EVALUATION OF ANTIPROLIFERATIVE ACTIVITY OF CHALCONE AND DIHYDROCHALCONE EXTRACT FROM *Artocarpus lowii* LEAVES ON BREAST CANCER CELL LINES

by

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TABLE OF CONTENT

CHAPTER 3.0: MATERIAL AND METHOD

LIST OF TABLES

LIST OF FIGURE

LIST OF ABBREVIATION

BSA	Bovine Serum Albumin
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
FBS	Fetal Bovine Serum
HCl	Hydrochloric acid
IC_{50}	Half maximal inhibitory concentration
NaCl	Sodium chloride
$NaHCO_3$	Sodium bicarbonate
Pen-Strep	Penicillin-Streptomycin
PBS	Phosphate buffer saline

LIST OF SYMBOLS

%	Percentage
µg/mL	Microgram per mililitre
cm^3	Cubic centrimetre
g	Gram
h	Hour
M	Molarity
mg/mL	Miligram per mililitre
rpm	Regulation per minute
v/v	Volume per volume
µL	Microlitre

ABSTRAK

Artocarpus sp kaya dengan sebatian fenolik seperti flavonoid, stilbenoid, arylbezofurons dan jacalin yang menyokong ciri farmakologinya sebagai sumber perubatan. Kajian sebelum ini menunjukkan bahawa beberapa penyelidikan farmakologi produk semulajadi daripada *Artocarpus* sp telah memberikan tindak balas dalam merawat pelbagai penyakit dan mempunyai faedah kesihatan yang banyak. Oleh yang demikian, dua sebatian flavonoid daripada Artocarpus lowii 2',4-dihydroxy-4-methoxy-3prenyldihydrochalcone (dihydrochalcone) dan 2',4-dihydroxy-3',4'-(2,2-dimethylchromene)chalcones (chalcone) diuji untuk aktiviti antiproliferasi terhadap sel-sel kanser payu dara manusia iaitu MCF-7 dan MDA-MB-231. Hasil menunjukkan kedua-dua sebatian chalcone dan dihydrochalcone menghasilkan IC_{50} di bawah 20 µg/mL yang merupakan satu nilai terapeutik kanser yang baik apabila diuji ke atas sel kanser MCF-7 dan MDA-MB-231. Walaubagaimanapun, sebatian dihydrochalcone memberikan nilai IC_{50} yang terbaik iaitu 1.023 µg/mL terhadap sel MCF-7. Sebatian ini juga merencat pertumbuhan sel normal, L929 tetapi pada nilai IC_{50} yang lebih tinggi iaitu 1.659 µg/mL. Sebatian ini kemudiannya dikaji mod kematian sel melalui pengesanan apoptosis menggunakan pewarnaan Hoechst 33258. Ciri-ciri sel yang mengalami apoptosis dapat dilihat dengan jelas pada sel kanser tersebut. Sel yang mengalami apoptosis juga dianalisis menggunakan teknik flositometri yang menunjukkan hanya sebahagian kecil jumlah sel mengalami apoptosis. Sebagai kesimpulan, sebatian dihydrochalcone daripada *A.lowii* menunjukkan kesan antikanser terhadap sel kanser payu dara MCF-7 melalui tindak balas apoptosis. Kesan yang dihasilkan juga boleh dijadikan sebagai petanda yang baik bagi melanjutkan penyelidikan

ini ke tahap yang lebih terperinci pada masa hadapan untuk menjadikan sebatian ini sebagai salah satu ubat antikanser.

ABSTRACT

Artocarpus species are rich in phenolic compounds including flavonoids, stilbenoids, arylbenzofurons and Jacalin which support their pharmacological properties as a medicinal sources. Previous pharmacological studies of the natural products from *Artocarpus* sp have established their mode of action in treatment of various diseases and other health benefits. Therefore, in this study two flavonoid compounds from *Artocarpus lowii*, 2,4-dihydroxy-4-methoxy-3prenyldihydrochalcone (dihydrochalcone) and 2,4-dihydroxy-3,4-(2,2-dimethylchromene)chalcones (chalcone) were tested for antiproliferative activity on human adenocarcinoma breast cancer cell lines, MCF-7 and MDA-MB-231. The findings showed that both of the compounds, dihydrochalcone and chalcone had IC_{50} lower than 20 µg/mL which were of good cancer therapeutic value on the above mention cancer cell lines. However, dihydrochalcone had the best IC_{50} value which was 1.023 µg/mL on MCF-7 cell line. This compound also inhibits the growth of normal cell line, L929 but at higher IC_{50} value which is 1.659 µg/mL. Mode of cell death caused by the compound was further tested by using Hoechst 33258 staining for apoptosis . The apoptotic features were seen in the treated cancer cells. Flow cytometry analysis also had been done as complement to Hoechst staining which revealed a small number of apoptotic cells. It is concluded that dihydrochalcone compound from *A.lowii* exert anticancer effect on MCF-7 breast cancer cell line through apoptosis. The results inspire future advanced studies in order to produce an anticancer drug from this plant.

