CHAPTER 1
INTRODUCTION

Cancer is a disease characterized by uncontrolled cellular division of abnormal cells and the potential of these cells to invade other local and distant tissue. This unregulated growth is usually triggered by a series of acquired or inherited DNA mutations within cells that damage genetic material necessary for normal cell function and control of cellular division (Hanahan & Weinberg, 2000, 2011). The global burden of cancer continues to increase rapidly with about 12.7 million cancer cases and 7.6 million cancer deaths estimated in 2008 (Jemal et al., 2011). For developing countries such as Malaysia, cancer is the second leading cause of death after coronary artery disease (CAD). NCR reported that a total of 18,219 of new cases were diagnosed in 2007 and breast cancer is one of the ten leading cancer among the Malaysia population in 2007 (Zainal & Nor Saleha, 2011).

Apoptosis is a highly regulated death process occurring in all normal cells. Upon receiving apoptosis signals, a series of proteins will activate other proteins in this process. It will create a cascade downstream pathway that will involve several proteins including the pro-apoptotic and anti-apoptotic proteins. These proteins then will activate caspases which are proteases and finally lead to apoptotic morphological changes (Petros et al., 2004; Kumar, 2006; Lamkanfi et al., 2006). However, this process was evaded by majority of cancer cells and progression of the cells showed dysregulation of apoptosis regulatory proteins. Furthermore, the cancer cells could produce their own growth factor in order for the cells to proliferate unlimitedly (Sporn & Roberts, 1985). Studies also showed that there was an imbalance in the production of anti-apoptotic and pro-apoptotic proteins in cancer cells (Juin et al., 2004; Vogler et al., 2008). Therefore, proper regulation of apoptosis is important for proliferation in normal
cells together with regression of cancer cells, with the majority of both natural and synthetic anti-cancer products possessing the ability to induce apoptosis.

Malaysia is positioned near the Equator making it a typical tropical climate country, characterized by high temperature, humidity and rainfall throughout the year which are conducive for a rich and thriving flora (Turner, 1997). Natural products and their by-products represent most of the current anti-cancer drugs in clinical use. Anti-tumor drugs derived from a natural product with semi-synthetic modification “ND” (Natural product Derivation) represent approximately 28.0% of all anti-cancer drug from 1940s to 2010 (Newman & Cragg, 2012). Taxol is one such anti-cancer drugs, collected from bark of Taxus brevifolio in 1971 and approved by the Food and Drug Administration (FDA) (Wani et al., 1971). Since then, the mechanism of its action was investigated and in 1978, Funch and Johnson, 1978 proposed that this compound acts as a mitotic spindle inhibitor. In recent years, Polyphenon E® is another example of a natural product approved by FDA in 2007 for treatment of genital warts linked to human papilloma viruses (HPV) and recent studies suggest it may prevent bladder, prostate, and lung cancer (Graff, 2009).

The tropical plant Chisocheton tomentosus from the Meliaceae family is a medium-sized tree that can grow up to 21 m in height (Mabberly & Pannell, 1989). Chisocheton species were known to produce bioactive compounds having complex molecular structures such as erythrocarpine E and chisomecine A (Awang et al., 2007b; Najmuldeen et al., 2011). Plants from this family has been known to be a rich source of secondary metabolites including various sterols, terpenoids and alkaloids with medicinal and pesticidal properties such as anti-fungal, anti-bacterial, anti-viral, anti-inflammatory and anti-plasmodial agents (Joshi et al., 1987; Mahmood et al., 1991; Agbedahunsi et al., 2004; Boeke et al., 2004; Mohamad et al., 2009). In tropical countries, this plant has been used as a form of traditional medicine against several
diseases including diabetes, malaria, liver and cancer diseases (Oliver-Bever, 1986; Omar et al., 2003; Nagoor et al., 2011).

Plant-derived sterols or phytosterols are structurally similar to cholesterol with a slight difference at the C-24 position containing an additional ethyl group (Rubis et al., 2008). About 44 phytosterols have been identified to date, with major forms existing in higher plants constituted by β-sitosterol, campesterol and stigmasterol (Ju et al., 2004; Paniagua-Perez et al., 2008; Woyengo et al., 2009; O'Callaghan et al., 2010). Past studies have also shown that β-sitosterol possesses a polar or hydrophilic nature which is a desirable trait in most in vivo drug applications (Kurban et al., 2010). β-sitosterols have also been reported to bind various carrier proteins such as human serum albumin mainly by hydrophobic and hydrogen bond interactions, thus making protein-drug combination complexes a viable option for chemotherapy (Sudhamalla et al., 2010). Sitosterols often undergo oxidation process to form sitosterols oxidation products (SOP) (Ryan et al., 2005; Koschutnig et al., 2009). These form of sterol are structurally different from its parent with an additional steroid ring group of either hydroxy- (OH-), keto (=O), expoxy or triol (Hovenkamp et al., 2008). Forms of SOP that exist in human plasma are β-epoxysitostanol, sitostanetriol, campestanetriol, α-epoxysitostanol, 7-ketositostanol and 7β-hydroxysitosterol with β-epoxysitostanol, sitostanetriol and campestanetriol are most abundant (Otaegui-Arrazola et al., 2010).

