IN VITRO INVESTIGATION OF CYTOTOXICITY AND APOPTOSIS INDUCTION BY METHANOL EXTRACT OF *Calophyllum inophyllum* FRUIT IN MCF-7 HUMAN BREAST CANCER CELL LINE

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by

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µg/mL	Microgram per milliliter
µM	Micro molar
AA	Arachidonic acid
ACS	American Cancer Society
ADP	Adenosine diphosphate
AIF	Apoptotic inducing factor
ANOVA	Analysis of variance
ANT	Adenine nucleotide translocase
Apaf-1	Apoptotic protease activating factor-1
APC	Anaphase promoting complexes
As_2O_3	Arsenic trioxide
ATP	Adenosine triphosphate
B-CLL	B-cell chronic lymphocytic leukemia
BSA	Bovine serum albumin
C. inophyllum	*Calophyllum inophyllum*
CAM	Complementary and Alternative Medicines
CARD	Caspase-recruitment domain
CCCP	Carbonyl cyanide m-chlorophenyl hydrazone
CDC	Centers for diseases control and prevention
CDK	Cyclin-dependent protein kinase
CEN	Chicken erythrocyte nuclei
cIAP	Cellular IAP
COX	Cyclooxygenase

CTN	Calf thymocyte nuclei
DAPI	4',6-diamidino-2-phenylindole
dATP	Deoxyadenosine triphosphate
DCF	Dichlorodihydrofluorescein
DCFH	2', 7'-Dichlorodihydrofluorescin
DCFH-DA	Dichlorodihydrofluorescin diacetate
ddH_2O	Double distilled water
DI H_2O	Deionised water
DISC	Death-inducing signalling complex
DMEM	Dubelcco's Minimum Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EBCTCG	Early Breast Cancer Trialists' Collaborative Group
EBV	Epstein-Barr virus
EBV-EA	Epstein-Barr virus early antigen
EEC	European Economic Community
ESI	Electrospray ionization
$FADH_2$	Flavin-adenine dinucleotide
FBS	Fetal bovine serum
FDA	Food and Drug Administration
GAE	Gallic acid equivalent
GSHPx	Glutathione peroxidase
H_2O_2	Hydrogen peroxide
HIV-1	Human immunodeficiency virus type 1

HIV-IR	HIV-1 integrase
HIV-PR	HIV-1 Protease
HPV	Human papilloma virus
IAP	Inhibitors of apoptosis protein
IC_{50}	50% Inhibitory concentration
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolo carbocyanine iodide
KB cells	Keratin forming Hela tumor cell line
MSDS	Material Safety Data Sheet
MTS	5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)- 3-(4-sulfophenyl)tetrazolium
MTT	3-(4,5-dimethylthiazolyl)-2,5- diphenyltetrazolium bromide
Na_2CO_3	Sodium carbonate
NAACCR	North American Association of Central Cancer Registries
NADH	Nicotinamide adenine dinucleotide
NCI	National Cancer Institute
NF-κB	Nuclear factor-kappa B
OD	Optical density
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PCD	Programmed cell death
PEITC	Phenethyl isothiocyanate
PI	Propidium iodide
pmf	Proton motive force

*p*NA	*p* nitroanilide
PS	Phosphatidylserine
PT	Permeability transition
PTP	Permeability transition pore
PTPC	Permeability transition pore complex
QTOF-MS	Quadrupole time of flight mass spectrometer
RB	Retinoblastoma
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutases
TNF	Tumor-necrosis factor
TNFR	TNF receptor
TRAIL-R	TNF-related apoptosis-inducing ligand-receptor
UV	Ultraviolet
VDAC	Voltage-dependent anion channel
WHO	World Health Organization
WST-1	(4-[3-4-iodophenyl]-2-(4-nitrophenyl)-2H-5-tetrazolio)- 1,3-benzene disulfonate
XIAP	X chromosome-linked IAP
XTT	2,3-bis(2-methoxy- 4-nitro-5-sulphophenyl)-5-carboxanilide-2H-tetrazolium

KAJIAN SITOTOKSIK SECARA *IN VITRO* DAN INDUKSI APOPTOSIS OLEH EKSTRAK METANOL BUAH *Calophyllum inophyllum* TERHADAP TITISAN SEL PAYUDARA MANUSIA MCF-7

ABSTRAK

Calophyllum inophyllum adalah sejenis tumbuhan bakawali yang kaya dengan nilai-nilai perubatan yang telah dimanfaatkan secara meluas dari zaman dahulu lagi untuk menyembuhkan pelbagai penyakit. Walaupun *C. inophyllum* termahsyur dengan sejarah perubatan tradisionalnya, tetapi hanya sedikit bukti saintifik yang melaporkan tentang ketoksikan *C. inophyllum* khususnya buah *C. inophylum,* dimana tiada kajian langsung yang melaporkan tentang mekanisma tahap molekul yang komprehensif. Oleh itu, penyelidikan ini telah dilaksanakan untuk menentukan ketoksikan ekstrak buah *C. inophyllum* secara *in vitro* terhadap titisan sel kanser manusia MCF-7 dengan penjelasan terperinci mengenai mekanisma tahap molekular disebalik kematian sel kanser. Dalam kajian ini, pengesahan spesies tumbuhan serta analisis jumlah kandungan fenol telah dijalankan. Kesan sitotoksisiti ekstrak buah *C. inophyllum* terhadap titisan sel kanser MCF-7 telah dinilai dengan menjalankan ujian-ujian MTT dan CyQuant yang menunjukkan perencatan pertumbuhan sel MCF-7 dengan nilai IC_{50} sebanyak 19.63 µg/mL dan 27.54 µg/mL masing-masing. Penelitian terhadap morfologi sel MCF-7 setelah dirawat dengan ekstrak buah *C. inophyllum* pada kepekatan IC_{50} (23.59 µg/mL) telah memaparkan ciri-ciri histologi apoptosis yang jelas. Analisis Sitometri-Aliran dengan pewarnaan Annesin V/ Propidium Iodida ke atas sel MCF-7 yang dirawat dengan ekstrak buah *C. inophyllum* telah menunjukkan penginduksian apoptosis yang bergantung kepada dos kepekatan ekstrak. Selain itu, analisis Sitometri-Aliran tentang kitaran sel