CHAPTER 1

INTRODUCTION

1.1 Research background

Consumption of plant derived medicines is wide spread and increasing significantly in both traditional and modern medicine. According to The World Health Organization, more than 80% of the world population in developing countries depends primarily on plant based medicines for basic healthcare needs. One of the plants of interest is from genus *Artocarpus* sp which belong to Moraceae family. Hakim *et al* (2006) states that many members of the genus *Artocarpus* have been used as traditional folk medicine in Southeast Asia for the treatment of inflammation, malarial fever, ulcers, abcess, and diarrhea. Most of the pharmacological effects can be explained by the presence of phenolic compounds including flavonoids, stilbenoids and arylbenzofurons in *Artocarpus* sp.

As many biological activities of *Artocarpus* sp. were reported, it is therefore important to investigate the anticancer properties of natural flavonoids extracted from *Artocarpus lowii*. This plant, locally known as ''miku,'' is a rare species in Malaysia. Based on study done by Jamil *et al* (2008), there is one new prenylated dihydrochalcone extracted from *A.lowii* leaves which is 2,4-dihydroxy-4-methoxy-3-prenyldihydrochalcone. The compound together with two other known compounds, 2,4,4-trihydroxy-3-prenyl-chalcone and 2,4-dihydroxy-3,4-(2,2-dimethylchromene)chalcones were reported to have free radical scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Jamil *et al.*,2008)

Ubeda *et al.* (1995) stated that many flavonoids have the ability to scavenge free radicals. The free radicals has been known to play an important role in the cancer development through oxidative DNA damage (Dreher and Junod, 1996). Cancer is a term used for disease with abnormal dividing cells that can invade other tissue. There are many types of cancer and the name depends on the origin of the cancer. The growth of cancer cell is different from the growth of normal cells. Instead of dying, cancer cells tend to grow uncontrolled and continuously and later become a mass or so called tumor. Cancer cell form when its genetic material (DNA) had undergones mutation or damage. Unlike the normal cells, genetically damagr cancer cells do not undergo repair or death program but keep on dividing.

There are two classifications of tumour which are benign and malignant. Benign is not cancerous and do not spread to other parts of the body. However, some could be precancerous and may progress to cancer if untreated. Malignant is cancerous and if not treated early, may spread and affect other parts of the body and thus becoming invasive cancer. Cancer can be further categorized into primary and secondary cancer. The site where the cancer started is called the primary cancer. The site the cancer spreads to is called the secondary cancer.

Currently throughout the world, the most common cancer among women is breast cancer. According to College of Radiology Breast Health Information Centre, in 2000, there were 1,050,346 cases reported with 372,969 deaths from breast cancer worldwide. The incidence ranged from an average of 95 per 100,000 in more developed countries to 20

per 100,000 in less developed countries. The National Cancer Institute estimates that by age 50, one out of every 50 women will develop breast cancer. By age 80, it will rise to one in 10. According to Nirmala *et al.* (2011), the incidence of breast cancer has escalated in most Asian countries over the past two decades.

Recent methods to treat cancer patient include surgery, chemotherapy, radiotherapy and hormonal therapy. Usually, surgery will come first to treat the breast cancer. There are two types of surgery to remove cancerous tissue; a lumpectomy which removes the breast lump while mastectomy which removes all or part of the breast and possible nearby structure. Meanwhile, chemotherapy prevent cancer cells from growing and spreading by destroying the cells or stopping them from dividing. Chemotherapy weakens and destroys cancer cells at the original tumor site and throughout the body. In many cases, chemotherapy medicines are given in combination, which means two or three different medicines are given at the same time. However, since chemotherapy is very toxic to the cells, it has high chances to kill normal quickly dividing cells also. Some of the common side effects of chemotherapy include anemia, hair loss, fatigue, appetite change, diarrhea, infertility, pain and infection (Cancer Research, United Kingdom 2013)

Therefore, it is important to look for other alternatives which can minimize or prevent side effects of chemotherapeutics. In this study, two flavonoid compounds, 2,4-dihydroxy-3,4-(2,2-dimethylchromene)chalcones (chalcone) and 2,4-dihydroxy-4-methoxy-3-prenyldihydrochalcone (dihydrochalcone) from *A.lowii* leaves had been tested for anticancer effect towards breast cancer cell line (MCF-7 and MDA-MB-231).