In recent years, researchers have been focusing on the effects of phytosterols toward various cancer cell lines and its implication in multidrug resistance (Bradford & Awad, 2007; Baskar et al., 2010; Rubis et al., 2010). To date, several apoptotic pathways mediated by phytosterols have been proposed in cancer culture models such as the Ras/ERK and the PI3K/Akt pathway (Awad et al., 2007; Moon et al., 2007; Moon et al., 2008; Park et al., 2008; Chien et al., 2010; Hsu et al., 2011). In contrast, investigation on the mechanistic effects of phytosterol oxides is still scarce. Despite
several reports on the cytotoxicity of these compound, their cytotoxic mechanism of action is, however, still unclear (Koschutnig, et al., 2009; O’Callaghan, et al., 2010). Roussi et al., 2004; 2006; 2007 suggested that 7β-hydroxysitosterol, one of the phytosterol oxides could target the mitochondria, leading to loss of mitochondrial membrane potential to induce cytochrome c release. However, the mechanism of cytochrome c release and caspase activation is not well characterized.

In this study, the cytotoxic effects of 7α-hydroxy-β-sitosterol isolated from Chisocheton tomentosus (Meliaceae) was investigated on human breast, liver, cervical and oral cancer cell lines and the apoptotic potential and anti-cancer mechanism of 7α-hydroxy-β-sitosterol, CT1 was elucidated on MCF-7 human breast cancer cell line for the first time (Figure 1.0).

**Figure 1.0:** Chemical structure of 7α-hydroxy-β-sitosterol (CT1) isolated from Chisocheton tomentosus (Meliaceae family).
1.1 **Objectives of study**

i. To investigate the cytotoxic effect of CT1 against various human tumor cell lines and human normal cell line using MTT assay and Live/Dead cytotoxicity/viability assay.

ii. To investigate the anti-migratory effect of CT1 against cytotoxic-induced cancer cell lines using wound healing assay.

iii. To determine the mode of cell mediated-death of CT1 on cytotoxic-induced cancer cell lines and human normal cell line using flow cytometric analysis and DNA fragmentation.

iv. To identify the apoptotic protein regulation expression mediated by CT1 using western blotting.
CHAPTER 2
LITERATURE REVIEW

2.1 Cancer Overviews

About 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008 worldwide (Jemal et al., 2011). In 2008, approximately 1.4 million women were diagnosed with breast cancer worldwide with corresponding 460 000 deaths (Ferlay et al., 2010). Meanwhile, cancer of cervix or cervical cancer is the second most common cancer in women worldwide, with about 500 000 new cases and 250 000 deaths each year (WHO, 2006). Liver cancer is the fifth most common cancer in the world and the second most common cause of cancer mortality. An estimated 748 300 new liver cancer cases and 695 900 cancer deaths occurred worldwide in 2008. Although oral cavity cancer representing only nearly 3% of all cancer cases worldwide, however it is estimated that 127 459 deaths are caused by this cancer annually (de Camargo Cancela et al., 2010).

In the developing nations of Asia and Africa, the risk of cancer burden increase rapidly per year. Developing populations still account for more than half of the world’s cancer burden, since they represent almost seventy five percent of the world population (Thun et al., 2010). Oral cavity cancer caused deaths about 96 720 cases in less developed countries which represent 75% of countries worldwide. Number of new cases of liver cancer in developing countries is estimated 600 000 cases in 2002, which represent 82% of countries worldwide and this numbers are expected to increase every year (Chuang et al., 2009). It seems that developing countries contribute to high percentages in cancer occurrences worldwide. In Malaysia a total of 18 216 new cases were diagnosed in 2007 and registered at the National Cancer Registry (NCR) (Zainal & Nor Saleha, 2011).
Cancer is being seen differently, the general public sees it as a modern day plague. Meanwhile for a patient, cancer is a terrifying alien entity that invades the body and can be treated only if it is detected in early stages. However, cancer is a collection of well-established cell lines that conveniently take up foreign DNA and proliferate endlessly (Green & Evan, 2002). Since 1998, most oncologist and basic scientists proposed the vast catalogue of cancer cell genotypes. Hanahan & Weinberg, 2000 had listed six alterations that occur in cancer cells such as self-sufficiency in growth signals, insensitivity to growth-inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), unlimited replication potential, sustained angiogenesis, tissue invasion and metastasis.

