MECHANISMS OF ANTI-PROLIFERTATIVE EFFECT OF *GARCINIA HOMBRONIANA* ESSENTIAL OILS LEAVES IN MCF-7 AND MCF-7/TAMR-1 HUMAN BREAST CANCER CELL LINES

BY

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DECLARATION

I hereby declare that this research was sent to universiti sains malaysia (USM) for the degree of Master of Science in Health Toxicology. It has not been sent to other Universities. With that, this research can be used for the consultation and can be photocopied as reference.

Sincerely,

ALAA TAHA YASIR AL KANAN

(P-IPM0060/18)

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ATCC	American type culture collection
ATP	Adenosine triphosphate
BARD1	Breast cancer Associated RING Domain 1
BRCA1	Breast-Cancer Susceptibility Gene 1
DBTRG	Human glioblastoma cell line
DMEM/F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
EDTA	Ethylenediaminetetraacetic acid
EO	Essential oils
EO-L	Essential oils leave
ER	Estrogen receptor
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GH	Garcinia hombroniana
GH-EO-L	Garcinia hombroniana essential oil leaves
hEGF	human Epidermal Growth Factor
HER2	human epidermal growth factor receptor 2
IC50	Inhibitory concentration at 50%
LDL	Low – density lipoprotein
MCF-10A	Human mammary epithelial breast cell line

MCF-7	A designated nomenclature for a human breast adenocarcinoma cell line
MCF-7/TAMR-1	A designated nomenclature for a human breast adenocarcinoma cell line resistant to tamoxifen
MTT	Microtiter tetrazolium
^{o}C	Celsius
OD	Optical density
PBS	Phosphate buffered saline
Pen-Strep	Penicillin Streptomycin
PI	Propidium iodide
PR	Progesterone receptor
PS	Phosphatidylserine
RNS	Reactive nitrogen species
ROS	reactive oxygen species
RPMI-1460	Roswell Park Memorial Institute 1640 Medium
SD	Standard deviation
SPSS	Statistical Package for the Social Sciences

ABSTRAK

Latar Belakang: Kanser payudara adalah merupakan kanser yang paling biasa menyerangi kaum wanita dan kadar insiden semakin meningkat setiap tahun di seluruh dunia. Tamoksifen ialah ubat kemoterapeutik utama digunakan dalam merawat pesakit kanser payudara. Walaubagaimanapun, komplikasi ginekologi dan rintangan terhadap ubat merupakan antara kesan buruk yang utama terutamanya selepas 10-15 tahun rawatan dengan tamoksifen. Kemudian, kemungkinan berlakunya perulangan kanser payudara yang juga boleh menyumbang kepada penyebab utama kepada kematian yang berpunca daripada kanser tersebut. *Garcinia hombroniana* (GH) (manggis hutan) ialah sejenis pokok yang telah dilaporkan mempunyai kesan sitotoksik yang baik dalam melawan pertumbuhan pelbagai jenis sel titisan kanser, termasuklah MCF-7. Oleh sebab itu, tujuan kajian ini adalah untuk melanjutkan penyiasatan kesan-kesan anti-proliferasi dan apoptosis minyak pati yang telah diekstrak daripada daun GH terhadap dua jenis sel titisan kanser payudara yang berbeza, iaitu MCF-7 dan MCF-7/TAMR-1.

Kaedah: Minyak pati telah diekstrak daripada daun GH melalui proses penghidrosulingan. Kesan anti-proliferasi minyak pati terhadap sel-sel titisan MCF-7 dan MCF-7/TAMR-1 ditentukan menggunakan asai MTT dan dinilai melalui pembaca plat mikro. Mekanisme kematian sel ditentukan menggunakan asai pewarna Aneksin V-FITC/propidium iodida dan diukur secara kuantitatif melalui aliran sitometri. Sel titisan payudara bukan kanser iaitu MCF-10A juga dinilai dalam kedua-dua asai sebagai sel kawalan untuk dibandingkan kepada sel MCF-7 dan MCF-7/TAMR-1.

Keputusan: Keputusan telah menunjukkan bahawa minyak pati GH mempamerkan kesan anti-proliferasi melawan pertumbuhan kedua-dua jenis sel MCF-7 dan MCF-7/TAMR-1 yang bersandarkan kepada dos dan masa, dengan nilai IC_{50} masing-masing 35.22 µg/mL and 17.67 µg/mL. Utamanya, minyak pati GH menunjukkan kesan toksik yang rendah terhadap sel titisan payudara bukan kanser, MCF-10A, dengan nilai IC_{50} 76.11 µg/mL. Tambahan pula, analisis aliran sitometri juga selanjutnya mengesahkan kematian yang diaruh oleh minyak pati GH berlaku melalui mekanisme apoptosis.

Kesimpulan: Kajian ini menyimpulkan bahawa minyak pati GH menunjukkan kesan anti-proliferasi yang kuat terhadap kedua-dua sel kanser payudara manusia iaitu MCF-7 dan MCF-7/TAMR-1. Berdasarkan nilai IC_{50}, MCF-7/TAMR-1 yang mewakili sel titisan payudara yang berfenotip rintangan terhadap tamoksifen, adalah lebih sensitif terhadap rawatan minyak pati GH. Paling utama, minyak pati GH menunjukkan aktiviti sitotoksik yang rendah terhadap sel titisan payudara bukan kanser, MCF-10A. Keputusan-keputusan ini dapat menjelaskan sebahagian kepada tindakan minyak pati yang bersifat selektif terhadap sel-sel titisan kanser payudara tetapi tidak kepada sel normal. Di samping kesan sitotoksik yang selektif, tindakan perencatan minyak pati GH dalam melawan pertumbuhan sel-sel kanser payudara manusia MCF-7 dan MCF-7/TAMR-1 juga diperantarakan melalui apoptosis. Oleh itu, minyak pati GH sangat berpotensi untuk dimajukan sebagai agen anti-kanser yang baru, selektif dan kuat di masa hadapan.

