ANTICANCER EFFECT OF ARTONIN E AND CHAPLASHIN FROM *ARTOCARPUS* SPECIES ON BREAST CANCER CELLS, MDA-MB-231 AND MCF-7

by

CHOO YEN LING

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LIST OF SYMBOL

%	percentage
<	less than
=	equal to
°C	degree celsius
µg/ml	microgram per milliliter
µl	microliter
cm	centimeter
cm^2	centimeter square
g	gram
h	hour
mg	milligram
mg/ml	milligram per milliliter
ml	milliliter
rpm	relative centrifugal force
v/v	volume per volume
w/v	weight per volume

LIST OF ABBREVIATION

BSC	Biosafety cabinet
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
HCl	Hydrochloric acid
IC_{50}	Median inhibition concentration
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
OD	Optical density
Pen-Strep	Penicillin-streptomycin
PBS	Phosphate buffer saline
PI	Propidium iodide
SEM	Standard error mean
SD	Standard deviation
WHO	World Health Organization

ABSTRAK

Kanser payudara merupakan antara masalah kesihatan global yang utama. Rawatan kanser payudara yang utama ialah kemoterapi. Walau bagaimanapun, kajian telah menunjukkan bahawa kemoterapi mempunyai kesan jangka panjang boleh membawa kepada kerintangan terhadap dadah dan menunjukkan kesan sampingan yang menyebabkan beberapa jenis pengubahsuaian genetik seperti mutasi gen, metilasi DNA dan pengubahsuaian histon. Kajian epidemiologi menunjukkan bahawa pengambilan makanan seperti buah-buahan dan sayur-sayuran yang kaya dengan flavonoid mungkin boleh mengurangkan risiko mendapat kanser termasuk kanser payudara. Oleh itu, kajian ini dijalankan untuk menilai kesan anti-kanser oleh dua jenis flavonoid yang diekstrak daripada *Artocarpus* sp, iaitu Artonin E and Chaplashin, terhadap titisan sel kanser payudara, MCF-7 and MDA-MB-231. Objektif projek ini adalah untuk menentukan kesan anti-kanser Artonin E and Chaplashin terhadap titisan sel kanser payudara MDA-MB-231 and MCF-7 dan menentukan mod kematian sel dengan melihat fragmentasi nukleus. Kesan perencatan bersandar dos oleh pertumbuhan sel dengan IC_{50} bagi sel kanser payudara MDA-MB-231 and MCF-7 ditentukan oleh pewarnaan metilena biru. Nilai IC_{50} kemudiannya digunakan untuk kajian fragmentasi nuklear melalui penggunaan pewarna fluoresen Hoeschst 33342. Mod kematian sel dipastikan dengan pewarnaan Annexin V/PI melalui analisis sitometri aliran. Hasil menunjukkan Artonin E mempunyai aktiviti anti-kanser yang lebih baik terhadap sel MDA-MB-231 dengan nilai IC_{50} 4.95 μg/ml. Nilai ini menunjukkan Artonin E adalah lebih poten berbanding dengan IC_{50} Cisplatin, 5.88 μg/ml. Asai fragmentasi nuklear telah menunjukkan sel yang dirawat dengan Artonin E menyerap warna fluoresen biru dengan lebih jelas jika dibandingkan dengan sel yang tidak dirawat. Morfologi perwarnaan ini menunjukkan

ciri-ciri apoptosis kerana fragmentasi nuklear jelas kelihatan di dalam sel yang dirawat. Sebagai kesimpulannya, titisan sel kanser payudara MDA-MB-231 yang telah dirawat dengan sebatian flavonoid Artonin E mempunyai kesan antiproliferasi yang kukuh untuk mengaitkan kepotenan kesan anti-kanser. Mod kematian sel mengesahkan induksi apoptosis telah berlaku apabila fragmentasi nuklear kelihatan dalam sel-sel kanser yang dirawat. Oleh itu, sebatian Artonin E perlu dikaji secara mendalam untuk menyokong aktiviti anti-kansernya supaya dapat dijadikan satu lagi alternatif dadah anti-kanser yang berpotensi pada masa depan.

ABSTRACT

Breast cancer appears to be a major global health problem. The most common treatment approach of this disease is chemotherapy. However, studies showed that long-term use of chemotherapy can lead to drug resistance and implies side effect that may cause several genetic alteration, such as gene mutation, DNA methylation and histone modification. Epidemiologic studies revealed that consuming flavonoid rich fruits and vegetables might reduce all types of cancer incidences including breast cancer. Therefore, this research was conducted to evaluate the anticancer effect of two types of flavonoid extracted from *Artocarpus* sp, Artonin E and Chaplashin towards breast cancer cell lines, MCF-7 and MDA-MB-231. The objectives of this project were to determine anticancer effect of Artonin E and Chaplashin towards MDA-MB-231 and MCF-7 breast cancer cell lines and to determine mode of cell death of cells treated with most potent compound by assessing nuclear fragmentation effect. Dose-dependent inhibitory effect of cell growth on MDA-MB-231 and MCF-7 breast cancer cells with a median inhibition concentration (IC_{50}) was determined by methylene blue assay. The IC_{50} concentration was then used to analyse nuclear fragmentation event using fluorescence dye Hoeschst 33342. Mode of cell death was confirmed by flow cytometry analysis using Annexin-V/PI double staining. It was found that Artonin E showed better anticancer activity towards MDA-MB-231 cells with IC_{50} of 4.95 μg/ml compared to Cisplatin with IC_{50} of 5.88 μg/ml. Chaplashin showed the best activity towards MDA-MB-231 breast cancer cells with IC_{50} of 24.80 μg/ml. Nuclear fragmentation assay showed the cells treated with Artonin E absorbed more distinct fluorescent blue glow compared to the untreated cells. The stained morphology indicated apoptosis characteristic as nuclear fragmentation appeared clearly in the

treated cells. Thus, we postulated that anticancer effect in the treated cells underwent apoptosis. In conclusion, Artonin E exerted strong antipoliferative effects to attribute anticancer effect potential towards MDA-MB-231. Mode of cell death confirmed the induction of apoptosis when nuclear fragmentation occurred in the treated cancer cells. Therefore, these plant-derived compounds need further investigation to support the anticancer effect. Thus it might become a potential anticancer drug in the future.