menunjukkan bahawa rawatan ekstrak buah *C. inophyllum* mengakibatkan rencatan kitaran sel MCF-7 di fasa G_0/G_1 dan G_2/M. Aktiviti spesies oksigen reaktif (ROS) telah dikaji dan didapati pengasilan ROS lebih tinggi dalam sel-sel MCF-7 yang telah dirawat dengan kepekatan IC_{50} (23.59 μg/mL) eksrak buah *C. inophyllum* berbanding sel-sel yang tidak dirawat. Tambahan pula, ekstrak buah *C. inophyllum* didapati menyebabkan hilangnya potensial membran mitokondria ($\Delta\psi_m$) dalam sel-sel MCF-7 yang bersandarkan kepekatan ekstrak. Aktiviti enzim *caspase-3* dalam sel MCF-7 yang dirawat dengan nilai kepekatan IC_{50} (23.59 μg/mL) ekstrak buah *C. inophyllum* telah mendedahkan hidrolisis substrat yang tinggi berbanding dengan sel-sel yang tidak dirawat. Data ini menunjukkan bahawa ekstrak menginduksi apoptosis melalui pengaktifan *caspase-3*. Di samping itu, ujian comet menunjukkan fragmentasi DNA genomik berlaku bersandarkan kepekatan ekstrak yang telah dinilai melalui penilaian panjang ekor, moment ekor dan % kandungan DNA dalam ekor secara kolektif. Kesimpulannya, berdasarkan data yang diperolehi daripada keseluruhan kajian ini menunjukkan ketoksikan buah *C. inophyllum* secara *in vitro* dan juga penjelasan menyeluruh tentang mekanisma tahap molekul berkenaan kematian sel yang jelas diaruhkan melalui laluan ‘apoptosis-dalaman’ yang bergantung pada *caspase*. Memandangkan apoptosis sebagai pendekatan klinikal yang diterima untuk mengaruhkan kematian sel kanser, ekstrak buah *C. inophyllum* boleh menjadi sumber drug antikanser yang baru.

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ABSTRACT

Calophyllum inophyllum is an exquisite plant species with rich ethnomedicinal values have been diversely utilized to heal several diseases. In spite of its long-established sophisticated traditional medicinal properties, only a few investigations have reported its cytotoxicity especially fruit extract, with absolutely no means of scientific evidence of its comprehensive molecular mechanism. Hence, this study was conducted to determine the *in vitro* cytotoxicity of *C. inophyllum* fruit extract against MCF-7 human breast cancer cells with an intricate elucidation of the molecular mechanism of the cell death. In this study, authentication of the plant species and the determination of total phenolic content were carried out. The cytotoxic effect of *C. inophyllum* fruit extract against MCF-7 cancer cells was evaluated through MTT and CyQuant assays which demonstrated the inhibition of cell viability with the IC_{50} values 19.63 µg/mL and 27.54 µg/mL respectively. The preliminary time-based morphological investigation of MCF-7 cells treated with the IC_{50} value (23.59 µg/mL) of *C. inophyllum* fruit extract revealed prominent histological characteristics of apoptosis. Flow cytometric analysis of Annexin V/ Propidium Iodide assay ascertained the induction of apoptosis in *C. inophyllum*-treated MCF-7 cells in a dose-dependent manner. Moreover, flow cytometric cell cycle analysis demonstrated cell cycle arrest at G_0/G_1 and G_2/M phases simultaneously. Reactive oxygen species (ROS) activity revealed that *C. inophyllum* fruit extract induces the generation of ROS in treated MCF-7 cells when compared to

the untreated cells. Furthermore, *C. inophyllum* fruit extract dose-dependently decreases the mitochondrial membrane potential ($\Delta\psi_m$) in MCF-7 cells. The enzymatic activity of caspase-3 in MCF-7 cells treated with the IC_{50} value (23.59 µg/mL) of *C. inophyllum* fruit extract in comparison with untreated cells revealed an elevated hydrolysis of the substrate in treated cells, upholding that the extract induced apoptosis via activation of caspase-3. In addition, comet assay showed a dose-dependent genomic DNA fragmentation indicated by the evaluation of tail length, tail moment and tail DNA collectively. Conclusively, based on the data obtained from this overall study not only determined the *in vitro* cytotoxicity of *C. inophyllum* fruit extract but also shed light on its comprehensive molecular mechanism which clearly indicated a caspase-dependent intrinsic apoptotic pathway of cell death. Considering apoptosis as a clinically admissible approach of cancer cell death, *C. inophyllum* fruit extract could be a promising novel anticancer drug candidate.