1.1 Researh Objectives

General Objective:

The aim of this study is to evaluate anticancer properties of natural flavonoids from *A.lowii* leaves extract.

Specific objectives:

- To determine anticancer activity of natural flavonoids isolated from leaves *A.lowii* towards breast cancer cells MCF-7 and MDA-MB-231
- To elucidate mode of cell death of the cancer cells treated with the most potent anticancer agent

1.2 Experimental Design

In this study, two flavonoid compounds derived from *A.lowii* leaves were screened for antiproliferative activity towards breast cancer cell lines (Figure 1.1). The two compounds used were chalcone and dihydrochalcone and the selected breast cancer cell lines were MCF-7 and MDA-MB-231. A type of non-cancer cell or normal cell, L929 was also used as a control. Anticancer effect of the plant extract on cancer cells were studied using methylene blue assay. All the cell lines were cultured in 96 well plates in the presence of the compound and dimethylsulfoxide (DMSO) as negative control for 72 hours. Tamoxifen was used as positive control. The viable cells were stained with methylene blue and the absorbance was read at 655 nm using microtiter plate reader. The IC_{50} value was determined from the graph percentage of viability against final concentration of the compounds tested. The best IC_{50} of the compound on selected cancer cell was chosen to be directed to death mechanism analysis. Nuclear fragmentation assay using Hoechst 33258 staining was carried out for apoptosis detection. The cells treatred with flavonoid compound, DMSO and tamoxifen for 72 hours were analyzed for nuclear morphology with fluorescent microscopy. Flow cytometry analysis was also had been done in order to identify the stage of apoptosis.