CHAPTER 1

INTRODUCTION

1.1 Introduction towards research project

Cancer is one of the most life threatening risks in a person's life. In spite of considerable progress in its understanding and challenges, treatment is not yet the correct word to apply on this disease and losing life is the most foreseeing adventure in many patients. Although new gene therapy based approaches are looking for the cure of malignant cells, but using cytotoxic agents is currently the main chemotherapy approach to fight this problem. Still, effective chemotherapy treatment of cancer requires targeting the pathways that support the cell growth and proliferation.

However, these therapies cannot totally prevent cancer patients from recurrence and metastasis, thus new drugs and new therapies are in great need for cancer patients. Therefore nowadays, there are a great interest in the developments of bioactive compounds from medicinal plants which have potential and selective anticancer activity, especially in the candidate compound which have ability to trigger apoptosis.

Therefore, the search for alternative or complementary drugs that are effective on cancer cells with, less or no toxicity to normal cells is an active area of

research (Tang *et al.* 2003). Many of these investigations are plant-based, folkloric medicine from various societies around over the world. Moreover, a report from WHO (World Health Organisation, 1996) stated that about 80% of the world population is wholly or partially dependent on plant-based drugs (Ahmad *et al.*, 2010).

Thus, this study was conducted to analyse the anti-cancer potential of two flavonoid rich compounds Artonin E and Chaplashin from *Artocarpus* sp.

1.2 *Artocarpus* Plant Species

1.2.1 Family Moraceae

The plants of Moraceae family usually consists of trees, shrubs, vines, or rarely hearbs, where they are frequently with milky or watery latex, sometimes they are also spiny. Economically, the most important species are those of *Morus* and *Maclura* associated with the production of silk. Some species in *Broussonetia*, *Maclura*, and *Morus* are important for paper making and some species *Artocarpus*, *Ficus*, and *Morus* have edible fruit, and some species *Artocarpus* and *Broussonetia* are used for furniture or timber (Wu *et al.*, 2003).

1.2.2 Genus *Artocarpus*

Artocarpus comprises nearly 60 species (Jarrett 1959a, b, 1960; Kochummen 2000), including two widely cultivated throughout the tropics, breadfruit (*A. altilis*) and jackfruit (*A. heterophyllus* Lamarck). The remaining species are primarily restricted to Malaysia and Southeast Asia and include several utilized on a regional scale for food or timber (Zerega *et al.*, 2005). Besides that, this genus is widely distributed throughout subtropical and tropical region of the Indian subcontinent south of the Himalayas, Sri Lanka, Burma, Thailand, Indo-China, Southern China, Taiwan, Hainan, Malesia, and Melanesia (Hashim *et al.*, 2012).

Most plant of genus *Artocarpus* are trees, either evergreen or deciduous, with latex. They are monoecious. Their leaves are spirally arranged or distichous, where the leaf blades are simple to pinnatifid, rarely pinnate. Their flowers and bracts fused laterally to form a syncarp. Syncarp is fleshy throughtout or at least at the basal portions of calyx, where sometimes they can be very large. The flowers and bracts fused at their tips to form an areolation surface or free and forming variously shaped processes on surface. About 50 species can be found in tropical and subtropical Asia, and also in Pacific Islands (Forster and Forster, 2003).

Most importantly, *Artocarpus* species are rich in phenolic compounds including flavonoids, stilbenoids and arylbenzofurons. The chemical constituents of *Artocarpus* species have earlier been reviewed (Hakim *et al.*, 2006). Most of the pharmacological effects can be explained by the phenolic compounds including flavonoids, stilbenoids, arylbenzofurons (Hakim *et al.*, 2006) present from leaves,

bark, stem and fruit. Therefore, it is said that the extracts and metabolites of *Artocarpus* possess several useful bioactive compounds where recently additional data are available on exploitation of these compounds in the various biological activities including antibacterial, antitubercular, antiviral, antifungal, antiplatelet, antiarthritic, tyrosinase inhibitory and cytotoxicity (Jagtap and Bapat, 2010). Furthermore some parts of the plants are also used in traditional medicine preparations for the treatment of various diseases such as diarrhea, fever, liver cirrhosis, hypertension, diabetes, inflammation, malaria, ulcers, wound, and for tapeworm infection (Hashim *et al.*, 2012).

1.2.2.1 *Artocarpus teysmannii Miq.*

Artocarpus teysmannii is one of the *Artocarpus* plant genus where its family is Moraceae. Miq. is the author name of this plant. It was determined by Jarrett, F.M. where its collector was Teysmann, hence the name *teysmanii* is given (Research Center for Biology, 2008). Commonly it is known by a few local names such as "chempedak ayer", "sali saling", "tipulu", and "terbak kechil". Similarly, *Artocarpus peduncularis* Kurz is a synonym of *Artocarpus teysmannii* Miq.

Figure 1.1: Leaves and fruit of *Artocarpus teysmannii* (Cempedak air)

(Source:
http://www.europeana.eu/portal/record/11614/8B20C9545FCF81586DCB4ABA929D928D66DAB63A.html)

1.2.2.2 Artocarpus anisophyllus Miq.

Another type of *Artocarpus* plant is *Artocarpus anisophyllus* Miq, which is one of the endangered species of *Artocarpus*. In Latin, it means by "unequal leaves" as the unique feature of this tree is the large compound leaf containing leaflets of different sizes. Commonly it is known as Tree of Glory or "Keledang Babi". Furthermore, it has quite a number of synonyms, which are *Artocarpus anisophyllus* var. *sessifolius* Kochummen, *Artocarpus klidang* Boerl, *Artocarpus superba* Becc, *Artocarpus superbus* Becc, *Saccus anisophyllus* Kuntze. Whereas in Borneo, it is known as a few local names such as "Bakil", "Bintawak", "Danging", "Entawah", "Mantawa", "Mentawa", "Pepuan", "Puan", and "Tarap ikal" (Fl. Ned. Ind. Suppl.,1861).