CHAPTER 1: INTRODUCTION

1.1 Overview and Rationale of Study

Cancer which is also known as malignancy or malignant neoplasm can be characterized by the abnormal or uncontrolled growth of cells with the potential to invade to other parts of the body. According to the International Agency for Research on Cancer GLOBOCAN database and the World Health Organization (WHO), cancer is one of the leading causes of morbidity and mortality rate globally with approximately 8.2 million cancer-related deaths and 14.1 million new cases of cancer in 2012 (Ferlay *et al.*, 2015). There are more than 100 types of cancer which include breast cancer, lung cancer, prostate cancer, colon cancer, stomach cancer and liver cancer. Among all types of cancer, breast cancer is the most commonly diagnosed invasive cancer among women in the United States of America (USA) and is one of the leading causes of death due to cancer (Sun and Liu, 2006). An estimation of 234,190 new cases of invasive breast cancer and 40,730 breast cancer deaths are expected to occur among the population of the USA in 2015 (American Cancer Society, 2015; Siegel *et al*., 2015). In fact, according to National Cancer Registry, Ministry of Health, Malaysia (2011) breast cancer is the leading cause of death in Malaysia.

There are several types of breast cancer including the ductal carcinoma, lobular carcinoma and the rarely reported inflammatory breast cancer, Phyllodes tumor, angiosarcoma and Paget disease of nipples. Therefore, breast cancer is extremely difficult to treat due to several distinct classes of tumors that exhibit different treatment responses (Sun and Liu, 2006). Numerous drugs have been discovered for the treatment of breast cancer such as trastuzumab (Herceptin),

lapatinib (Tykerb), bevacizumab (Avastin), doxorubicin (Adriamycin), docetaxel (Taxotere), fluorouracil (Adrucil), paclitaxel (Taxol), methotrexate (Trexall), cyclophosphamide (Cytoxan) and tamoxifen (Hamilton, 2014; Hirsch, 2014; Mates *et al*., 2015). However, each of the chemotherapeutic drugs has its own limitations as well as side effects and in the case of non-steroidal anti-estrogen medicine, tamoxifen, it has been reported to be effective in only one-third of the breast cancer patients. In addition, resistance to artificial anticancer drugs is also a foremost problem to be taken into consideration in the treatment of this disease. Thus, searching for new alternative agents for the prevention and treatment of breast cancer is in great need of producing better novel drugs.

It has been reported that over 60% of the novel drugs discovered for the treatment of cancer were originated from natural products such as plants, marine organisms and microorganisms (Newman *et al*., 2003; Val'ko *et al*., 2007). There is a significant biological and ecological foundation behind the production of new bioactive secondary metabolites with potent anticancer properties which also have a lengthy history in sophisticated traditional medicine systems (Khazir *et al*., 2014). Our earth is rich in plant species in which only a fraction of the plants have been scientifically studied and reported to have chemical and pharmacological properties such as the antitumor activity due to the presence of a diverse range of anticancer compounds such as flavonoids, alkaloids, terpenoids, phenylpropanoids (Talib and Mahasneh, 2010). Fruits, vegetables, and spices are also known to be important therapeutic candidates which have been widely used in traditional medication since ancient times and believed to suppress cancer due to the presence of numerous anticancer components such as curcumin, genistein, resveratrol, isoflavones, saponins, beta-carotene, phytosterols, indole-3-carbinol, folic acids, selenium, and

flavonoids (Bhanot *et al*., 2011). Thus, it is necessary to explore and study the medicinal values of plant species for the discovery of novel anticancer agents as an alternative treatment for cancers.

The combination of knowledge of botanical, phytochemical, biological, and molecular techniques have lead to the discovery and development of anticancer drugs from plant extracts where the process begins with the identification of plant species with medicinal properties followed by the isolation and characterization of the bioactive compounds that is responsible for the biological activity through an appropriate bioassay fractionation (Balunas and Kinghorn, 2005). The recent developments in technology in the drug discovery field through experimental and computational approaches have expanded the range of research area. Discovery of anticancer drugs from plant extracts generally employ the cell-based screening of the drug for anti-proliferative effects through cytotoxicity assays followed by the determination of the mechanisms of action of the prospective anticancer agent in selective cancer cell lines by utilization of several bioassays.

Therefore, this study would contribute to the development of anticancer agents from the natural product of local medicinal plant namely *Calophyllum inophyllum*. C. inophyllum is commonly known as Alexandrian Laurel, Tamanu, Pannay Tree and Sweet Scented Calophyllum in English (Dweck and Meadows, 2002). Local Malay names of this *C. inophyllum* include bintagor, penaga or kamani (Friday and Okano, 2006). *C. inophyllum* is a well known ornamental plant species with a long history of medicinal value in which its leaves, barks, flowers, fruits and seeds are widely used in traditional practices. A study conducted by Yimdjo *et al.* (2004) on the chemical constituents of the root bark and nut of *C. inophyllum*, reported that this specific species contains antimicrobial and cytotoxic compounds

especially xanthone derivatives which are claimed to be the leading compounds in anticancer drugs (Goh and Jantan, 1991). Besides xanthone, previous scientific studies have also identified compounds such as biflavonoids, benzophenones, neoflavanoids, and coumarin derivatives in *C. inophyllum* and these are reported to have anticancer, antitumor and lipid peroxidation properties (Kathiresan *et al*., 2006). Moreover, all parts of this plant have been employed as antiseptics, astringents, expectorants, diuretics, purgatives, which emphasize the high medicinal properties of this plant (Ali *et al*., 1999). A scientific study conducted by Narayan and his colleagues (2011) revealed that the extract of *C. inophyllum* bark inhibited HIV-1 protease (HIV-PR) and HIV-1 integrase (HIV-IN) enzymes, which provides the scientific evidence for AIDS treatment. However, the pharmacological activity of *C. inophyllum* fruit extract is least investigated and reported. Hence, further studies on the methanol extract of *C. inophyllum* fruits are required to have a better understanding of their cytotoxicity mechanisms in breast cancer cells. The present study has been conducted with the incorporation of cytotoxicity assays such as MTT and CyQuant assays. Preliminary studies on the morphology of cells have also been deliberated through Giemsa staining with the IC_{50} value evaluated through the former assays and demonstrated the morphological criteria of apoptosis. In addition, several bioassays were also performed to study its mechanisms of action which includes Annexin V-FITC/PI assay, cell cycle assay, reactive oxygen species (ROS) assay, mitochondrial membrane potential analysis, comet assay and caspase 3 assay.