Flowchart

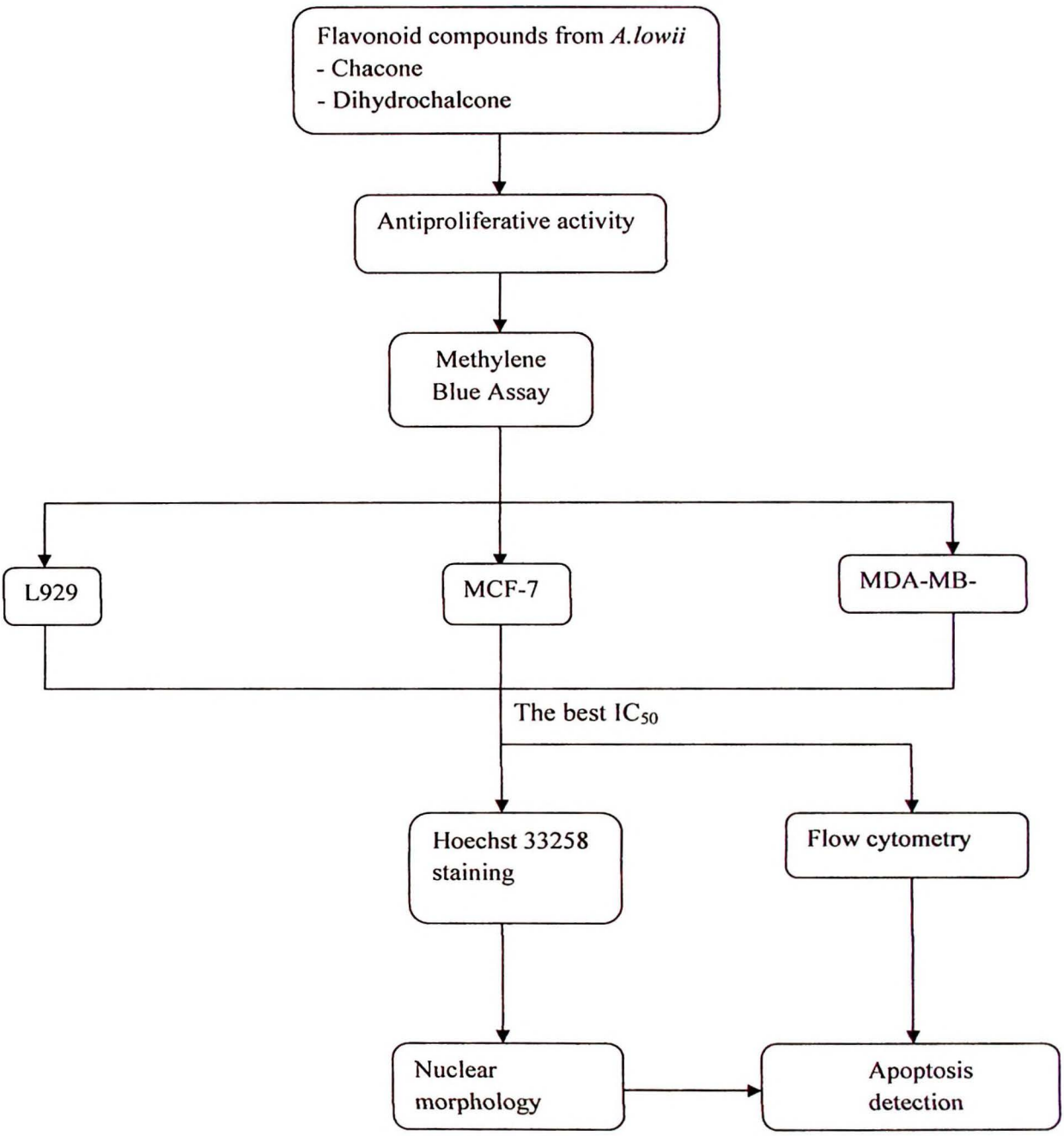

Figure 1.1: The flowchart of experimental design

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer has become health burden and arouses worldwide great concern . According to World Health Organization (WHO), 7.6 million people worldwide died from cancer in 2008 and approximately 70% of cancer deaths occur in low- and middle-income countries. Among racial and ethnic groups, the highest cancer incidence rates and death rate between 2004 and 2008 were among black men and white women. In estimation, new cases of cancer in the United State in 2012 is 1,638,910 with 577,190 death (National Cancer Institute)

Malaysia's Cancer Statistics reported that a total of 18,219 new cancer cases were diagnosed in 2007. It comprises of 8,123 (44.6%) males and 10,096 (55.4%) females. The ten leading cancers among population of Malaysia in 2007 were breast, colorectal, lung, nasopharynx, cervix, lymphoma, leukaemia, ovary, stomach and liver. The National Cancer Registry of Malaysia (NCR) records 21,773 Malaysians being diagnosed with cancer but estimates that almost 10,000 cases are unregistered every year. Increasing population and longer life spans contributes to rise of cancer. Less than 10% of cancers happen in children compared to over 50% in men and 35% in women aged 50 and above.

2.1.1 Breast Cancer

Breast cancer is the most common malignancy among females affecting approximately one out of ten women (Imyanitov *et al.*,2004). Therefore, it becomes the most burden disease among women in the world. One epidemiological study based on Malaysia and Singapore hospitals have found that approximately 50% of the patients were diagnosed before the age of 50 years and 15% of patients were aged younger than 40 years. The majority of patients were of Chinese ethnicity (72%), followed by Malays (16%), Indians (8%), and other races (4%). There are two main types of breast cancer, which are ductal carcinoma which starts in the ducts that deliver milk from the breast to the nipple and lobular carcinoma which starts in the parts of the breast called lobules that produce milk.

MCF-7 is a cell line that was first isolated in 1970 from the breast tissue of a 69-year old Caucasian woman. The first removed tissue was revealed to be benign but years later, a second operation revealed malignant adenocarcinoma in a pleural effusion from which MCF-7 cell was taken. MCF-7 cells are used widely for studies of tumor biology and mechanism of action of hormone (Osborne *et al.*,1987). MCF-7 cells also are useful for *in vitro* breast cancer studies because the cell line has retained several ideal characteristics particular to the mammary epithelium. These include the ability for MCF-7 cells to process estrogen via estrogen receptors or in other word MCF-7 cell is Estrogen-Receptor (ER) positive. MCF-7 cells are also sensitive to cytokeratin. When grown in *vitro*, the cell line is capable of forming domes and the epithelial like cells grow in

monolayers. Growth can also be inhibited using tumor necrosis factor alpha (TNFα). A study done by Coezy *et al* (1982) support the hypothesis that antiestrogens control the growth of breast cancer by acting directly on the estrogen-receptor located in cancer cells.

The MDA-MB-231 breast cancer cell line was obtained from a patient in 1973 at M. D. Anderson Cancer Center. The MDA-MB-231 breast cancer cells appear phenotypically as spindle shaped cells with epithelial-like morphology. *In vitro*, the MDA-MB-231 cell line has an invasive phenotype. The cell line is thought to be more aggressive due to its capability of metastasis. A study done by Abdelkarim *et al* (2011) showed that invading basement membrane matrix is sufficient for MDA-MB-231 breast cancer cells to develop a stable *in vivo* metastatic phenotype.

2.2 Plant-derived Anti-cancer Agent.

In general, cancer treatment nowadays include surgical, radiotherapy and chemotherapy. Surgery is not a definite curative treatment as the disease can recur. On the other hand, chemotherapy has many side effects and is toxic to the normal cells. Radiotherapy is not very effective for all types or stages of cancer. Therefore, world nowadays has turn to explore a new field about natural products which are thought to be more safe. Many researches had been done on use of plant extraction as anticancer agent because many of them are proven to have that kind of capability (Cragg and Newman, 2005).