ABSTRACT

Background: Breast cancer is the most common cancer affected women and the incidence rate is increasing yearly throughout the world. Tamoxifen is the first-line chemotherapeutic drug used in treating breast cancer patients. However, gynaecological complications and drug resistance are among the major drawback effects of tamoxifen, particularly at 10-15 years of post-treatment. Thereafter, breast cancer recurrence may occur which also contribute to the major causes of breast cancer-related deaths. *Garcinia hombroniana* (GH) (seashore mangosteen) is a plant that has been reported to possess good cytotoxic effect against the growth of various human cancer cell lines, including the MCF-7. For this reason, the aim of this study was to further investigate the anti-proliferative and apoptotic effects of the essential oil extracted from the leaves of GH against two different types of breast cancer cell lines, MCF-7 and MCF-7/TAMR-1.

Methods: The essential oil was extracted by hydrodistillation process from the leaves of GH. The anti-proliferative effects of the essential oil against MCF-7 and MCF-7/TAMR-1 cancer cell lines were determined using MTT assay and measured by a microplate reader. The mechanism of cell death was determined using Annexin V-FITC/propidium iodide staining assay and quantitatively measured by flow cytometry. The human non-cancerous breast cell line, MCF-10A was also included in both assays as comparative control cells to the MCF-7 and MCF-7/TAMR-1 cells.

Results: The results showed that the GH essential oil exhibited anti-proliferative effect against the growth of both MCF-7 and MCF-7/TAMR-1 cells following dose- and time-dependent manners, with an IC_{50} of 35.22 µg/mL and 17.67 µg/mL, respectively.

Importantly, it exhibited low toxicity effect against the non-cancerous human breast cell line, MCF-10A, with an IC_{50} of 76.11 µg/mL. Additionally, flow cytometric analysis also further confirmed that the cell death induced by GH essential oil occurred via the mechanism of apoptosis.

Conclusions: This study concluded that the essential oil of GH exhibited potent antiproliferative effects against both MCF-7 and MCF-7/TAMR-1 human breast cancer cells. Based on the IC_{50} values, the MCF-7/TAMR-1, which represents tamoxifen-resistant phenotype breast cancer cell line, was more sensitive towards the GH essential oil treatment. Importantly, GH essential oil demonstrated low cytotoxicity towards the non-cancerous breast cell line, MCF-10A. These findings may at least in part explain to the selectivity of GH essential oil in killing breast cancer cell lines but not in normal counterpart. Besides its selective cytotoxic effect, the growth inhibitory action of GH essential oil against MCF-7 and MCF-7/TAMR-1 human breast cancer cells also mediated by apoptosis. Therefore, GH essential oil could be developed as a new, selective and potent anticancer agent in future.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Cancer is defined as a group of disease that is characterized by abnormal cell growth which tends to proliferate in an uncontrollable way and metastasize to distant sites of the body (National Cancer Institute, 2015). GLOBOCAN database indicated that the worldwide cancer morbidity has increased to more than 18 million cases and mortality has increased to more than 9.5 million cases in 2018 (Bray et al., 2018). Breast cancer is now leading cause of cancer incidence among females and it is the second most common cause of death after lung tumour in both males and females (GLOBOCAN, 2018). All over the world, both in developed and developing nations, breast cancer is becoming a major health problem among females. In South-Eastern countries, Malaysian women have the largest mortality rate of breast cancer (Nordin et al., 2018). Although chemotherapies are commonly prescribed for the control of breast cancer, the drugs can also harm normal cells, causing significant adverse effects. Consequently, cancer patients are increasingly seeking out for alternative and complementary medicins such as herbs and medicinal plants (Gomez et al., 2016).

Breast cancer is a disease which has diverse etiologies classified by histological, molecular and phenotypes. Estrogen receptor-positive (ER+) breast cancer is the most frequent reported breast cancer subtypes which depend on the estrogen to support the growth and scattered off the cancer cells (Hon et al., 2016). Tamoxifen and aromatase

inhibitors, are examples of the most effective endocrine therapies, to help delay or prevent the growth of ER+ breast cancer by blocking the actions of estrogen. However, resistance to these agents has become a crucial clinical challenge in the management of ER+ breast cancer. The MCF-7/TAMR-1 breast cancer cell line provides a model cell system for studying tamoxifen resistance (Viedma-RodriGuez et al., 2014).

Medicinal plants have been widely used and explored for their good potential as new anticancer agents. Moreover, these plants may exhibit a lesser or minimal level of adverse effects on healthy cells when compared with synthetic drugs (Roy et al., 2017). Species of genus *Garcinia* could affect the development and progression of breast cancer. Specifically, *Garcinia* species has been demonstrated to attenuate breast cancer progression through its anti-proliferative, anti-metastasic of breast tumour cells, induction of apoptosis and synergistic activity with chemotherapeutic drugs (Li et al., 2017). *Garcinia hombroniana* (GH), known as "seashore mangosteen" in Malaysia, is commonly used in traditional Malay medicine to treat different disorders such as abdominal pain and gonorrhoea, however little is known about its toxicological properties (Dyary et al., 2016). Therefore, the use of GH could be a practical attempt as a potential agent in preventing and treating breast cancer.