This plant is a medium-sized tree growing to 30 m tall. The bole is dark grey, smooth to dippled bark and has spreading buttresses to 2.5 m tall. It is the only species of *Artocarpus* with pinnately compound leaves that are about 30–90 cm long and with leaflets of two different sizes. In total, there are 8–12 pairs of leaflets, with the small leaflets alternately arranged with the larger ones. The leaf is glabrous and the leaflets have unequal base. The fruit head is globose, 10–12 cm across with blunt thick spines (Raffles Museum of Biodiversity Research, 2013).

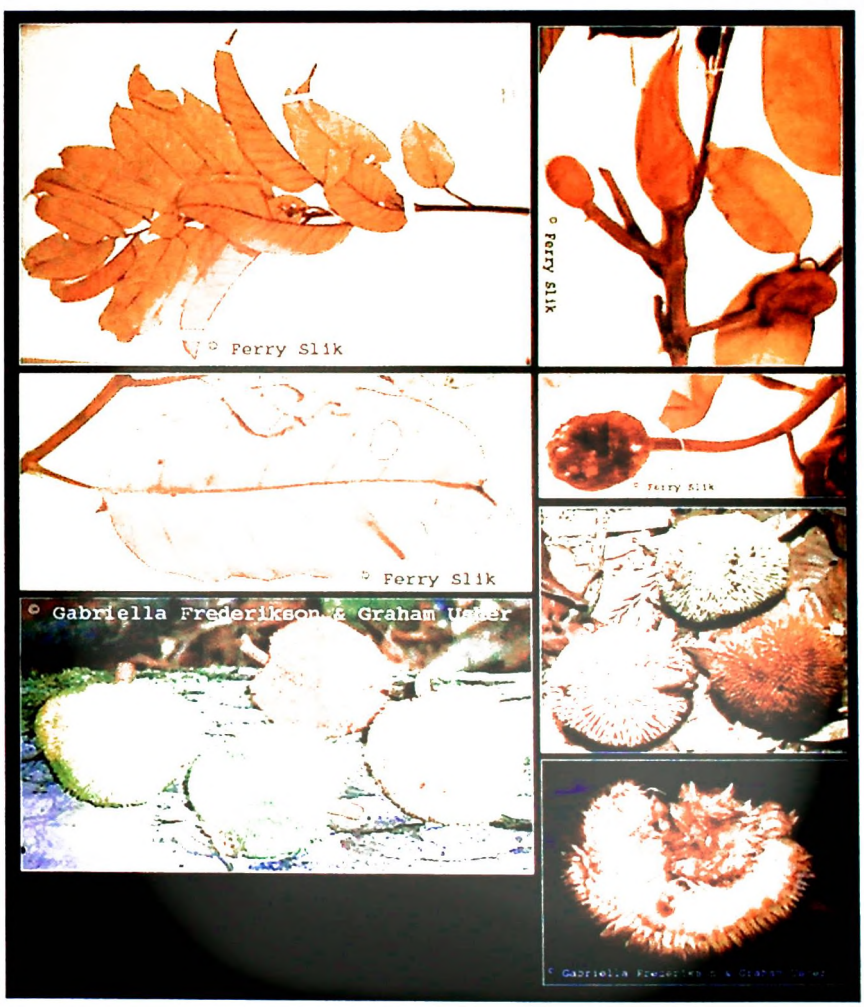

Figure 1.2: Leaves and fruits of *Artocarpus anisophyllus* (Keledang babi)

(Source: http://www.asianplant.net/Moraceae/Artocarpus_anisophyllus.htm)

This tree is unique in being the only Malayan tree that sheds its compound leaves as a whole and with such leaflet arrangements. It is being distributed in Peninsula Malaysia, Singapore, Sumatra, and Borneo. For localities, it is found in Bukit Timah Nature Reserve and MacRitchie Reservoir. Its ecology can be in undisturbed to slightly disturbed mixed dipterocarp forests up to 700 m altitude. It is also found on alluvial sites near rivers and streams, hillsides and ridges, on sandy to clay soils, and also on limestone. Furthermore, in secondary forests it is usually present as a pre-disturbance remnant (Raffles Museum of Biodiversity Research, 2013).

The wood (keledang wood) is used where it is fairly good quality and used for gun stocks, hilts and parang sheaths. The bark is locally used as rope for backpacks. The leaves are burned, and mixed with coconut oil used against boils and itch. The fruits and seeds are edible (Fl. Ned. Ind. Suppl.,1861). The Selako native community in Sarawak hang leaves on the door to prevent evil spirits from entering the premises. Little is known about its nutritional value and medical uses but the Ibans in Sarawak mix ash from the burnt leaves in a little cooking oil and apply this mixture to wounds, cuts, boils and itching scalp in children (Lim, 2012).

1.3 Flavonoid and Breast Cancer

Flavonoids are naturally occurring substances in plants (Peterson and Dwyer, 1998). Flavonoids constitute the largest group of plant phenolics, accounting for over half of the eight thousand naturally occurring phenolic compounds (Harborne *et al.*, 1999). Flavonoids are low molecular weight compounds, consisting of fifteen carbon atoms, arranged in a C6–C3–C6 configuration. Essentially the structure consists of

two aromatic rings A and B, joined by a 3-carbon bridge, usually in the form of a heterocyclic ring, C.

As several flavonoids have antioxidant properties, as well as antimutagenic and antiproliferative properties *in vitro* (Kandaswami *et al.*, 1992; Franke *et al.*, 1998; Takahashi *et al.*, 1998; Le Marchand *et al.*, 2000), these compounds have been investigated for possible inverse associations with various chronic diseases, including cardiovascular diseases and several forms of cancer. In numerous laboratory studies, flavonoids have demonstrated the ability to inhibit aromatase activity and thus lower estrogen biosynthesis and circulating estrogen levels, inhibit tumor cell proliferation, and inhibit the formation of reactive oxygen species, all of which are mechanisms thought to influence breast cancer development (Brian *et al.*, 2007).