2.2.2 Clinically used compound

Plants had created its own story in the medical history. The study for anti-cancer agents from plant sources started in the 1950s with the discovery and development of the vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins (Cragg and Newman, 2005). A research done by Graham *et al* (2000) reported that over 350 species were used against cancer all over the world. The study was done as an extension of the work of Jonathan Hartwell which previously listed over 3000 plants species that being used against cancer in his series of articles work.

According to Nirmala *et al* (2011) until now there are four major structural classification of plant-derived anti-cancerous compounds which are vinca alkaloids, epipodophyllotoxin lignans, caxane diterpenoids and camptothecin quinoline alkaloid derivatives. The first agents introduced into clinical use were the vinca alkaloids, vinblastine and vincristine which were isolated from the Madagascar periwinkle, known as *Catharanthus roseus* G. Don. (Apocynaceae) (Cragg and Newman, 2005; Shah *et al.*, 2013). The mechanism of action of vinca alkaloids is that they inhibit the cell proliferation by affecting the microtubular dynamics during mitosis and leading to apoptosis. They showed potential activity against leukemia, lymphomas, advanced testicular cancer, breast cancer, lung cancer and Kaposi's sarcoma when treated in combination with other chemotherapeutic drugs (Cragg and Newman, 2005).

Other agent that is clinically used is podophyllotoxin. The compound was obtained from the roots of *Podophyllum* species, namely, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich. It was isolated in 1880s, and its structure was elucidated in 1950s. Epipodophyllotoxin is an isomer of podophyllotoxin. The two clinically important semi-synthetic analogs generated from epipodophyllotoxin are etoposide and teniposide which were found very potential in treating lymphomas, bronchial and testicular cancers (Shah *et al.*, 2013).

Another group of plant-derived chemotherapeutic agent is the taxanes. Paclitaxel (taxol) initially was isolated from the bark of the Pacific Yew, *Taxus brevifolia* Nutt. Due to poor water-soluble and toxic, Docetaxel (Taxotere), a semi-synthetic analog was derived from paclitaxel and found to be more effective (Nirmala *et al.*,2011). Paclitaxel is used in the treatment of the breast, ovarian, and non-small cell lung cancer (NSCLC). Paclitaxel has also attracted attention in the potential treatment of multiple sclerosis, psoriasis and rheumatoid arthritis and has also shown efficacy against Kaposi sarcoma. Whereas, docetaxel is primarily used in the treatment of breast cancer and NSCLC (Cragg and Newman, 2005). The mechanism of action is that these active agents bind to the polymerized microtubules which prevent the normal mitosis to occur. Paclitaxel inhibits the cell-cycle traverse at the G2-M phase junction while docetaxel produces its maximum cell-killing effect against cells in the S phase (Nobili *et al.*,2009)

Camptothecin is an active agent isolated from the Chinese ornamental tree, *Camptotheca acuminate.* It is a cytotoxic alkaloid and because it showed poor solubility

and severe toxicity, a few analogs are synthesized. Two semi-synthetic analogs of camptothecin clinically used are topothecan and irinothecan. The analogs work by inhibiting DNA Topoisomerase I which later interrupt the replication and transcription of DNA. Topothecan is used for the treatment of ovarian and small cell lung cancers, while irinotecan is used for the treatment of colorectal cancers (Cragg and Newman, 2005; Nirmala *et al.*,2011)

2.2.3 Flavonoids

Flavonoids are one of the phenolic compounds which are widely distributed in most plants. It refers to a plant pigment and gives colors to the plant itself. Flavonoids are claimed to have a beneficial medical effect by many medical practitioners. One reason is that flavonoids are abundant in the natural products that are used for treatment (Havsteen , 2002). One of the most useful properties of many flavonoids is their ability to scavenge free radicals (Gyorgy *et al.*,1992; Ubeda *et al.*,1995).

Free radicals are chemical species with unpaired electrons in outermost orbit, very reactive and are harmful to the body. The source of free radical can be endogenous or exogenous. Body metabolism, stress, lifestyle, infection and radiation are some example of some situation that produce free radicals. According to Agati *et al* (2012), stress-responsive dihydroxy B-ring-substituted flavonoids have great potential to inhibit the generation of reactive oxygen species (ROS) and reduce the levels of ROS once they are formed.