1.2 Objectives

The current study of undertaken with the objective:

1) To study the cytotoxic activitiy of *C. inophyllum* fruit extract against MCF-7 breast cancer cells.

2) To investigate the possible anticancer mechanism of action of *C. inophyllum* fruit extract against MCF-7 breast cancer cells.

CHAPTER 2.0: LITERATURE REVIEW

2.1 Cancer

Genetically programmed cell division and differentiation occur in the process of formation of specific tissues and eventually functional organs. However, intermittently the above events may give rise to tissue masses called tumors, or neoplasms. A single mass of benign tumor is usually not life threatening since it can be cured completely by surgical removal. However, when the cells of a tumor start to invade and interrupt the surrounding tissues, the tumor is said to be malignant and is identified as cancer which can consequently lead to death due to injury to vital organs, secondary infection, metabolic problems, secondary malignancies, or hemorrhage (Russell, 2010). The place where cancer begins is known as the original or primary site. A malignant tumor can break away from its original location and invade far-away sites through the lymphatic system, forming new tumors. This process is known as metastasis. The uncontrollable growth of cells may occur in any parts of the body leading to more than 100 types of cancer including lung cancer, breast cancer, cervical cancer, stomach cancer, prostate cancer, bowel cancer and ovarian cancer.

According to the American Cancer Society (2015), risk factors for cancer include genetic factors as well as the lifestyle of a person such as tobacco use, alcohol use, diet, and physical activity. Other disposing factors to cancer are certain type of infections such as human papilloma virus (HPV), Epstein-Barr virus (EBV), hepatitis B, hepatitis C and *Helicobacter pylori*. Environmental exposures to diverse range of chemicals, radiations and even overexposure to ultraviolet (UV) light from the sun may also lead to cancer. Smoking and alcohol intake can be associated with

several cancers such as the mouth, oral cavity, pharynx, larynx, esophagus, lung, stomach, pancreas and even colon (Schmidt and Popham, 1981). Besides, viral infection can be related to cancer because of their capability to integrate into the DNA of the human stem cell where it mutates and transforms the cell to be the parent of the malignant clone (Doll and Peto, 1981).

The severity of the disease depends on the degree of primary tumor and its competence of invading to other parts of the body. Therefore, the stage of the cancer is identified prior to proceed with any sort of treatments. Generally, there are four stages of cancer as described in Table 2.1.

There are several types of treatments available for cancer including surgery, radiation therapy, chemotherapy, immunotherapy, hyperthermia, and stem cell transplant. However, these treatments have excruciating side effects that vary from person to person depending on the frequency of treatment, the age of the person and other health conditions. Commonly occurring side effects generated by cancer treatments include anemia, alopecia (hair loss), constipation, edema, fatigue, memory problems, peripheral neuropathy, nausea and vomiting (Nordqvist, 2014; National Cancer Institute, 2015). Chemotherapy is one of the popular cancer treatments from the 1960s as the degree of curing cancer elevated at approximately 33% through radical local treatments. Eventually Cancer Chemotherapy National Service Centre was established in the effort of developing methods to screen chemicals using transplantable tumors in rodents (Devita and Chu, 2008).

Table 2.1: Stages of cancer

STAGE	DEFINITION
Stage 0: *In-situ*	Cancer is located in place and have not spread to nearby tissues which carry little or no threat to life with a high probability of curing
Stage I: Localized cancer	Cancer cell grows and obtain its competency to pass through basement membrane where it begins to spread to nearby tissues
Stage II & III: Regional spread	Tumor grows larger in size and its daughter cells spreads through lymph vessel to adjacent tissues of the primary tumor
Stage IV: Distant spread	Tumor invades to other parts of the body which is known as the secondary or metastatic cancer

Source: American Society of Clinical Oncology, 2015

2.1.1 Breast Cancer

Breast cancer is a type of cancer that evolves in cells of the breast which can invade to other parts of the body. Approximately one out of ten women is affected by breast cancer. According to the Annual Report collaboratively presented by the American Cancer Society (ACS), Centers for Diseases Control and Prevention (CDC), Surveillance, Epidemiology, and End Results Program of National Cancer Institute (NCI), and North American Association of Central Cancer Registries (NAACCR), incidence rate for breast cancer was the highest among females for 2007 to 2011 while the death rates caused by breast cancer is at second place after lung cancer (American Cancer Society, 2015).

This heterogeneous disease can be categorised by its histological patterns where specific architectural and cytological patterns are identified; or by its molecular features when gene expression profiling is studied (Weigelt and Reis-Filho, 2009). Among the histological types of breast cancer, the lobular and ductal/lobular carcinoma cases are more likely to be diagnosed with stage III/IV in contrast to ductal carcinoma cases, while other breast cancers such as mucinous, tubular and papillary tumours occur in small number (Li *et al.*, 2005). Gene expression study conducted by Perou *et al.* (2000) further classified the breast cancer based on their pervasive differential gene expression patterns, and divided the breast cancer into the basal-like, *HER2*, normal breast-like, luminal, luminal A and luminal B. Figure 2.1 shows the anatomy of the breast.