1.2 Problem statement

Breast cancer is the most common malignancy in women around the world. Globally, it is estimated that the ratio of breast cancer is that might occur among women one in four (Bray et al., 2018). Tamoxifen, which is a selective ER regulator, has contributed to the decline in mortality rate among patients with hormone receptor-positive

breast cancer. However, development of resistance to tamoxifen has led to disease progression and death (Fagan et al., 2017). Medicinal plants have been regarded as one of the valuable sources of bioactive agents that may contribute to the anticancer activity. This includes *Garcinia* species, which also produces essential oils. The essential oil can be extracted from different parts of the plant including leaves by various methods. These essential oils contain a mixture of mostly volatile and triterpenoids constituents such as α-copaene, germacrene D and β-caryophllene (Jamila et al., 2015; Tan et al., 2018). Furthermore, essential oils extracted from *Garcinia atroviridis* have been demonstrated to induce cytotoxicity against MCF-7 human breast cancer line (Tan et al., 2018). Therefore, for this study, GH leaf extract was chosen to study its anti-proliferative effect on breast cancer cell lines. Two models of breast cancer cell lines were used in this study, namely MCF-7 and MCF-7/TAMR-1. Besides that, the dose-response effects and the IC_{50} values of essential oils extracted from leaves of GH were determined and established by using MTT assay. Furthermore, the mode of cell death via apoptosis and/or necrosis was determined by using Annexin-V FITC and propidium iodide staining assay by flow cytometry. The hypothesis of this study was GH essential oils leaves (GH-EOL) may exhibit anti-proliferative effect against both cell lines through the induction of apoptosis.

1.3 Research objectives

For this study, the main objective was to investigate the anti-proliferative effect of GH-EOL in human breast cancer cells. To achieve this, sub-objectives were constructed and listed as below:

1. to extract essential oils from GH leaves by using hydrodistillation technique.

2. to determine the dose-response effects and to establish the IC_{50} values of GH-EOL on MCF-7 and MCF-7/TAMR-1 human breast cancer cell lines by using MTT assay.
3. to determine the apoptotic or necrotic effects of GH-EOL in MCF-7 and MCF-7/TAMR-1 human breast cancer cell lines by flow cytometry.

1.4 Research question

In general this study need to investigate the anti-proliferative effect of GH-EOL in human breast cancer cells. Following questions had been developed: Is there an anti-proliferative effect of GH-EOL in tamoxifen resistant human breast cancer cell line (MCF-7/TAMR-1)? What are the mechanism of GH-EOL in regards to cell death?

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

According to the World Health Organization (2018), cancer is defined as a complex disease involving abnormal cells grow uncontrollably and, in some cases, to metastasize (spread) nearby tissues. Worldwide, cancer is the second dominant cause of death and 9.6 million deaths from cancer were estimated in 2018. Globally, about 1 in 6 deaths is due to cancer. It is also stated that cancers of the males, lung, prostate, colorectal, stomach and liver are the most frequently diagnosed cancers, whereas among females are breast, colorectal, lung, cervix, and thyroid cancers (International Agency for Research on Cancer, 2018).

The most common or suspected factors increase the risk of developing cancer are advancing age, drinking alcohol, exposure to cancer-causing substances in the environment (carcinogens) such as tobacco smoking and UV radiation from sunlight, chronic inflammation, dietary components or nutrients, hormones such as estrogens, immunosuppressive drugs, infectious agents including viruses, bacteria and parasites, and obesity (National Cancer Institute, 2015). Cancer therapies are being continually developed as increasing knowledge of molecular and tumour biology. There are many therapeutic strategies to prevent and/or cure cancer such as surgery, radiation, chemotherapy, immunotherapy, targeted therapy (drug only acts on cancer cell), and

hormone therapy (Zugazagoitia et al., 2016). However, interest is also being shown in alternative treatment using natural products (Shaikh et al., 2016).

2.2 Breast cancer

Breast cancer is the most commonly diagnosed cancer and also the second dominant cause of cancer death among women in the world. In 2018, approximately 2.1 million women diagnosed with breast cancer, accounting for approximately 11.6% of the cancer cases (Bray et al., 2018). In South-East Asia, the incidence rate of breast cancer in Malaysia is 18 per 100,000 populations in comparison to Singapore and Thailand which at 15 and 11 per 100,000 populations respectively. On the other hand, the median survival time for breast cancer patients diagnosed in stage III is 50.77 months in North-East Peninsular Malaysia (Nordin et al., 2018).

2.3 Breast cancer subtypes

Breast cancer cells often have different types of hormonal receptors including estrogen (ER), progesterone (PR), and human epidermal growth factor receptor 2 (HER2). These receptors mediate cell growth signalling. Breast cancer is divided into four molecular subtypes according to these receptors (Figure 2.1). Luminal A breast cancers are ER+, PR+, HER2-, Luminal B breast cancers are known as triple positive, are ER+, PR+, HER2+, HER2 enriched breast cancers are ER-, PR-, HER2+, and Triple Negative (basal-like) breast cancers are ER-, PR-, HER2-. Luminal A subtype account for 70 % of all cases, whereas Basal-like, Luminal B, and HER2 make up 15 %, 10 %, and 5 % of all breast cancer cases respectively (Dai et al., 2016).