As cancer is caused by the disturbance in growth metabolism, flavonoids are also capable of influencing the growth regulation of human cells, but they must be encapsulated to be effective (Havsteen, 2002). The inhibition of the topoisomerase II by flavonoids, shows that quercetin and similar flavonoids can induce a mutation, a single-strand break that releases the operation of the repair enzyme. Besides, flavonoids also act on the cancer cells by inhibiting the glucose transporter in the plasma membrane, which furnishes such cells with glucose for glycolysis (Salter *et al.,*1978; Hume *et al.,*1979). Two examples of herbal preparations that recognize as effective anticancer drugs from flavonoids are propolis and Essiac (Grunberger *et al.*,1988; Havsteen, 2002).

2.3 *Artocarpus* sp

2.3.1 Ethnopharmacological and phytochemical studies

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Artocarpus sp. is one of the genus members in Moraceae family. According to Hakim *et al* (2006), the genus *Artocarpus* comprises about 50 species of evergreen and deciduous trees, with all parts containing white latex. The *Artocarpus* plant has been recognized as economic sources of edible fruit such as *Artocarpus heterophyllus* (jackfruit), *Artocarpus altilis* (breadfruit) and *Artocarpus chempeden* (Chempedak) and yielding fairly good timber (Jagtab and Bapat, 2010). Other than that, some of *Artocarpus* sp are used as traditional folk medicines in South-East Asia, Indonesia, Western part of Java and India.

Hakim *et al* (2006) stated that over 60 phenolic constituents have been discovered and characterized, including 27 new compounds from 13 Indonesian taxa of Artocarpus, namely *A. champeden, A. lanceifolius, A. teysmanii, A. scortechinii, A. rotunda, A. maingayi, A. kemando, A. bracteata, A. altilis, A. fretessi, A. gomezianus, A. reticulatus and A. glaucus.* Many studies had been done on various species of *Artocarpus* plant due to their promising constituents as a new treatment for various diseases.

Artocarpus altilis also known as breadfruit, is a widely known food source but is also commonly used as a folk medicine in Indonesia where it is locally called sukun. Traditionally, the leaves of sukun are used for the treatment of various kinds of diseases such as liver cirrhosis, hypertension and diabetes (Arung *et al.,*2009). Scientifically, some biological activities of the extract of this plant have been reported. The methanol or dichloromethane extract from bud covers of sukun was shown to have activity in a cathepsin K inhibition assay (Arung *et al.,*2009). Delaisse *et al* (1980) reported that cathepsin K inhibitors are very effective in preventing bone resorption, and therefore, be a potential treatment option for osteoporosis. A study done by Bhoonphong *et al* (2007) showed antimalarial activity of the roots of *Artocarpus altilis* which led to the isolation of nine prenylated flavones which exhibited moderate antiplasmodial activity with IC_{50} values ranging from 1.9 to 4.3 μg/mL

Artocarpus heterophyllus or jackfruit is rich in protein and the protein content is higher than those from high protein animal sources such as beef and marine fishes. Also, its seeds were found to be good sources of minerals including iron (Jagtab and Bhapat,

2010). The methanolic plant extract consisting of two active isoprenyl flavones artocarpin and artocarpesin were isolated from *Artocarpus heterophyllus.* These inhibited the growth of primary cariogenic bacteria and also exhibited the growth inhibitory effects on plaque-forming *Streptococci.* This finding showed that phytochemicals from *Artocarpus heterophyllus* would be potent compounds for the prevention of dental caries (Sato *et al.,* 1999).

Chalcone and dihydrochalcone are another form of flavonoids which were just recently isolated from *A.lowii* leaves. Very few researches have been done on this plant and it was believed that the first study was conducted by Jamil *et al* (2008). The study showed that, the compounds extracted from this plant have strong free radical scavenging activity towards 2,2-diphenyl-1-picrylhydrazyl (DPPH) when measured by electron spin resonance (ESR) spectrometry.

2.4 Tamoxifen

Tamoxifen has become a drug of choices for breast cancer therapy. A study carried out by Yu *et al* (2002) have found out that both E2 and E1 receptors could be activated by epoxidation resulting in their ability to inhibit DNA-dependent RNA synthesis and to bind to DNA forming DNA adducts both in vitro and in vivo . These experimental results have therefore provided a strong molecular basis for an initiation role of E2 and E1 in breast cancer carcinogenesis. Since tamoxifen is an antiestrogen, it is used in breast cancer therapy because it competes with E2/E1 at the estrogen-receptor sites and blocks the promotional role of E2/E1 in breast cancer carcinogenesis . A study conducted by Alkner

et al (2009) on premenopausal women have found that untreated women, regardless of age, had a 12% risk, and women with less than 40 years of age had a 20% risk of developing a contralateral metachronous breast cancer with a median follow-up time of 14 years and adjuvant treatment with tamoxifen for 2 years reduced this risk by 50% in all women, and by 90% in women with less than 40 years of age.