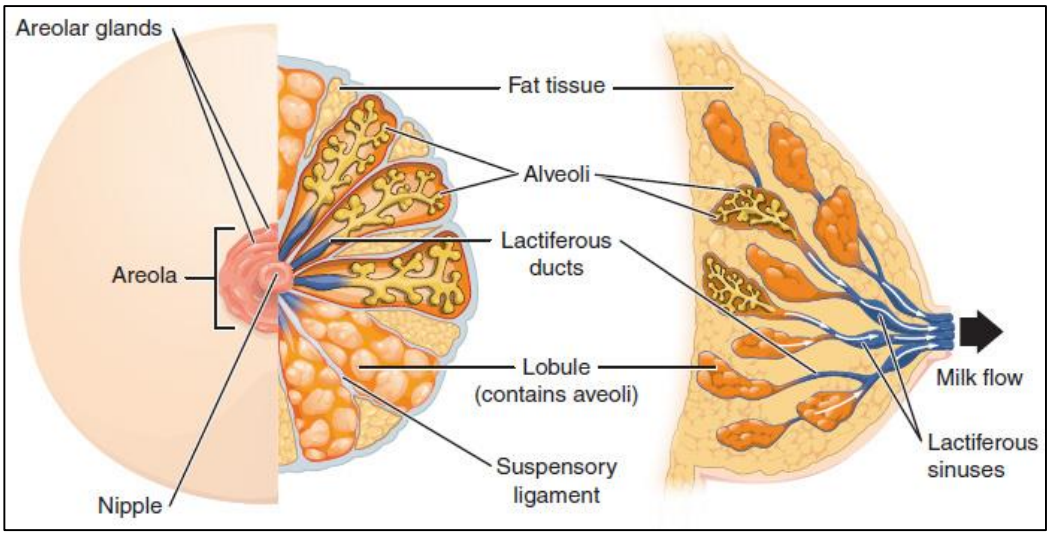

Figure 2.1: Anatomy of the breast
Source: Anatomy of Physiology of the Female Reproductive System, 2015

Hormonal risk factors are determinant for the development of breast cancer. Excessive exposure to estrogen is believed to be one of the mechanisms of carcinogenesis in the breast cancer and this cancer can be correlated with life style factors including low parity, late age at first delivery, lack of breast feeding, increased intake of alcohol, use of hormonal contraceptives, and incidence of obesity (Imyanitov and Hanson, 2004; Yager and Davidson, 2006). Besides hyperestrogenia, several researches confirmed that constitutional genomic instability is also associated with breast cancer susceptibility. Mutations in *BRCA1* and *BRCA2* have been reported to attribute to breast cancer based on an evaluation of genetic heterogeneity in 145 breast-ovarian cancer families (Narod *et al.,* 1995a and 1995b). There are epidemiological and molecular evidences for mutations in *ATM* gene to be linked with breast cancer susceptibility (Ahmed and Rahman, 2006). Germ line mutations also include *p53, CHEK2* polymorphism and *NBS1* gene that confer elevation in breast cancer risk (Imyanitov *et al.,* 2003; Imyanitov and Hanson, 2004). These mechanisms of carcinogenesis in breast cancer are responsible for the subsequent genetic instability as well as the alteration of specific genetic pathways either by activating the oncogenes or by inactivating the suppressor genes that lead to the hallmarks of cancer.

Commonly practiced treatments for breast cancer are surgery such as lumpectomy, mastectomy, and sentinel node biopsy, followed by radiation therapy, hormone therapy and chemotherapy. According to Early Breast Cancer Trialists' Collaborative Group (EBCTCG), the earliest systemic therapy found to have a significant improvement in survival with prevention from recurrence is tamoxifen, an anti-estrogen (Fisher *et al.,* 1998). Disparate number of drugs have been discovered with a promising survival perk for breast cancer patients such as the trastuzumab

(Herceptin) that targets and blocks the human epidermal growth factor receptor 2 (HER2) protein (Fischer *et al.,* 2003); anthracyclines like doxorubicin and epirubicin as well as the taxanes like paclitaxel and docetaxel which have been reported to be potential therapeutic agents for advanced breast cancer (Esteva *et al.,* 2001).

Therapeutic strategies utilized in the discovery of novel therapeutic agents for breast cancer are the properties of cancer cells, in which, genetic instability and uncontrollable cell growth are taken into consideration; molecular targets where tumor-specific anchors are targeted for the delivery of cytostatic substances; and finally some genuine targets which mainly focus on suppressing the molecules essential for breast cancer maintenance (Imyanitov and Hanson, 2004).

Recently, complementary and alternative medicines (CAM) have drawn an extra interest among oncologists and breast cancer patients, and therefore CAM has been generally practiced in combination with the conventional therapy. CAM have associated with natural herbal medicines which are believed to protect the body from malignancy by inhibiting the growth of cancer through various biological and molecular mechanisms and also by reducing the lethal side effects and complications caused through conventional treatments (Shahid, 2013). Therefore, discovery of novel anticancer cytotoxic agents from natural herbal plant parts such as roots, flowers, seeds, fruits, leaves or branches are exclusively anticipated for the treatment of breast cancer.

2.2 Anticancer

The evolution of the life threatening disease, cancer alarmed the whole world since it is known to be difficult to cure due to uncontrollable multiplication of abnormal cells leading into an invasive malignant tumor. The development of anticancer drugs began ever since the discovery of the anticancer properties in nitrogen mustard and the folic acid analogue amino protein in the 20^{th} century that gradually led to the identification of clinically effective novel genotoxic drugs through cytotoxicity screening (Baguley, 2002).