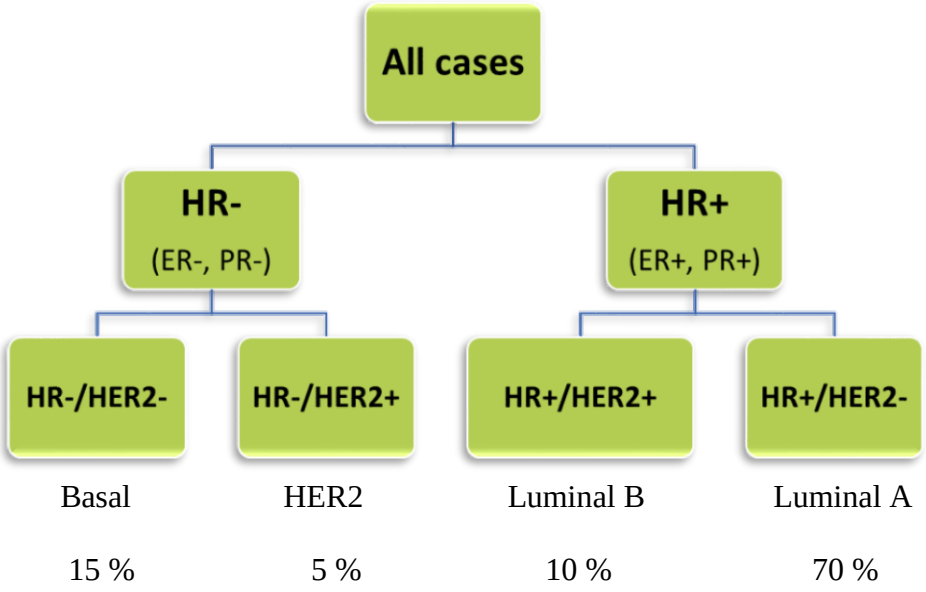

Figure 2.1: Molecular subtypes of breast cancer

2.4 Breast cancer risk factors

Breast cancer is more commonly associated with family history, obesity, sex, ageing, prolonged exposure to estrogen and in postmenopausal women. The family history of breast cancer in a first-degree relative increased the risk for both ER-positive and ER-negative invasive breast cancer, but the level of risk varied by age and more common for women older than age 50 years. Similarly, postmenopausal women who were overweight were at risk of ER-positive and ER-negative cancer (Kerlikowske et al., 2016). The number of breast cancer cases occurred in women is 100 times higher than that in men (Sun et al., 2017).

2.5 Treatment of breast cancer

There is a variety of treatment for women diagnosed with breast cancer which include surgery, radiation therapy, chemotherapy, hormonal therapy, and targeted therapies. The most appropriate treatment depends on the stage and type of breast cancer, characteristics of the cancer cells, menopausal status, and the patient's state of health (Nounou et al., 2015).

Surgical treatment to remove breast cancer involve two basic types lumpectomy and mastectomy. Lumpectomy, surgically removing the tumour and a small margin of surrounding normal epithelial tissues, but not the entire breast. Mastectomy, surgical removing the all breast includes nipple and areola (American Cancer Society, 2016).

Radiation therapy is often started after lumpectomy to kill any remaining cancer cells in a particular area by damaging DNA via radiation (Balaji et al.,2016). Chemotherapy is a treatment which uses drugs to enfeeble and damage tumour cells in the body, including cells at the primary site of cancer and any cancer cells that break away and spread throughout the body parts. Chemotherapy is sometimes given before surgery (neoadjuvant therapy) to shrink larger tumours (Pathak et al., 2018).

Hormone therapy slows or stops the growth of hormone receptor-positive breast cancer cells by blocking hormones from binding to receptors on tumour cells. Tamoxifen is one of the most common hormone therapy medications used to block estrogen receptors in breast cancer cells. It is often given to decrease the size of breast tumour before surgery to remove it, to reduce the risk of breast cancer coming back after surgery, and to treat breast cancer that has already spread. Another examples of hormonal therapies used in the

management of metastatic hormone receptor-positive breast cancer are fulvestrant act as an selective estrogen receptor downregulator and Letrozol act as irreversible non-steroidal aromatase inhibitor in postmenopasual women diagnosed with hormone receptor-positive breast cancer (Drãgãnescu and Carmocan, 2017).

Targeted cancer therapies are drugs that block the action of a specific protein (molecular target) that allows the cancer cells to grow in a rapid or abnormal way without harming normal cells. Hormone replacement therapies, signal transduction inhibitors, modulators of gene expression, apoptosis inducers, angiogenesis inhibitors, immunotherapies, and toxin delivery molecules are types of targeted therapies have been approved for use in cancer treatment (National Cancer Institute, 2019).