2.5 Apoptosis

Apoptosis also known as programmed cell death, is a normal component of the development and health of multicellular organisms. Cell undergoes apoptosis in response to a variety of stimuli in a controlled and regulated manner. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death which is often referred to as cell suicide. A family of proteins known as caspases are typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNAses, which begin to cleave the DNA in the nucleus.

Apoptosis is different to necrosis morphologically and physiologically. The terms 'apoptosis' is to mean active cell death occurring via physiological or pathological stimuli whereas 'necrosis' being the cell death process involving catastrophic failure of cellular homeostasis seen after major pathological cellular injuries (Raffray and Gerald, 1997). Table 2.1 lists some morphological comparisons between apoptosis and necrosis.

Table 2.1: Comparison between apoptosis and necrosis

Source: Baba, 2009

	Apoptosis	**Necrosis**
Histology	Isolated cells affected in healthy tissues	Cells that die together, with structural disintegration
Cytology	Pyknotic nuclei, condensed cytoplasm, round cell fragments	Cellular edema, intact but poorly stained nuclei
Exclusion test by staining agents	The cell membrane is not permeable to staining agents	The cell membrane is permeable to staining agents
Cytoplasmic ultrastructure	Intact, compact organelles, intact cytoplasmic membrane	Significantly increased, swollen mitochondria and matrixdensification, rupture of plasma and internal membrane
Nucleus	Capsular and toroidal chromatin condensation	The primary chromatin pattern is maintained with a normal distribution

circumstances	Frequently in 'programmed death', atrophy, immunomediated cell death	Never physiological, hypoxia
Tissue effects	Non-inflammatory, phagocytosis induced by adjacent cells, rapid involution without general tissue collapse	Acute inflammatory, subsequent scarification,

CHAPTER 3

MATERIAL AND METHOD

3.1 Compounds

Two flavonoid compounds, 2',4-dihydroxy-4-methoxy-3-prenyldihydrochalcone (ALLD-721) and 2',4-dihydroxy-3',4'-(2,2-dimethylchromene)chalcones (ALBD-2C) were supplied by Dr. Shajarahtunnur Jamil from Natural Product Research Laboratory, School of Chemistry, Universiti Teknologi Malaysia. Both of the compounds were isolated from leave of *Artocapus lowii* whereas Tamoxifen was purchased from Merck Sdn. Bhd.

3.2 Materials

Phosphate Buffer Saline (PBS) was purchased from Zymed Invitrogen, Dulbecco's Modified Minimum Essential Medium, Hoescht 33258, Tryphan blue and Triton X-100 purchased from SIGMA, Penicillin-Streptomycin from Gibco Invitrogen, Sodium chloride, methanol, sodium hydrogen carbonate were brought from Merck, Dimethyl sulfoxide, Hydrochloric acid 0.33 M solution, gluteraldehyde 25 % aqueous solution and Methylene Blue from Sigma Co. and Annexin V-FITC, BD 556420 kit from BD Pharmingen™. Other components such as cell culture flask and filter tips was purchased from Greiner-Bio-One, microtube 1.5 ml from Genuine AXYGEN Quality and 5 mL polystyrene Round-Bottom Tube from BD Falcon™.

3.3 Cell lines

The breast cancer cell lines that are used in this research were MCF-7 and MDA-MB-231 which are adenocarcinoma breast cancer cells. As for normal cell, L929 fibroblastic cell from mouse has been choosen. All the cell lines were brought from American Type Culture Collection (ATCC)

3.4 Instrumentation and Appliances

NUAIRE^{TM} IR Autoflow CO_2 Water-Jacked Incubator, Type II Biohazard Safety Cabinet Series Erla Technologies, Inverted Microscope Leica DMIL, Centrifuge (Hettich Zentrifugen), Shaker (Heidolph-Rotamax 120, Germany), Water Bath (Memmert wb22), BD FACSCalibur^{TM} Flow Cytometer and Microplate Reader Biorad Moedel 680.

3.5 Antiproliferative Assay

3.5.1 Methylene Blue Assay

Methylene Blue Assay was done to count the residual live cells after the treatment of selected compounds which the percentage of reduction in cell number was calculated to get the IC_{50}. It was utilized to estimate the antiproliferative effect of compound. The assay depends on the binding of Methylene Blue to the fixed monolayer at pH 8.5 and, after washing the monolayer, release of dye by lowering pH. Elution solvent containing acidified ethanol was used ensures a linear correlation between absorbance of the dye and cell number, as the number of cell increases, the intensity of the dye also increases.The assay is rapid, highly reproducible and easy to perform, making it ideal for screening large numbers of samples. It was shown to be applicable to a number of foetal and adult cell lines derived from man and experimental animals (Oliver *et al.,*1989)