Few epidemiologic studies have examined whether flavonoid intake is associated with breast cancer in humans. Therefore, the objectives of this research done was to determine anticancer effect of Artonin E and Chaplashin towards MDA-MB-231 and MCF-7 breast cancer cell lines and also to determine mode of cell death of the treated cells with most active compound by assessing nuclear fragmentation effect.

1.3.1 Artonin E

Four known compounds were isolated from the bark of *A. teysmanii* Miq., which were identified as Artonin E, artobiloxanthone, artonol B and cycloartobiloxanthone. IUPAC name for Artonin E is 5-Hydroxy-8,8-dimethyl-3-(3-

methylbut-2-enyl)-2-(2,4,5-trihydroxyphenyl)pyrano[2,3h]chromen-4-one. With a chemical molecular formula of $C_{25}H_{24}O_7$, it has a molecular weight of 436.15.

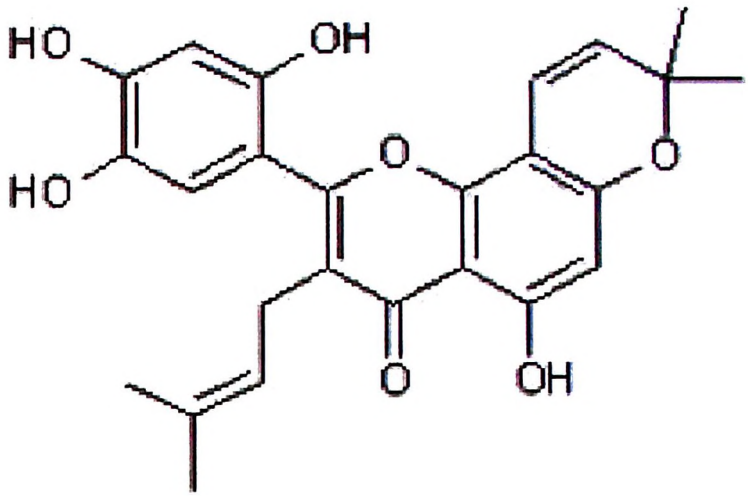

Figure 1.3: Molecular structure of Artonin E

(Source: http://www.imaginechem.com/product/prodetail-809.html)

In a study, it was mentioned that Artonin E were found to be active against breast cancer cell line, MCF-7 (Jamil, 2006). In another study done, Artonin E (5'-hydroxymorusin) was found to be a potent and fairly selective inhibitor of porcine leukocyte 5-LO (Reddy *et al.*, 1991). In other studies, Artonin E (5'-hydroxymorusin) was a potent and fairly selective inhibitor of porcine leukocyte on 5-LO activity (Reddy *et al.*, 1991) where it had increased the resultant anti-inflammatory activity.

1.3.2 Chaplashin

On the other hand, Chaplashin used in this research project is being extracted from the hexane crude extract of the heartwood of *Artocarpus anisophyllus* Miq. It

has an IUPAC name which is 6,7-Dihydro-3,9-dihydroxy-6-(1-hydroxy-1-methylethyl)-11-methoxy-10-(3-methyl-1-butenyl)-8H-[1]benzopyrano[3,2-d][1]benzoxepin-8-one.

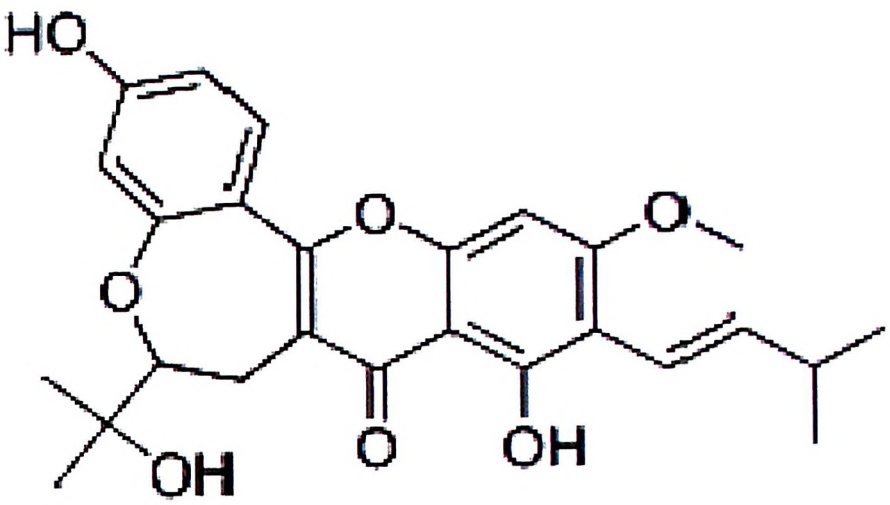

Figure 1.4: Molecular structure of Chaplashin

(Source: http://www.chemicalbook.com/ChemicalProductProperty_EN_CB22186968.htm)

The bioactivity study of the isolated Chaplashin can be found in this study done where it revealed that all nine compounds tested, including Chaplashin, exhibited interesting antitubercular and antiplasmodial activities, whereas the cytotoxic activity towards breast cancer cell lines was moderate (Boonphong *et al.*, 2007).

1.4 Objectives of the Research Project

The purposes of conducting this research are:

General objectives:

1. To determine the anticancer effect of Artonin E and Chaplashin towards MDA-MB-231 and MCF-7 breast cancer cell lines and its mode of cell death.

Specific objectives:

1. To determine the IC_{50} of Artonin E and Chaplashin treated on MDA-MB-231 and MCF-7 breast cancer cell lines.
2. To investigate the mode of cell death of MDA-MB-231 and MCF-7 breast cancer cell lines treated with the most potent IC_{50} concentration of flavonoid compound used.

1.5 Research Design

After obtaining the most potent IC_{50} (Artonin E) for MDA-MB-231 from methylene blue assay, nuclear fragmentation assay was conducted where Hoeschst 33342 staining showed the morphology and characteristic of apoptosis induced by Artonin E. Flow cytometry was then done to investigate if the apoptosis occurred at an early or late stage or if there was an occurrence of necrosis by Artonin E treatment. Further investigation of Chaplashin was not done on nuclear fragmentation assay as it was less potent than Artonin E.