The evolving knowledge on cancer mechanisms has expedited the expansion of novel anticancer approaches. One of the most extensive conventions is to slow down or to inhibit the prime characteristic of cancer cells that grow uncontrollably. This can be correlated with the elevation of tendency of the cells to go through the process of cell suicide, or apoptosis. This effective route is eventually achieved through a mechanistic manner where the cytotoxic drugs are designed so as to impede the DNA replication by damaging the DNA of the cancer cells, subsequently inducing apoptosis. Besides cytotoxic drugs, cytosolic drugs are also used to fight cancer in which the drugs are designed to specifically modify the biochemical pathways that facilitate the fast growth of cancer cells (Denny, 1988). Although these drugs do not kill the cancer cells, they prevent the cancer cells from reproducing by exclusively interrupting their growth signalling.

The most notable limitation and challenge encountered in the development of anticancer drug is the tendency of the cancer cells to achieve resistance against these compounds. This in turn creates a great urgency for the discovery and designing of anticancer drugs with improved molecular structure and mechanism of action.

Consequently, the abundantly available natural products including plants, microorganisms, and marine organisms, provide an enormous structural diversity of compounds for the development of novel anticancer agents (Rocha *et al.,* 2001; Kinghorn *et al.,* 2009).

2.2.1. Plants as a Source of Anticancer Agents

Beyond 60% of recognized drugs for the treatment of cancer to date are originated from natural products in which huge number of plant species have been proclaimed to possess promising anticancer effects (Rocha *et al.,* 2001; Newman *et al.,* 2003; Cragg and Newman, 2005; Val'ko *et al.,* 2007). The breakthrough in chemical field such as the discovery of vinca alkaloids, vinblastine and vincristine, and the isolation of the cytotoxic podophyllotoxins in 1950 initiated the new aeon of exploration of plant sources as chemotherapeutic candidates for cancer (Cragg and Newman, 2005). This led to the plant collection program by the United States National Cancer Institute (NCI), followed by screening of plant species for anticancer activity which resulted in a revelation of enormous number of new anticancer agents such as taxanes and camptothecin (Cassady and Douros, 1980; Shoeb, 2006).

The natural ability of plants to produce toxins against microorganisms like fungi gained attention for the development of anticancer agents since recent evolutionary researches have demonstrated that microorganisms such as yeast and fungi are closely associated with mammalian cells at biochemical level (Cardenas *et al.,* 1999; Hanlon and Hodges, 2013). On account of this, the chemical compounds produced by plants for their defence against microorganisms are postulated to have an inhibitory effect on human cells correspondingly. Crude extracts from plant samples have been established to be selectively toxic to cancer cells after passing

through various bioassays including *in vitro* and *in vivo* screenings which eventually accelerated to fractionation processes for the isolation of specific active compound responsible for the anticancer effect as exemplified in the discovery of approved drugs namely Camptothecin and Taxol.

The bioactive compounds of plants responsible for its anticancer properties are known as the secondary metabolites which are classified based on their biosynthetic pathways. The major groups of these secondary phytochemicals include the terpenoids, phenolic metabolites, alkaloids, flavonoids, polyketides and other nitrogen-containing metabolites (Harborne, 1999; Oksman-Caldentey and Inze, 2004). Unlike primary metabolites such as carbohydrates, proteins and lipids, secondary metabolites are not involved in the growth and metabolism of plants but are considered as the end products of primary metabolism, that have a role as defence chemicals.

The advancement in analytical technology and biological sciences have allowed an impressive number of naturally occurring secondary metabolites from plants to be isolated and studied for their cancer chemopreventive effects. Examples of as such phytochemicals and derivatives which are clinically accessible include vinca alkaloids (vinblastine, vincristine, vinorelbine), podophyllotoxin derivatives (etoposide, etoposide phosphate, teniposide), taxanes (paclitaxel and docetaxel), campothecin derivatives (irinotecan and topotecan) and homoharringtonine (Chabner *et al.,* 2005; DeVita *et al*., 2008; Asif, 2015; Biswas *et al.,* 2015). These bioactive compounds were demonstrated to possess significant anticancer activity against lung cancer, breast cancer, ovarian cancer, testicular cancer, colorectral cancer, lymphoma, and several other cancers. This can be exemplified by etoposide that inhibits topoisomerase II (Liu, 1989), campothecin that inhibits topoisomerase I (Liu

et al., 2000), taxanes that causes mitotic arrest via stabilization of microtubules (Wani *et al.*, 1971), flavopiridol that inhibits cyclin-dependent kinase (Kelland, 2000), and homoharringtonine that inhibits protein synthesis and blocks cell-cycle progression (Zhou *et al.*, 1995).

2.3 *Calophyllum inophyllum*

2.3.1 Botany

C. inophyllum Linn. is commonly as Alexandrian Laurel, Laurel Wood, Tamanu, Pannay Tree, Sweet Scented *Calophyllum*, Beach *Calophyllum*, and Borneo Mahogany in English (Dweck and Meadows, 2002). It is known as Bitaog in Tropical Asia. Local Malay names of this species are bintagor, penaga or kamani (Friday and Okano, 2006).

Synonyms of *C. inophyllum* includes *Calophyllum bintagor* Roxb., *Mesua ferrea* Linn, *Balsamaria inophyllum* Lour., *Calophyllum apetalum* Blanco [Illegitimate], *Calophyllum blumei* Wight, *C. inophyllum* var. *blumei* (Wight) Hassk., *C. inophyllum* forma *oblongata* Miq., *C. inophyllum* forma *obovata* Miq., *C. inophyllum* var. *takamaka* Fosberg, *C. inophyllum* var. *wakamatsui* (Kaneh) Fosberg & Sachet, *Calophyllum ovatifolium* Norona *Calophyllum spurium* Choisy and *Calophyllum wakamatsui* Kanehira (The plant List, 2013).