3.5.2 Culturing the cell

Cryopreserved cells were thawed first and then transferred into falcon tube filled with 5 mL complete medium and centrifuged at 1200 rpm for 5 minutes. Complete medium contains Dulbecco's Modified Eagle's Medium (DMEM), 5 % Fetal Bovine Serum (FBS) and 1% antibiotics (penicillin-streptomycin). FBS is globular protein which acts as nutrient for the cells to survive and proliferate. Meanwhile, antibiotic was supplied to prevent bacterial contamination. After centrifugation, pellet containing cells settled down and the supernatant was discarded. 1 mL complete medium was added to re-suspend the cells. The cell suspension

then was transferred into the new flasks filled with 5 mL fresh complete medium. Cells were incubated in a humidified atmosphere of 5% CO_2 incubator at 37°C. All culturing work were handled using sterile techniques and done in Biosafety Cabinet Class II.

3.5.2 Cell sub-culture

In this procedure, cell lines must be at least 80-90% confluent. When the confluency was achieved, old medium was discarded. Then, the cells were washed with PBS three times before trypsinization process. Next, 1 mL trypsin-EDTA was added and the flask was incubated in 5% CO_2 incubator at 37°C. After 5 minutes, the flask was tapped to dislodge the attached cell and observed under the inverted microscope. The detached cells seen were round in shape. Neutralization of trypsin was done by adding 1mL of medium into the flask. Too much of trypsin or too long exposure to trypsin can kill the cell. The cell suspension was pipetted into 15 mL falcon tube and spun at 1000 rpm for 5 minutes. Supernatant was discarded from the tube and 2 mL of fresh medium were added. The cells suspension was resuspended gently. Cells suspension was aliquot into few new 25 cm^3 flasks filled with 5 mL fresh complete medium. The flasks were then incubated in a humidified atmosphere of 5% CO_2 incubator at 37°C.

3.5.3 Cell treatment

A. Cell collection

The cells were cultured in 75 cm^3 flask until it reached 80% confluence for seeding into 96 well plate. When the cells reached 80% confluency, old medium was discarded. Then the cells were washed with PBS three times before trypsinization. Then, 2 mL trypsin-EDTA was added into the flask and will be incubated in the incubator for 5 minutes. After 5 minutes, the flask was taken out and observed under microscope for cell detachment. Detached cells would appear round and shining under microscope. Once the cells were detached, 2 mL fresh complete medium was added into the flask to inactivate the trypsin in the cell suspension. Gentle pipetting of this suspension would then help to break up any cells clusters into single cells. Cell suspension was pipette into 15 mL falcon tube and spun at 1000 rpm for 5 minutes. Pellet containing cells settled down and the supernatant was discarded. The viable cells were resuspended and counted using tryphan blue and hemacytometer.

B. Cell Seeding and Treatment

Only cells that have at least 80% viability will be seeded into 96 wells microtiter plate. For microtiter plate, media needed around 6 ml (100 µL x 60 wells). The exact volume of cell suspension containing 5 X 10^4 cell/mL was transferred into a petri dish followed by pouring 6-8ml media into the same petri dish. The petri dish was shaken in

rotating motion to ensure even spreading. The petri dish was held slant and resuspended again with multichannel pipette. After that, 100 µL medium that contained the cell was seeded into central 60 wells of microtiter plate. The well at four edges of the plate was filled with distilled water to prevent the plate from drying during incubation. Next, the microtiter plate was incubated overnight to allow cell attachment onto the surface of the wells. After 24 hours incubation, the cells were checked under microscope for the attachment and 80 % confluency. After confluency was achieved, the old media was discarded and 200 µL of new medium will be added. Then the cells in the first column of the seeded wells were treated with 2 µl of 10 mg/mL compound followed by 5 mg/ml in the second column until 0.0391 mg/ml in the 9^{th} column. 2 µL of DMSO (negative control) at 1% concentration was to treat cells in the 10^{th} column. The last column was left blank which contained media only. The procedure was done to the first three rows. Another three rows were treated with tamoxifen (positive control). Three 96 well plates were set up for MCF-7, MDA-MB-231 and L929 cell lines.

Table 3.1 : Final concentration of tamoxifen and compounds extracted from *A.lowii* used for treatment of cancer cell lines

Extract concentration (mg/ml)	**Final concentration in well (mg/ml)**	**Final concentration in well (µg/ml)**	**Log $_{10}$ final concentration (mg/ml)**
10.0000	990.00×10^{-4}	99.00	-1
5.0000	495.00×10^{-4}	49.50	-1.31
2.5000	248.00×10^{-4}	24.80	-1.61