2.6 Tamoxifen resistance in breast cancer

Around 2 out of 3 breast cancers are hormone receptor-positive. Estrogen signalling plays a critical role in the proliferation of breast cancer, and therefore reducing the amount of estrogen or blocking its action using endocrine therapy can reduce the risk of early-stage hormone-receptor-positive breast cancers coming back. The selective estrogen receptor modulator (SERM), tamoxifen, is the standard treatment options for estrogen receptor-positive breast cancer patients. Tamoxifen treatment of estrogen receptor (ER)-positive breast cancer reduces the annual breast cancer death rate by 31%. However, about half of patients with advanced ER-positive disease immediately fail to respond to tamoxifen and approximately 40% will acquire the resistance during the treatment (Hultsch et al., 2018). Acquired resistance to hormone therapy remains a major challenge for women with estrogen receptor-positive metastatic breast cancers. Multiple

mechanisms responsible for endocrine resistance may include deregulation of various components of the ER pathway itself, alterations in cell cycle and cell survival signalling molecules, and the activation of alternative signalling pathways that can provide tumours with alternative proliferative and survival stimuli (Hayes and Lewis-wambi, 2015). For instance, breast cancer cells resist to tamoxifen treatment by overactivation the phosphatidylinositol-3-kinase (PI3k)/Akt and the mammalian target of rapamycin (mTOR) intracellular signalling pathway through cross-talk between the estrogen receptor and this growth factor signalling pathway. In consequence, reducing apoptosis and allowing the proliferation of cancer (Won et al., 2016). Another example, tamoxifen-resistant breast cancer cells are resistant to genotoxicity mechanism of tamoxifen (DNA damaging through oxidative stress) by a mutation in tumour suppressor genes BARD1 and BRCA1 (gens to protect normal cells from conversion to cancer cells). This will lead to loss or reduction in gens functions and then normal cells progress to cancer cells (Zhu et al., 2018). Due to severe side effects and multidrug resistance, these treatment approaches become increasingly ineffective. However, turning to complementary treatment approach can be a big solution for this situation, as it is evident that compounds derived from the natural source have a great deal of anticancer activity (Mitra and Dash, 2018).

2.7 Natural products derived from the plant as an alternative treatment for breast cancer

Natural plants have been used to prevent and treatment of breast cancer. Huge number of natural compounds from plants are identified and showed very promising anti-cancer properties with less toxic side effects compared to current treatments such as

chemotherapy. Anticancer properties of natural, synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression. Natural compounds reduced the aggressiveness of breast cancer through various mechanisms of action, such as downregulating ER-α expression and activity, inhibiting proliferation, migration, metastasis and angiogenesis of breast tumour cells, inducing apoptosis, cell cycle arrest and sensitizing breast tumour cells to radiotherapy and chemotherapy (Mitra and Dash, 2018).

The plant kingdom produces naturally occurring secondary metabolites which are being attracted to the interest of scientific and research to investigated and designed cancer therapeutics from natural compounds especially from phytochemicals (Greenwell and Rahman, 2015). Many dietary natural products could affect the development and progression of breast cancer, such as mangosteen (*Garcinia mangostana* L.) known as "queen of fruits", Pomegranate (*Punica granatum* L.), Mango (*Mangifera indica* L.), and Jujube (*Ziziphus jujube*) were used to prevent and treatment of breast cancer in South-East Asia (Li et al., 2017). Substantial experimental studies on the effectiveness of Garcinia hombroniana extract indicated that this plant is an anti-cancer agent (Dyary et al., 2016; Jamila et al., 2015).

2.8 Taxonomy of *Garcinia hombroniana* plant

Table 2.1: Taxonomy hierarchy of *Garcinia hombroniana* plant

Kingdom	*Plantae*
Phylum	*Tracheophyta*
Class	*Magnoliopsida*
Order	*Malpighiales*
Family	*Clusiaceae*
Genus	*Garcinia* L.
Species	*Garcinia hombroniana Pierr*

Adapted from (GBIF Backbone Taxonomy)

2.9 *Garcinia hombroniana*

Garcinia is the major genus in the family *Clusiaceae* with over 400 species found throughout the tropics of Africa and Asia and about 50 species found in the lowland and mountains of Peninsular Malaysia (Tan et al., 2018). The genus *Garcinia* is reported to possess pharmacological activities such as antimicrobial, anti-inflammatory, anticancer, hepatoprotective and anti-HIV activities. Some studies have reported anti-cholinesterase activity of the plant on the nervous system disorders (Jamila et al., 2017). Traditionally, numerous parts of *Garcinia* plant (fruits, leaves, flowers, stem and bark) have been utilized to treat various ailments such as abdominal pain, leucorrhoea, gonorrhoea, diarrhoea, dysentery, wound infection, suppuration, and chronic ulcer in Malaysia, Thailand, Indonesia, Sri Lanka, Philippines and China (Jamila et al., 2015).

Figure 2.2: *Garcinia hombroniana* leaves

Adapted from (plantsystematics.org)

Garcinia hombroniana plant belongs to family *Clusiaceae*. It is a small or medium evergreen tropical tree which produces globose fruit in a bright red colour. The plant is native to the tropical rainforests of Southeast Asian countries such as Vietnam, Cambodia, Malaysia and Thailand (Chew and Lim, 2018). It is known as “Manggis hutan” (seashore mangosteen) in Malaysia or “Waa” in Thailand. In Malaysia, it is found in the longshore area, from the lowland forests near the sea to the lower mountain forests and the highlands.