2.3.1. (a) Classification

Kingdom	Plantae
Subkingdom	Tracheobionta
Phylum	Trachephyta
Class	Magnoliopsida
Subclass	Dilleniidae
Order	Theales
Family	Clusiaceae-Guttiferae
Subfamily	Kielmeyeroideae
Tribe	Calophylleae
Genus	Calophyllum
Species	*C. inophyllum* L.

(Stevens, 1998)

2.3.1. (b) Distribution

The native of *C. inophyllum* is East Africa, through southern coastal India to Malesia, northern Australia and the Pacific islands which further extended to Philipines, Taiwan and the Marianas through Southeast Asia (Dweck and Meadows, 2002). The geographical distribution of this species also includes the coastal region of Polynesia and Madagscar (Friday and Okano, 2006). During the early migrations of Polynesian settlers, Tamanu has been introduced in Hawaii from the south Pacific islands (Dweck and Meadows, 2002). This species was also brought and successfully cultivated in southern China. The habitat of this species is mainly in the coral sands and on the sea shores while some samples may be established in valleys and low land forests (Lim, 2012).

2.3.1. (c) Botanical Description

The *C. inophyllum* is a slow growing, medium to large sized tree reaching a height of about 8 to 20 m. The canopy of the tree is widely spread to an irregular crown shape. It has a thick, fissured and grey trunk with a rough and cracked textured bark. Its sap is milky white.

Leaves of *C. inophyllum* are opposite with largely elliptical or oval lamina of 10 to 20 cm long by 6 to 9 cm wide. The blunt ended leaves are strong and deep shiny green in colour with intimately positioned thin parallel veins organized perpendicularly from a prominently elevated yellowish green midrib to the rounded leaf boundary (Orwa *et al*., 2009).

C. inophyllum bears pleasantly scented white flowers in clusters of 4 to 15 flowers and each flower has 4 to 8 delicate oblong petals. Flowers are usually bisexual with a puff of golden yellowish stamens and a pink pistil with a thin, long style and a superior ovary. The flowers are 2.5 cm across and 8 to 14 mm long and positioned on long sturdy stalks at leaf axils. The flowering is heaviest in late spring or early summer.

Fruits also grow in clusters, with each fruit having a diameter of 2 to 5 cm. The ball shaped light green fruit has a thin compact outer layer with a smooth texture. When the fruit ripens, the skin turns yellow to brown and the smooth texture becomes wrinkled. The fruit holds a large brown seed with a diameter of 2 to 4 cm which contains a pale yellow kernel. The trees usually bear fruits twice a year and these periods are from April to June and October to December (Friday and Okano, 2006). Figure 2.2 shows the *C. inophyllum* plant morphology.

(A) **(B)**

(C)

(D)

Figure 2.2: *C. inophyllum* plant morphology

(A) Tree with its canopy spread widely to an irregular crown;
(B) Perpendicularly arranged leaves with blunt ends; (C) Flower clusters with yellow stamens; (D) Ball shaped light green fruits

2.3.1. (d) Propagation

C. inophyllum is usually propagated through seeds. However, the germination of the seed is initially slow. Cracking the shells or shelling the seeds entirely will eventually germinate the seeds faster where a study established that a fully shelled seed germinated in 22 days, seeds in a cracked shell took 38 days to germinate, whereas 57 days was needed for seeds still in their shells (Elevitch and Wilkinson, 2000; Prabakaran and Britto, 2012).

2.3.2 Ethnomedicinal uses

C. inophyllum is a well known ornamental plant species in which its leaves, barks, flowers, fruits and seeds are diversely used in traditional ethnomedicine. The high medicinal properties of all parts of this plant have also been employed as antiseptics, astringents, expectorants, diuretics and purgatives (Ali *et al.*, 1999). Traditionally, its emetic and purgative gum extracted from the wounded bark of the plant has been recorded to be used for treatment of wounds and ulcers. In Asia, mainly in India and Indo-china, the astringent bark which contains tannins and its purgative juices are widely used for vaginal discharge and the passing of blood after child birth and also for gonorrhea (Burkill, 1994). In addition, antineuralgic, diuretic, antiseptic and disinfectant properties of the bark are also well known and a preparation from bark acts as an expectorant when taken internally which is useful in chronic bronchitis, and phthisis (Prabakaran and Britto, 2012). The resin is helpful in unrelieved catarrh while the infusion of gum, bark and leaves are used on for sore eyes. In Fiji and Linga, the leaves soaked in water are applied to inflamed eyes. The leaf infusion has been reported to be useful for the treatment of heatstroke when taken internally and it has also been prescribed as an inhalation for migraine and vertigo in Cambodia. In

Philippines, macerated leaves are also used as astringent for haemorrhoids (piles). In Madagascar, Polynesia and Malaysia, bark is crushed into powder to be used for orchitis while the gum resin is a remedial, resolvent and antiseptic. The seed oil is also utilized against psoriasis and rheumatism (Prabakaran and Britto, 2012). The blond nut kernel in the fruits of *C. inophyllum* is responsible for the dark green, rich and pleasant smelling oil which is readily and completely absorbed when applied to skin, leaving no residue (Oil of Tamanu, n. d.). It is widely been used for treating skin diseases.

2.3.3 Pharmacological Activities

2.3.3. (a) Antiviral activity

Fractionation of *C. inophyllum* extract yielded several active compounds which play an important role against human immunodeficiency virus type 1 (HIV-1). According to Patil *et al.* (1993), inophyllum B and P were isolated from the methanol chloride extract of *C. inophyllum* and these compounds showed strong activity against HIV-1 by inhibiting the HIV reverse transcriptase with an IC_{50} value of 38 and 130 nM, respectively. These compounds were also reported to exhibit anti-HIV properties against cell culture with an IC_{50} value of 1.4 and 1.6 µM, respectively. Coumarin derivatives isolated from *C. inophyllum* extract such as castatolide and inophyllum P are potent HIV reverse transcriptase non-nucleoside inhibitors (Spino *et al.*, 1998). Studies reported that inophyllums have a novel mechanism of interaction with reverse transcriptase and has potential to play a role in combination therapy.