The progress of conceptual development in the last decade had created two new concepts which are reprogramming of energy metabolism and evading immune destruction in the list of cancer hallmark. Lists of the alterations are shown in figure 2.1 (Hanahan & Weinberg, 2011). Moreover it is accepted that cancers arise from the rare simultaneous acquisition of the two cooperating conditions that permit cell expansion, which is deregulated cell proliferation and suppressed apoptosis. To make sure that cancer cells undergo cell proliferation unlimitedly, these two processes must occur together in the same cell at the same time. Therefore cancer cells only propagate when received interlocking signal that simultaneously promote proliferation and suppress consequent apoptosis (Pardee, 2006).
Generally, cancer development consists of three major steps, initiation, promotion and progression. Initiation, which is an irreversible process, starts when normal cells are exposed to carcinogenic substances and their DNA undergo damage that remain unrepaird or misread. In chemical carcinogenesis, initiation involves the uptake of a given carcinogen, which is subsequently distributed to organs for metabolism. Metabolic activation leads to reactive species, which binds to DNA and cause coding errors at the time of replication which leads to mutation. The somatic mutation in a damaged cell can then be reproduced during mitosis to produce clones of mutated cells. The next stage in the carcinogenesis process, promotion, is the expansion of the damaged cells to form an actively proliferating multi-cellular premalignant tumor cell population. The last stage known as progression is the irreversible process, which produces new clone of tumor cells with increased proliferative capacity, invasiveness and metastasis (Pitot & Dragan, 1991).
The size, stage, rate of growth and other characteristics of the tumor determine the type of treatment. Treatments may include surgery, radiation, immunotherapy and drugs (hormonal therapy and chemotherapy) (Buchholz, 2009; Florescu et al., 2011). Radiation treatments show stimulating repopulation of tumor cells mediated by caspase-3 (Huang et al., 2011). Most of the chemotherapy treatment kill cells that are dividing rapidly anywhere in the body, and as a result it can cause temporary hair loss and digestive disturbance (Thao & Nelson, 2012). To avoid these adverse side effects, active researches on natural compounds to be used as treatments are being carried out. The use of natural compounds would certainly reduced toxicities.

Another important field of research is identifying drugs that can prevent cancer, known as chemoprevention. This includes the administration of pharmaceutical or dietary constituents that have been identified to have anti-cancer effects. The development of chemopreventive agents may help in inhibition of one or more stages of carcinogenesis which are initiation, promotion and progression (Walaszek et al., 2004).
2.1.1 MCF-7 Human Breast Cancer Cell

Breast cancer is the second most common cancer and the most common cancer among women worldwide, with an estimation of 1,152,161 new cases and 411,093 deaths per year (Kamangar et al., 2006). Currently, NCR reported that breast cancer is the most commonly diagnosed type of cancer among females in Peninsular Malaysia, with prevalence of 3242 female breast cancer cases with 18.1% of all cancer cases reported and 32.1% of all female cases in year 2007. Breast cancer was the most common cancer in all ethnic groups and the age pattern showed a peak at the 50-59 age group (Zainal & Nor Saleha, 2011).

Breast cancers originate from the inner lining of milk ducts or the lobules of breasts, and are known as ductal carcinomas and lobular carcinomas respectively (Sariego et al., 1995). However, there are also sarcomas but its occurrences are rare compared to carcinoma (Bloom & Richardson, 1957). Cumulative exposure to high estrogen levels is implicated as a causal factor. Breast cancer is most strongly associated with early menarche and late menopause, childlessness, late age at the birth of the first child, and increase in the total number of menstrual cycles in a woman’s life (McPherson et al., 2000; Key et al., 2001). Approximately 5% of cases are due to the BRCA1 and BRCA2 gene mutations (Peto et al., 1999).

