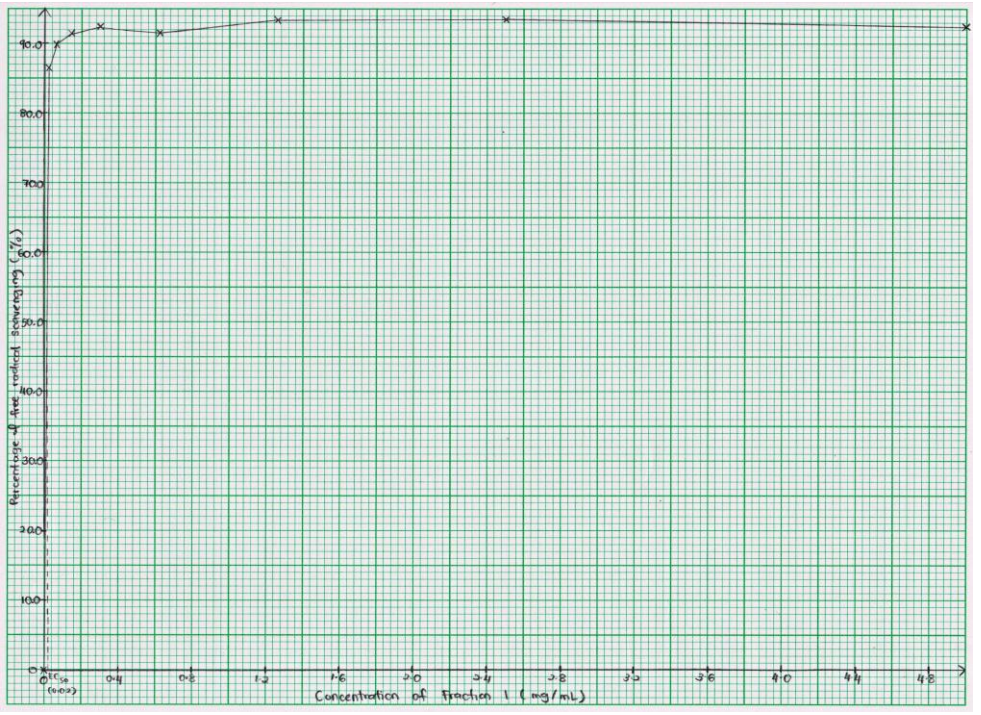

Figure A: The percentage of radical scavenging activity against various concentration of fraction 1.

APPENDIX C

Figure B: The percentage viability of HeLa cells against various concentration of fraction 1.