2.3.3. (b) Anticancer activity

Primary screening of ten 4-phenylcoumarins isolated from *C. inophyllum* was conducted by Itoigawa *et al.* (2001) in the search for antitumor-promoting agents and reported that calocaumarin-A displayed a significant inhibitory effect on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-O-tetradecanoylphorbol-13-acetate in Raji cells which was further confirmed by an *in vivo* two stage carcinogenesis test on mouse skin tumor promotion (Itoigawa, *et al.*, 2001). Although methanol extract of *C. inophyllum* leaves exhibited a weak anticancer activity against MCF-7 and HT-29 cell lines with 31.25% and 22.56% inhibition at 200 µg/ml tested dose, it is believed to show a higher cancer cell death with the identification and isolation of the potent active chemical constituent present in the extract (Aditya *et al.,* 2013).

2.3.3. (c) Antimicrobial activity

Investigation conducted by Ha *et al.* (2009) revealed a potential antimicrobial activities of methanol and n-hexane extract of *C. inophyllum* fruit peel against *Staphylococcus aureus*, and *Mycobacterium smegmatis* through the disc diffusion method. Methanolic crude extract exhibited higher zone of inhibition in both *S. aureus* and *M. smegmatis* which are 58.1% and 46.9%, respectively whereas the n-hexane crude extract demonstrated a slightly lower zone of inhibition for these microbes with values of 53.8% and 37.5%, respectively. Screening of ethanol and ethyl acetate extracts from various parts of *C. inophyllum* such as leaves, fruits, stems, flowers, and roots against *Salmonella typhi*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli* and *Vibrio colerea* have been studied through the cup-plate method and demonstrated promising antimicrobial properties

for the extracts, which can be used in the development of novel drugs for the treatment of contagious diseases caused by pathogens (Saravanan *et al.*, 2011). Several compounds have been isolated from the fractionation of the crude extract of the root bark and nut of *C. inophyllum* which were tested against various microorganisms such as *S. aureus*, *V. anguillarum*, *E. coli*, and *Candida tropicalis* through the classic agar disc dilution method at 20 µg per disk in which compounds such as caloxanthone A, calophynic acid, brasiliensic acid, inophylloidic acid, calophyllolide, as well as inophyllum C and E demonstrated strong inhibitory activity against *S. aureus* (Yimdjo *et al.*, 2004). The presence of phenolic compounds in *C. inophyllum* which give an acidic property is said to be responsible for the antimicrobial activities. Friedelin, canophyllol, canophyllic acid, and inophynone which are the known derivates of phenolic group in *C. inophyllum* demonstrated significant bactericidal and fungicidal action (Mahmud *et al*., 1998). The oil of *C. inophyllum* exhibited *in vitro* antibacterial activity against Gram negative bacteria. At 20 µg per disc, the *C. inophyllum* extract inhibited the growth of *S. aureus* (Bhat *et al*., 1954).

2.3.3. (d) Anti-inflammatory activity

Anti-inflammatory effect of ethanolic extract of leaf and stem bark of *C. inophyllum* have been studied on albino Wistar rats through carrageenan induced paw edema and cotton pellet granuloma method which consequently demonstrated strong activities on both acute and chronic models of inflammation which was also directly proportional to the dosage of extract dosage (Baig *et al*., 2014). Acetone extract of *C. inophyllum* leaves displayed potential anti-inflammatory effects against lipopolysaccharide-induced RAW 264.7 cells which successfully suppressed the

nitric oxide production and also the expression of iNOS, cyclooxygenase (COX-2) and nuclear factor-kappa B (NF-κB) in a dosage reliant behaviour (Tsai *et al*., 2012). The anti-inflammatory activity of *C. inophyllum* is somewhat due to the presence of friedelin and triterpenes of the friedelin group, specifically canophyllal, canophyllol and canophyllic acid, and the heartwood xanthones such as mesuaxanthone B and calophyllin (Saxena *et al.*, 1982). This inflammatory agent was found to be effective in both intra peritoneal and oral routes demonstrated in adrenalectomised rats (Gopalakrishnan *et al.*, 1980).

2.3.3. (e) Antioxidant activity

Antioxidant properties of aqueous and methanolic extracts of *C. inophyllum* leaf have been evaluated by Dutta and Ray (2014) and demonstrated a significant free radical scavenging activity and reducing power for the methanolic leaf extract in a concentration dependent manner. The strong antioxidant activity of the methanolic leaf extract was related to the high phenol and flavonoid contents which are reported to be 140.28 ± 17.1 mg/g and 177.06 ± 5.29 mg/g, respectively (Dutta and Ray, 2014). Several bioactive compounds have been isolated from the leaves of *C. inophyllum* and tested for antioxidant activity in hyperlipidemia model which revealed the compounds such as the combination of calophyllic acid and isocalophyllic acid, triterpene and canophyllic acid to have a strong antioxidant activity at the concentration of 200 µg/mL (Prasad *et al*., 2012). Methanolic leaf extract of *C. inophyllum* was analysed for its antioxidant activity through DPPH and hydrogen peroxide radical scavenging activity and reducing power activity which showed that the highest antioxidant activity was when the total content of an active compound called calocoumarin A is high (Sebastian and Britto, 2014). The oil of *C.*