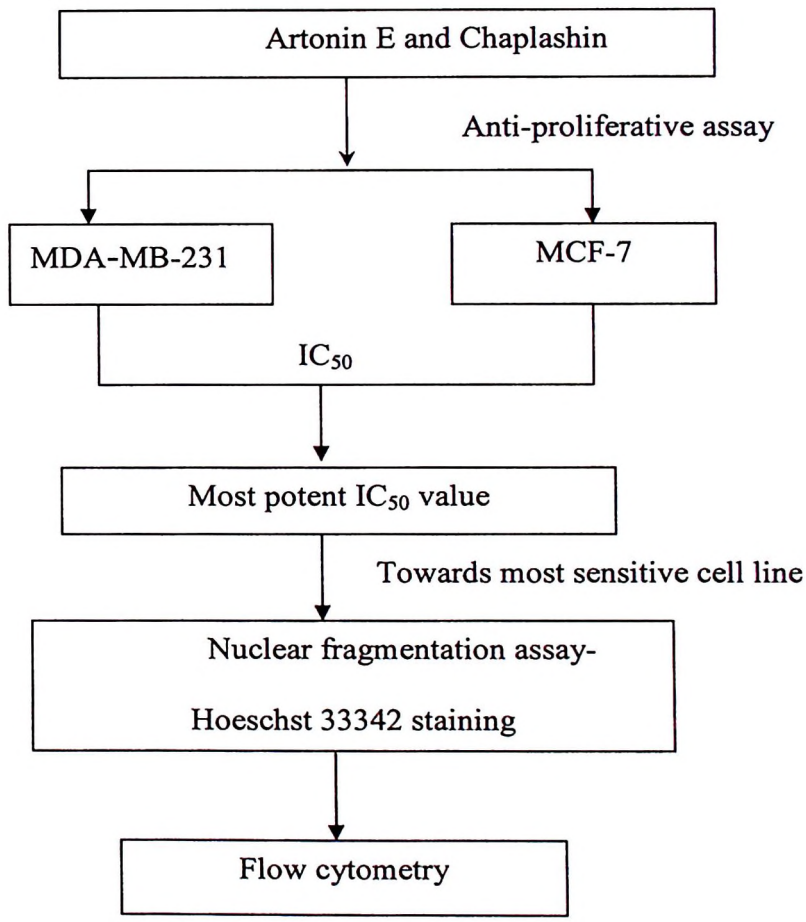

Figure 1.5: Flow chart of the antipoliferation activity of Artonin E and Chaplashin on *Artocarpus* plant extract on MDA-MB-231and MCF-7 breast cancer cells.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cell poliferation plays a role to replace worn out cells, heal damaged cells as well as aid in growth. Cancer, is known as a disease of the cells, where there are uncontrolled cell growth. Cancer can be benign or malignant. These cells are formed due to many factors, but it is still treated towards a certain extend. Still, cancer is becoming a leading cause of death in Malaysia due to avoidable risk factors like smoking and tobacco exposure, poor diet, alcohol, inadequate exercise or being overweight (National Cancer Society Malaysia, 2010).

Staging describes the extent or spread of cancer at the time of diagnosis. Proper staging is essential in determining the choice of therapy and in assessing prognosis. A cancer's stage is based on the size or extent of the primary (main) tumor and whether it has spread to other areas of the body. A number of different staging systems are used to classify tumors. A system of summary staging (in situ, local, regional, and distant) is used for descriptive and statistical analysis of tumor registry data. If cancer cells are present only in the layer of cells where they developed and have not spread, the stage is in situ. If cancer cells have penetrated beyond the original layer of tissue, the cancer is invasive and categorized as local, regional, or distant stage based on the extent of spread. Clinicians typically use the TNM cancer staging system, which assesses tumors in three ways: extent of the

primary tumor (T), absence or presence of regional lymph node involvement (N), and absence or presence of distant metastases (M). Once the T, N, and M categories are determined, a stage of 0, I, II, III, or IV is assigned, with stage 0 being in situ, stage I being early, and stage IV being the most advanced disease. Some cancers have alternative staging systems (e.g., leukemia). As the molecular properties of cancer have become better understood, tumor biological markers and genetic features have been incorporated into prognostic models, treatment plans, and/or stage for some cancer sites (American Cancer Society, 2013).

Globally, breast cancer incidence is rising but mortality is declining in high income countries due to earlier detection and more effective therapy. Incidence and mortality rates tend to be higher in high-resource countries compared to low-resource countries. However, in low resource countries fatality rates tend to be higher (Dahlui *et al.*, 2011).

2.1.1 Breast Cancer in Malaysia

Breast cancer is the most common cancer in women worldwide (World Health Organization, 2013). It is estimated that more than 1.6 million new cases of breast cancer occurred among women worldwide in 2010 (Forouzanfar *et al.*, 2011). Therefore, it is being the top five cancers affecting both male and female in Malaysia, which are breast, colorectal (bowel), lung, cervical and nasopharyngeal cancer (National Cancer Society Malaysia, 2010). Thus, breast cancer was the most common cancer in females and also the most common cancer among population regardless of sex and ethnic groups in Malaysia (Lim and Halimah, 2004). There

were 3,242 female breast cancer cases diagnosed in 2007 and reported to National Cancer Research, accounted for 18.1% of all cancer cases reported and 32.1% of all female cases. The age pattern in 2007 showed a peak age-standardized incidence rate (ASR) at the 50-59 age groups. The incidence of breast cancer was highest among Chinese where the ASR was 38.1 per 100,000 population followed by Indian and Malay with the ASR of 33.7 per 100,000 population and 25.4 per 100,000 populations respectively (Malaysia Cancer Statistics – Data and Figure 2007).