Two most common cell lines investigated are MCF-7 and MDA-MB-231. The MCF-7 cell line is from primary human breast cancer cell and is a estrogen receptor-positive human breast adenocarcinoma cells whereas MDA-MB-231 cell line is derived from metastatic human breast cancer cell line and usually formed at late stage of breast cancer (Brooks et al., 1973; Cailleau et al., 1978).
2.1.2 HSC-2 and HSC-4 Human Oral Cancer Cell

Oral squamous cell carcinoma of the oral cavity and pharynx (OSCC) are the fifth most common cancer in the world. It was estimated that two-thirds of the cases occurs in developing countries with a total of 14,160 new cases in 2008 (Camisasca et al., 2009). This statistic proved that this cancer was the third most common in developing countries. In Malaysia, the NCR reported that, in year 2007 oral cancer is ranked as the 21st most common cancer in the general population and the 17th most common cancer in male and 16th for females. This figure looks lower if compared to world incidence. However, this is because NCR separates oral cancer (buccal mucosa and others) from cancer of the lip and tongue (Zainal & Nor Saleha, 2011).

Oral squamous cell carcinoma (OSCC) is a malignant neoplasm affecting the lining epithelium of the oral mucosa. The most frequent neoplasia arising from oral epithelium is squamous cell carcinomas, representing more than 90.0% of all tumor of oral cavity. Most of oral squamous cell carcinomas involve in the area of the lips, lateral border of the tongue, floor of the mouth, and retromolar area (posterior to the third molars). Oral squamous cell carcinoma infiltrates local tissues and spreads via the lymphatics to the cervical lymph nodes, where it may escape into the tissues of the neck (Oliveira et al., 2009). Oral cancer is a major cause of morbidity and mortality in India (Subapriya et al., 2006). In Malaysia, the Indians were found to have the highest incidence of oral cancer (Zainal & Nor Saleha, 2011).

HSC-2 and HSC-4 have neither invasive nor metastatic potential. These two parent cell lines were established from tumors of metastatic lymph nodes originated in squamous cell carcinomas of two different patients. HSC-2 cell line regional lymph nodes metastatic primary site are from floor of mouth meanwhile HSC-4 are from tongue (Momose et al., 1989).
2.1.3 Ca Ski Human Cervical Cancer Cell

Global cancer statistics indicate more than 85% of cervical cancer cases and deaths occur in developing countries. High number of cervical cancer burden in developing countries is largely due to a lack of screening that allows detection of precancerous and early stage cervical cancer (Jemal et al., 2011). In Malaysia, cervical cancer is the third most frequent cancer among women and fifth most common in the entire general population. Cervical cancer incidence rate in women increased after the age of 30 years old and the percentage of cervical cancer detected at stage 1 and 2 was 55% (Zainal & Nor Saleha, 2011).

Cervical carcinoma is a cancer of the neck (cervix) of the uterus. The tumor may develop from the surface epithelium of the cervix (squamous carcinoma) which comprise 80 to 90% of this cancer type or from the epithelial lining of the cervical canal (adenocarcinoma) which made up the remaining 10 to 20% cases (Castellsagué et al., 2006). In both cases the tumor is invasive, spreading to the surrounding tissue and subsequently to neighboring lymph nodes and adjacent organs, such as the bladder and rectum. Cancer of the cervix can be detected in an early stage of development and the established diagnosis for it is biopsy.

Ca Ski is an HPV-positive cancer cell line. Majority of cases are due to the infectious of human papilloma virus (HPV) and estradiol level elevation (Singh & Singh, 2011). Studies found that the two oncogenes named as the E6 and E7 are capable of inducing epithelial cell immortalization in culture alone, and increase cellular transformation with other oncogenes. The E6 and E7 oncogenes work to promote cell transformation by binding to two important tumor suppressor genes p53 and pRB respectively and disrupting their normal cellular function. Both genes are found in high-risk HPV strains such as HPV16 and HPV18 (Crook & Vousden, 1992).
2.1.4 HepG2 Human Liver Cancer Cell

Liver cancer is the third leading cause of cancer death in developing countries with an estimate of 544,000 death occurring in 2007. Africans and Asians are main contributors to the increasing number of occurrences of primary liver cancer in the world (Thun et al., 2010). Current update of liver cancer statistic in Malaysia was 605 cases diagnosed in 2007, comprising of 443 males and 162 males. This cancer type is the fifth most common cancer in males (Zainal & Nor Saleha, 2011).

Most primary liver cancer starts in liver cells called hepatocytes and thus it is called hepatocellular carcinoma. Therefore, three-quarter of liver cancers are found to be hepatocellular carcinoma (HCC) and 50% of all death are caused by chronic infection with either HBV or HVC (Tomimatsu et al., 1993). Meanwhile, the secondary sites that liver cancer usually spread would be the lymph nodes, lungs and bones. Primary liver cancer is different with secondary liver cancer which is the metastasis is from another part of the body (Perz et al., 2006).