Figure 2.3: *Garcinia hombroniana* tree

Adapted from (toptropicals.com)

In Asian and West Africa, this plant had been used for its medicinal purpose. The plant roots are used to make a herbal decoction for women as an anti-infective agent after childbirth and the leaves are used to relieve itching. The previous studies show that twigs, stem bark, pericarp, and leaves *Garcinia hombroniana* to contain alkaloids, flavonoids, phenols, saponins, tannins, xanthones, benzophenones, and terpenoids. *Garcinia hombroniana* leaves aqueous extract shows little toxicity on the vital organs such as the liver, kidneys, heart, and spleen in laboratory animals (Dyary et al., 2016). Furthermore, *Garcinia hombroniana* leaves aqueous extract has numerous therapeutic effects such as anti-diabetic properties due to the à-glucosidase inhibitor agent, antioxidant and

lipoxygenase inhibitor activity, and potential antitrypanosomal activity (Marlin et al., 2017; Triadisti et al., 2017; Dyary et al., 2015). In addition to that, the methanol extract of the twigs of *Garcinia hombroniana* has strong antioxidant activity on human low-density lipoprotein (LDL) and antiplatelet aggregation activities, and antibacterial activity against methicillin-resistant *Staphylococcus aureus* and *S. aureus* (Saputri and Jantan, 2012; Klaiklay et al., 2013). Naturally, bioflavonoids from the bark of *Garcinia hombroniana* display significant antioxidant and antibacterial activities, and good dual inhibition on both acetylcholinesterase and butyryl cholinesterase (Jamila et al., 2014; Jamila et al.,2015). *Garcinia hombroniana* bark extract was also reported to possess good cytotoxic effect against human breast cancer (MCF7) and human glioblastoma (DBTRG) cell lines which might be due to the phenolic compounds (Jamila et al., 2014; Jamila et al.,2017).

2.10 Cell death

Cell death is a very well-organized fundamental activity that is equally complex in regulation as cell division and differentiation. Balance homeostasis in the body is a crucial factor in avoiding the growth of cancer cells. Thus, activation of the cell death mechanism is vital in maintaining the homeostasis of proliferative of health and normal cells (Green and Lambi, 2015). Cell death occurs when the cells are confronted with a process which is reversible at first before it becomes irreversible. Loss of plasma membrane integrity, cell fragmentation with its nucleus into apoptotic bodies, and engulfment its fragments by adjacent cells are the molecular or morphological criteria to define dead cell *in vivo*. Cell death, due to the presence of its genetic regulation, can be divided into programmed (or active) ones, such as apoptosis and autophagy, and not programmed (or nonactive) such

as necrosis (Galluzzi and Vitale, 2018). Figure 2.4 shows a summary of the classification of the cell death pathway.

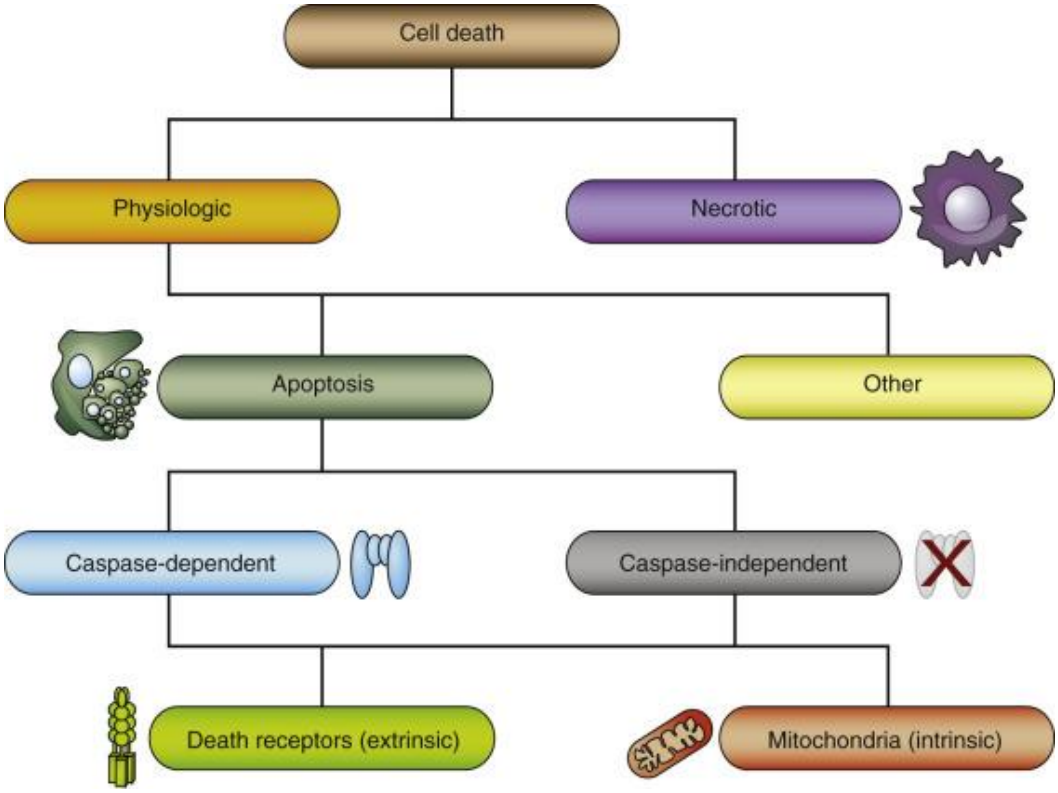

Figure 2.4: Classification of cell death pathway

Adapted from (Danial and Hockenbery, 2018)

2.11 Apoptosis

Apoptosis is programmed and an active form of cell death in which a highly specific and orderly set of biochemical changes underlie the unique morphologic changes and the ultimate disposition of the dying cell and its contents. It is distinguished by cell shrinkage, nuclear fragmentation, plasma membrane blebbing and finally by the separation of the cellular components into apoptotic bodies. These apoptotic bodies are removed by phagocytes which attracted by the "eat me" signal from the exposure of

phosphatidylserine on the plasma membrane. Apoptosis induction can be activated by two different pathways, the intrinsic and extrinsic pathways. The extrinsic pathway is mediated by death receptors, while the intrinsic or mitochondrial pathway is triggered by the release of apoptogenic proteins, such as cytochrome c, which activated caspase proteins that are the main effector molecules that induce this process (Hassan et al., 2014).