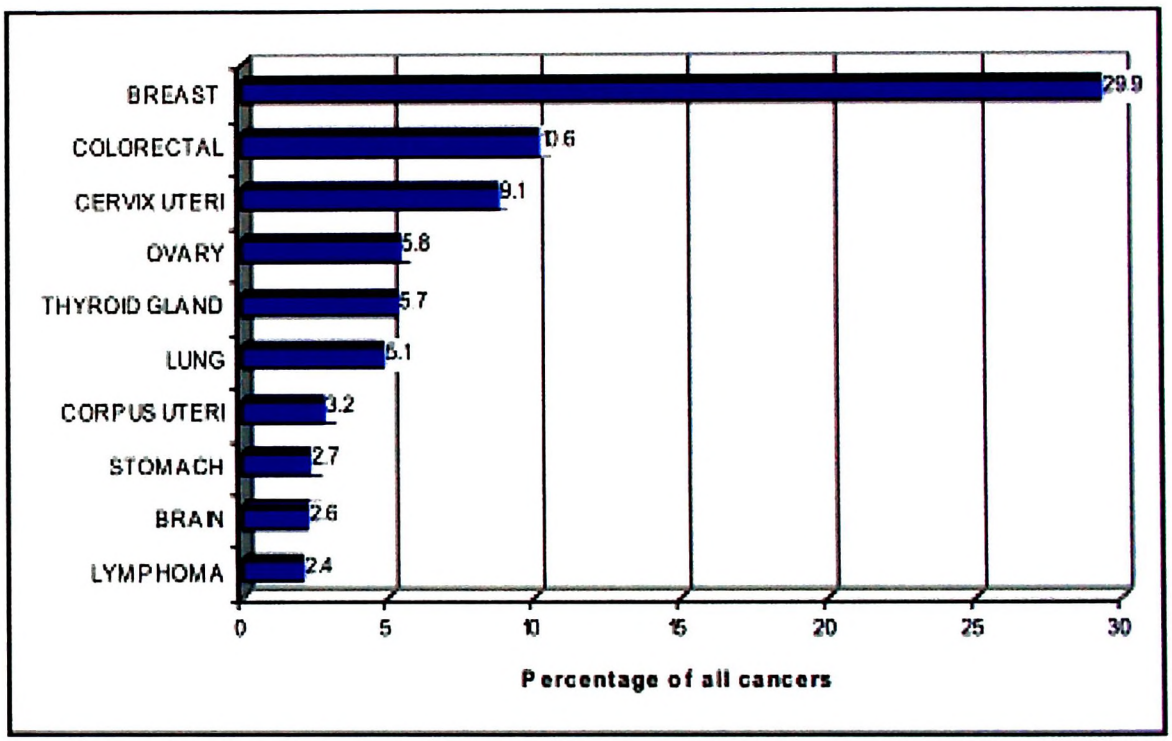

Figure 2.1: Ten most frequent cancers in females, Peninsular Malaysia 2006 (Source: Malaysian Cancer Statistics - Data and Figure, Peninsular Malaysia, 2006. http://www.makna.org.my/)

According to the Third National Cancer Registry Report (2008), the peak incidence for breast cancer is between the ages of 50 to 60 years of age except for

Indians where the peak is after the age of 60 years old. Generally, Malaysian women presents at later stages of breast cancer compared to other counterparts in the developing countries whereby 30 to 40% present at stage 3 to stage 4 involving mostly the Malays resulting poorer outcome compared to other ethnicities (Yip and Ibrahim, 2006). They reported the commonest presenting symptom was a lump in the breast (90%) and the mean size of the lump is 4.2cm (Dahlui *et al.*, 2011).

2.1.2 Treatment towards Breast Cancer

The old saying "Prevention is always better than cure" is particularly true in the case of cancer where a cure, if at all possible, is associated with high cytotoxic loads and/or invasive procedures. With our growing understanding of the molecular etiology of cancer, it has become apparent that strategies which limit DNA damage and/or increase the probability of DNA repair by inhibiting aberrant proliferation will decrease cancer incidence. Investigators have identified approximately 400 drugs, vitamins, hormones and other agents that might help in preventing cancer (Kaur *et al.*, 2011).

Although mutations in other known genes have also been identified, most of these mutations are located in BRCA1 and BRCA2 genes. Individuals with a strong family history of breast and certain other cancers, such as ovarian and colon cancer, should consider counseling to determine if genetic testing is appropriate. Prevention measures may be possible for individuals with breast cancer susceptibility mutations. In BRCA1 and BRCA2 mutation carriers, studies suggest that prophylactic removal of the ovaries and/or breasts decreases the risk of breast cancer considerably, though

not all women who choose this surgery would have developed breast cancer (American Cancer Society, 2013).

Nowadays, treatments of breast cancer involve radiation therapy, chemotherapy including before or after surgery, hormone therapy such as selective estrogen response modifiers, aromatase inhibitors, ovarian ablation, and targeted therapy. Until now, chemotherapy is one of the most widely used approaches for the treatment of many breast cancers, but the long-term use of chemotherapy can lead to drug resistance via several different mechanisms, such as gene mutation, DNA methylation and histone modification (Florea and Büsselberg, 2011). These resistance mechanisms have been reported to play important roles in the resistance of cancers to chemotherapeutic agents. Thus, development of anticancer agent from plant origin was targeted so as to combat the problems arose from the current drugs used.

Plants have been used for treating various diseases of human beings and animals since time immemorial. They maintain the health and vitality of individuals, and also cure diseases, including cancer without or less toxicity. More than 50% of all modern drugs in clinical use are natural products, many of which have the ability to control cancer cells (Madhuri and Pandey, 2009). A recent survey showed that more than 60% of cancer patients use vitamins or herbs as therapy (Sivalokanathan and Ilayara, 2005; Madhuri, 2008).

Therefore, a plant-based diet can help to shield us from cancer due to plants are rich in anti-inflammatory and antioxidant substances that help them to withstand

exposure to ultraviolet radiation, air pollution and other noxious substances .(White, 2012). Oxidative damage and inflammation promote cancer as well as a number of other chronic diseases (Reuter *et al.*, 2010). Furthermore, some plant chemicals enhance the body's detoxification systems, stimulate the immune system and have direct anticancer effects (White, 2012).

According to a study done, some examples of anti-cancer plants and food are being listed where some of them are cruciferous vegetables, asparagus, and *Alliums*, such as garlic and onions, which all contain sulfurous anticancer compounds. Furthermore, Lycopene, a carotenoid chemical found in high concentrations in tomatoes, pink grapefruit, watermelon and guava, has anticancer action. The same went to isoflavone genistein, which is found in soybeans (White, 2012). This study also mentioned that polyphenols, such as flavonoids, apart from contributing to the plant's colour, they also have anti cancer properties. For instance, fruits with deep red, purple and blue colors such as red grapes, cranberries, blueberries, pomegranates (White, 2012). Therefore this is another reason where why flavonoid is being chosen to be used as an anti-cancer plant in this study.

1.2 Apoptosis

Apoptosis, or physiologic cell death, is an endogenous cellular process whereby senescent, DNA-damaged or diseased cells are eliminated from the body. The term "apoptosis" is from Greek words meaning dropping off from falling of leaves from trees in autumn (Wong, 2011). This is in contrast to necrosis, where a

cell actively pursues a course toward death upon receiving certain stimuli (Kerr and Harmon, 1991).

Suppression of apoptosis in carcinogenesis plays a central role in the development and progression of cancer. Tumor cells use a variety of molecular mechanisms to suppress apoptosis (Elmore, 2007). The control of cell proliferation is crucial in maintaining cellular homeostasis and loss of this mechanism is a principle hallmark of cancer cells. Thus the inhibition of tumor cell growth without side effects is recognized as an important target for cancer therapy. Therefore, the research here we are searching for an alternative therapeutic approach towards the suppression of breast cancer cells.

Broadly, three main types of biochemical changes can be observed in apoptosis, which are the activation of caspases, DNA and protein breakdown and membrane changes and recognition by phagocytic cells (Kumar *et al.*, 2010). Apoptosis has been characterized biochemically by the activation of a nuclear endonuclease that cleaves the DNA into multimers of 180-200 basepairs and can be visualized as an 'oligosomal ladder' by standard agarose gel electrophoresis (Chompton, 1992). Therefore, morphological hallmarks of apoptosis in the nucleus are chromatin condensation and nuclear fragmentation, which are accompanied by rounding up of the cell, reduction in cellular volume (pyknosis) and retraction of pseudopodes (Kroemer *et al.*, 2005). From this morphological hallmark, therefore Hoechst stain and flow cytometry is conducted so as to detect the apoptotic activity of the flavonoid compound towards the breast cancer cell line.

2.3 Breast Cancer Cell Line

Generally, breast cancer cells can be divided into two categories according to their estrogen receptor (ER) status; the MCF-7 cell line is ER-positive and the MDA-MB-231 cell line is ER-negative. The former breast cancer cell line usually has a better prognosis, whereas approximately one-third of breast cancers are ER-negative with a worse prognosis (Zhang *et al.*, 2010). Moreover, MCF-7 cells were considered as a noninvasive cell line compared with the moderately invasive MDAMB-231 cells (Zhang *et al.*, 2010). Therefore, to find new anticancer agents that are effective in both ER-positive and ER-negative breast cancers is important, thus a reason for why MDA-MB-231 and MCF-7 breast cancer cell lines were used in this research.

2.3.1 MDA-MB-231 Cell Line

MDA-MB-231 is a type of epithelial cell isolated from pleural effusions of a breast cancer patient where it is a highly metastatic breast cancer cell line (Tu *et al.*, 2011). According to a study, it had mentioned that human metastatic breast cancer cells MDA-MB-231 are a well-known model for studying tumor aggressiveness, invasion and metastasis (Bachmeier *et al.*, 2011). Therefore it had served as a choice of breast cancer cell line to be studied here so as to see its association between the reductions of breast cancer tumor regression.

Furthermore, mitochondria derived from breast cancer cell line MDA-MB-231 with highly metastatic potency can up-regulate the baseline autophagic activities. Therefore, the ability of tumor mitochondria to increase autophagy may aid cancer cells in fulfilling high anabolic needs during rapid growth. Besides that, it also may

endorse cancer cells in order to survive under stressful cancerous environments (Tu *et al.*, 2011).

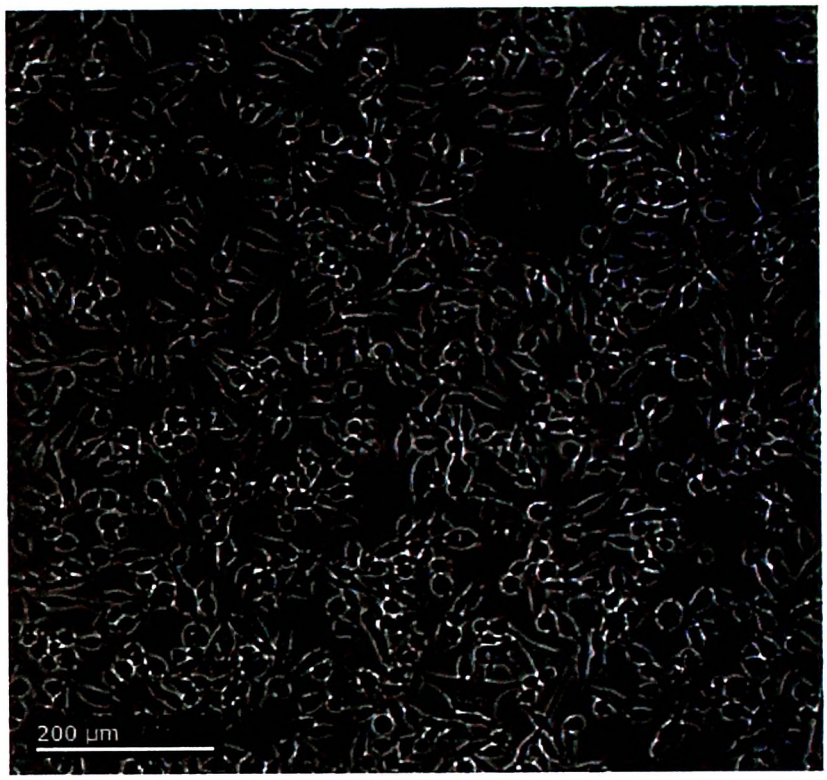

Figure 2.1: Cell morphology of MDA-MB-231 breast cancer cells

(Source: http://www.solentim.com/products/cell-gallery/cell-gallery-mdamb231/)