2.12 Necrosis

Necrosis has been considered as an accidental or not programmed and passive of cell death, and the endpoint commonly associated with very severe toxic damage. Necrotic cell death is characterized by an increase in cell volume, swelling of organelles, plasma membrane rupture and provoking an inflammatory response. The morphological features associated with necrosis include cellular energy depletion, damage to membrane lipids, and loss of function of homeostatic ion pumps/channels. It is considered as a toxic process because these necrotic cells are reported to release harmful chemicals that can induce damage to other cells. Necrosis is caused by factors external to the cell, such as lytic viral infection, physical trauma, complement-mediated lysis, depletion of ATP, loss of ionic homeostasis, and excessive ROS/RNS (Manning and Zuzel, 2010).

2.13 Essential oils and their bio-activities

The essential oil is a mixture of concentrated hydrophobic liquid containing volatile compounds synthesized by medicinal and aromatic plants as secondary metabolites. There are obtained from various parts of the plant such as flowers, fruits, leaves, twigs, and barks (Kumar et al., 2018). There are a variety of methods for obtaining essential oils from the plant. Steam distillation method was found to be one of the promising techniques for

extraction of essential oil from plants as reputable distiller will preserve the original quantities of the plant. The distillation was conducted in a Clevenger apparatus in which boiling, condensing and decantation were done (Rassem et al., 2016). Essential oils show a broad range of bioactivities, especially antimicrobial activity, and have long been utilized for treating various human ailments and diseases. Depending on type and concentration, essential oils possess antimutagenic, antiproliferative, antioxidant, and detoxifying capabilities acting on different routes in the cancer cell as well as cancer preventative potentialities. The cytotoxic activity of essential oils is mostly due to the presence of phenols, aldehyde and alcohol (Blowman et al., 2018).

CHAPTER 3

METHODOLOGY

3.1 Materials and chemicals

3.1.1 Chemicals and reagents

All chemicals and reagents used in this study are listed in Table 3.1

Table 3.1: List of chemicals and reagents

Chemicals and reagents	Supplier
Dimethyl Sulphoxide (DMSO)	Fisher Scientific, UK
Roswell Park Memorial Institute medium (RPMI-1640)	Gibco, USA
Phosphate Buffered Saline (PBS)	Gibco, USA
Penicillin-Streptomycin (Pen-strep) antibiotics solution	Gibco, USA
Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12), no phenol red	Gibco, USA
Fetal Bovine Serum (FBS)	Gibco, USA
Trypsin-EDTA	Gibco, USA
5-diphenyltetrazolium bromide dye (MTT)	Calbiochem, Germany
Trypan blue	Gibco, USA
Accutase	Millipore, USA

Garcinia hombroniana leaves	Penang Botanical Garden
MCF-7 cell line	American Type Culture Collection (ATCC), Virginia, USA
MCF-7/TAMR-1 cell line	American Type Culture Collection (ATCC), Virginia, USA
MCF10A cell line	American Type Culture Collection (ATCC), Virginia, USA
Annexin V FITC	Roche, Germany
Propidium Iodide (PI) dye	Roche, Germany
Tamoxifen citrate salt	Nacalai tesque, Japan

3.1.2 Consumables

All consumables used in this study are listed in Table 3.2

Table 3.2: List of consumables

Consumables	**Supplier**
Cryogenic vials and Cryoboxes	Nalgene, USA
Micropipette tips (10µl, 200µl, 1000µl)	Labcon, Germany
Serological pipettes (2ml, 5ml, 10ml)	Nunc, Denmark
Centrifuge tubes (15ml, 50ml)	Nest Biotechnology Co., Ltd., China
Tissue culture flasks (25 cm^2, 75 cm^2)	Becton Dickinson, USA
Syringes (10ml)	Terumo Corporation, Philippines
Syringes membrane filter (0.22 µm)	BD Plastipak, Spain
96-well microtiter plates	Essen Bioscience, USA
Aluminium foils	Reynolds Consumer Products Inc., USA

Coverslips	Deckglaser, Germany
Counting chamber	Becton Dickinson, USA

3.1.3 Laboratory equipment

All of the laboratory equipment used in this study are listed in Table 3.3

Table 3.3: List of laboratory equipment

Name and brand	Supplier
Airstream Class II Biological Safety Cabinet	ESCO, Singapore
Centrifuge	Hettich Zentrifugen, Germany
Incubator	ThermoScientific, USA
Water bath	Memmert, Germany
Inverted microscope	OLYMPUS, USA
Hemocytometer	LD-Laboroptik Ltd., UK
Microplate reader	Biotek, UK
Flow cytometer	Becton Dickinson, USA
Clevenger-type apparatus	Custom Made

3.2 Extraction of essential oils by hydrodistillation technique

The leaves of *Garcinia hombroniana* were collected at Penang Botanical Garden. The voucher specimen (USM 11748). The leaves of *Garcinia hombroniana* were washed with distilled water and cut into small pieces prior to hydrodistillation. The hydrodistillation was carried out for 5 hours using a Clevenger-type apparatus. The

extracts were carefully concentrated using gentle steam of nitrogen gas at room temperature, yielding pale yellow oils. The essential oils isolated from the leaves were kept at 4 ºc until analysis. A stock concentration 10 000 µg/ml was prepared by dissolving the oils in dimethyl sulphoxide (DMSO) and kept at -20 ºc until use.