2.3.2 MCF-7 Cell Line

MCF-7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman (Saab *et al.*, 2010). Of the two mastectomies she received, the first revealed the removed tissue to be benign. Five years later, a second operation revealed malignant adenocarcinoma in a pleural effusion from which was taken cells for MCF-7. The woman was treated for breast cancer with radiotheraphy and hormonotherapy. The cells were then proven to be of human origin, and cytogenetic studies indicated a distinct stem line of 88 chromosomes. Dr. Sam

Brooks, working with Dr. Soule (2), first described the ER3 in MCF-7 cells by both Scatchard and sucrose density gradient analysis (Levenson and Jordan, 1997).

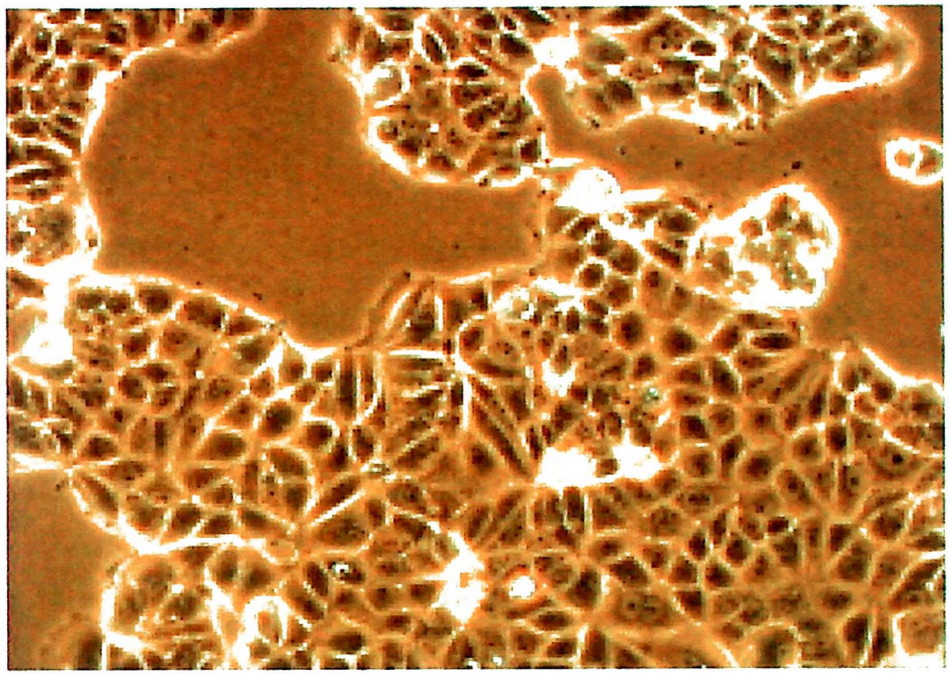

Figure 2.2: Cell morphology of MCF-7 breast cancer cells

(Source: http://www.tgrbio.com/cancer-cell-lines-primary-cell-cultures/cell-models-mcf7-cells.html)

MCF-7 cells are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium (Mohammadi *et al.*, 2013). These include the ability for MCF-7 cells to process estrogen, in the form of estradiol, via estrogen receptors in the cell cytoplasm (Coleman and Smith, 2001). This makes the MCF-7 cell line an estrogen receptor (ER) positive control cell line.

A study had identified the receptors for glucocorticoids, progestins, and androgens, as well as the estrogen receptor in MCF-7 breast cancer cells.

Furthermore in this study, it showed that MCF-7 may be an excellent in vitro model for studying the mechanism of tumor response to endocrine therapy as well as the complex relationships between binding and biological actions of these hormones (Horwitz *et al.*, 1975).

In addition to retaining their estrogen sensitivity, MCF-7 cells are also sensitive to cytokeratin. They are unreceptive to desmin, endothelin, GAP, and vimentin. When grown in vitro, the cell line is capable of forming domes and the epithelial like cells grow in monolayers. Growth can be inhibited using tumor necrosis factor alpha (TNF alpha), and treatment of MCF-7 cancer cells with anti-estrogens can modulate insulin-like growth factor finding protein's, which ultimately have the effect of a reduction in cell growth.

CHAPTER 3

MATERIALS AND METHOD

3.1 Materials

Artonin E and Chaplashin were supplied by Dr. Shajarahtum Jamil from Universiti Teknologi Malaysia. MDA-MB-231, MCF-7 breast cancer cells lines and L929 normal cell lines were obtained in cryopreservative form from Institute of Medical Research (IMR), Malaysia. Reagents such as Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotics mixture (penicillin-10,000 units/ml, streptomycin-10,000 µg/ml) were purchased from GIBCO Invitrogen (Auckland, New Zealand). Phosphate buffered saline (PBS) was purchased from Zymed Invitrogen (Carlsbad, USA). Trypsin, ethlenediaminetetraacetic acid (EDTA) and Hoeschst 33342 were purchased from Sigma Aldrich (Germany). 25 cm^2 cell culture flasks with standard red screw caps (without filter) were purchased from Greiner Bio One (Germany). 175 cm^2 flasks with 0.2 µm ventilated blue plug seal cap were purchased from Nunclon D (Denmark). 15 cm^3 and 50 cm^3 falcon tube were from BD Falcon (Franklin Lakes, USA). Glutaraldehyde, hydrochloric acid (HCl) 1.0 N solution, dimethyl sulfoxide (DMSO), and Cisplatin were purchased from Sigma-Aldrich Inc., USA, while methylene blue from BDH Chemical Ltd., Poole, England. Sodium chloride (NaCl) was purchased from Merck, Germany. FITC Annexin V Apoptosis test kit was purchased from BD Pharmingen.