3.3 Cell culture

3.3.1 Human breast adenocarcinoma cell lines

Two types of adenocarcinoma cell lines were used throughout this study. MCF-7 is an ER-positive cell line and MCF-7/TAMR-1 is an ER-positive tamoxifen resistance cell line, originated from human breast adenocarcinoma with epithelial morphology. MCF-10A is an ER-alpha negative normal breast cell line. These cells were obtained from American Type Culture Collection (ATCC, USA). These cells were grown in suitable condition and the medium was changed every 2 days. All cells handling and medium preparation were carried out using aseptic technique in class II safety cabinet.

3.3.2 Reagents for cell culture work

3.3.2.1 Medium

MCF-7 was cultured and maintained in RPMI-1640 growth medium (Gibco, USA). MCF-7/TAMR-1 and MCF-10A were cultured and maintained in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12) (Gibco, USA). Both medium stored

at 4 ºC. The reagents were thawed before used and filtered-sterilised with 0.22 µm disposable filter unit.

3.3.2.2 Heat-inactivated Fetal Bovine Serum (FBS)

A bottle of FBS (100 ml) (Gibco, USA) was stored at -20 ºC. The serum was filtered-sterilised with 0.22 µm disposable filter units and aliquoted into sterile 50 ml tubes and kept at -20 ºC. The FBS was thawed in a water bath at 37 ºC prior to use.

3.3.2.3 Phosphate-Buffered Saline (PBS)

Ten-time Phosphate-Buffered Saline (Gibco, USA) was filtered-sterilised with 0.22 µm disposable filter units and aliquoted into sterile 50 ml tube with distilled added to produce 1x working concentration. The PBS was stored at room temperature.

3.3.2.4 Penicillin-Streptomycin (Pen-strep) antibiotics solution

A bottle of ready-made containing Penicillin-Streptomycin (Pen-strep) solution (Gibco, USA) was filtered-sterilised with 0.22 µm disposable filter units and aliquoted into sterile 15 ml tubes and kept at -20 ºC. The antibiotic was thawed in a water bath at 37 ºC prior to use.

3.3.2.5 Complete growth medium

A complete growth medium was used in this study to culture and maintain the cell lines. The medium for MCF-7 cell line consist of RPMI-1640 solution supplemented with

10 % (v/v) FBS and 1 % (v/v) of Pen Strep. The 50 ml medium was prepared by adding 5 ml FBS, 50 µl Pen Strep and 45 ml RPMI-1640 media. The medium for MCF-7/TAMR-1 cell line consists of DMEM/F12 without phenol red solution supplemented with FBS, Bovine insulin and Tamoxifen. The 50 ml medium was prepared by adding 0.5 ml FBS, 15 µl Bovine insulin, 5 µl Tamoxifen and 49.5 ml DMEM/F12 without phenol red media. The medium for MCF-10A cell line consists of DMEM/F12 with phenol red solution supplemented with horse serum, insulin, hEGF enzyme, hydrocortisone, and Pen Strep. The 50 ml medium was prepared by adding 47.5 ml DMEM/F12 with phenol red solution, 2.5 ml horse serum, 125µl insulin, 50µl hEGF enzyme, 500µl hydrocortisone, and 50µl Pen Strep. All prepared media were kept at 4 ºC and thawed before used.

3.3.2.6 Trypsin (0.25%, w/v)/ EDTA (0.03%, w/v) solution

Ready-made trypsin of 0.25%, EDTA 0.03% was filter-sterilised, aliquoted into sterile 15 ml tubes and stored at -20 ºC until use. The trypsin was thawed in 37 ºC prior to use.

3.3.2.7 Cryogenic Medium

The cryogenic medium for MCF-7 cell line contained 95% RPMI complete medium and 5% DMSO. The 10 ml cryogenic medium was prepared by adding filtered-sterilised 9.5 ml RPMI complete media and 0.5 ml DMSO. The cryogenic medium for MCF-7/TAMR-1 and MCF-10A cell line contained 95% DMEM/F12 complete medium and 5% DMSO. The 10 ml cryogenic medium was prepared by adding filtered-sterilised 9.5 ml

DMEM/F12 complete media and 0.5 ml DMSO. Both cryogenic mediums were prepared fresh and kept cold prior to use.

3.3.3 Culture procedures and conditions

All tissue culture procedures were carried out in a sterile condition (Airstream Class II Biological Safety Cabinet) using aseptic techniques to avoid any contamination. Cells were cultured in growth medium and maintained in a humidified incubator at 37 ºC in an atmosphere in a 5 % CO2 and 95 % air.

3.3.4 Thawing of cells from frozen storage

The frozen cells were retrieved from the -80 ºC storage and thawed in a water bath at 37 ºC for 2 minutes. The entire frozen cells were gently pipetted into a 15 ml centrifuge tube containing 2 ml pre-warmed complete growth medium. The content centrifuged at 1000 rpm for 5 minutes to remove DMSO for MCF-7 cell and at 300 g for 4 minutes to remove DMSO for MCF-7/TAMR-1 and MCF-10A cells. The supernatant was aspirated and the cell pellet was then resuspended in 1-2 ml of complete growth medium. The cell suspension was then slowly pipetted into a 25 cm^2 tissue culture flask containing 4-5 ml culture media. The cell morphology was observed using an inverted phase-contrast microscope to check for cell viability. The culture flask was incubated in a humidified atmosphere containing 5 % CO_2 at 37 ºC. The growth medium was replaced every 2-3 days.