HepG2 is a cell line derived from a patient with primary liver cancer. This type of cell derived from a liver epithelial and the morphology is classified as hepatoblastoma. They are epithelial in morphology, produce a variety of proteins such as prothrombin, alpha-fetoprotein, C3 activator, and fibrinogen, and express a wide variety of liver-specific metabolic functions, including those related to cholesterol and triglyceride metabolism, making them a useful in vitro model system (Javitt, 1990). Currently, the cell is actively used for toxicity evaluations (Koschutnig et al., 2009).
2.2 Cell Death

2.2.1 Apoptosis

Apoptosis is a highly regulated process and is important for normal functioning in animal cells. This process is triggered in response to specific stimuli that help to remove unwanted cells during development (Metzstein et al., 1998). The term apoptosis was coined by Kerr and colleagues in year 1972 which described cells undergoing death process under physiological condition (Kerr et al., 1972). During frog development, increasing of thyroid hormone triggered tail regression which is a form of an apoptotic event (Tata, 1994). Viral infection is among the process that leads to trigger apoptosis as a defense mechanism in viral infected cells (Hilleman, 2004). Apoptotic cells are characterized by cell shrinkage, chromatin condensation, DNA fragmentation and engulfment by macrophages and neighboring cells, thus avoiding pro inflammatory response by immune cells (Savill, 1998; Häcker, 2000; Abbro & Dini, 2003; Doonan & Cotter, 2008). The inflammatory response is prevented in apoptotic cells because the cellular debris is contained rather than released (Ellis et al., 1991).

This kind of programmed cell death has been well studied and described in the nematode C. elegans model. By understanding this model, it helps the researchers to discover and reveal the mechanism responsible for this crucial process especially in mammals (Metzstein et al., 1998; Lettre & Hengartner, 2006). As mentioned previously, this process is a highly regulated process which involved a series of genes. The genes were first described by the Nobel Prize Laureates (Hedgecock et al., 1983; H. M. Ellis & Horvitz, 1986). Their studies revealed three genes; ced-4, ced-9 and ced-3 in C. elegans that are important for the proper functioning of apoptosis. These genes
have been found throughout the animal kingdom, which suggest that the apoptotic machinery is conserved across species (McCarthy & Dixit, 1998).

In humans, the protein encoded by CED-9 is a homolog of the Bcl-2 family of proteins, which have both pro and anti-apoptotic functions (Hengartner & Horvitz, 1994b). CED-4 is a protein with sequence homology to Apaf-1, a human protein involved in the formation of the apoptosome (Zou et al., 1997). Finally, ced-3 codes for a protein similar to a family of cysteine proteases known as caspases in humans, which function as the executioners of apoptosis (Hengartner & Horvitz, 1994a). Since apoptosis is a highly regulated process in healthy cells, impairment on its mechanism resulted in pathological condition such as autoimmune diseases, neurodegenerative and cancer (Yoshida et al., 1998; O'Reilly & Strasser, 1999).

There are two main apoptotic signaling pathways proposed which are known as intrinsic and extrinsic pathways. Figure 2.2 represents both pathways that appear in mammalian cells. In the intrinsic pathway, extracellular stimuli such as DNA damage, ROS and stress response will stimulate translocation of Bax from the cytosol to the mitochondria, or alternatively, Bak activation at the mitochondria, followed by cytochrome c release, activation of downstream caspases to induce apoptosis (Kuwana & Newmeyer, 2003; Elmore, 2007). In the extrinsic pathway, the stimuli which are death receptor ligands will bind to receptors on the cell surface and initiate the apoptotic cascade. Once the death receptor pathway is activated, procaspase-8 will be cleaved and activated, then the active caspase-8 cleaves Bid and translocation of tBid to the mitochondria will take place, this lead to cytochrome c release, downstream caspase activation, and finally apoptotic morphological changes (Wang & El-Deiry, 2003; Fulda & Debatin, 2006).
Alternatively, in the extrinsic pathway, active caspase-8 can also interact directly with downstream effector caspases, caspase-3 to initiate apoptosis. Inhibitor of apoptosis proteins (IAP) is functioning at this convergent point and inhibits the activation of effector caspases thus inhibiting apoptosis (LaCasse et al., 1998; Schimmer, 2004).

The molecular hallmark of apoptosis is activation of the caspase proteins. These enzymes constitute a family of cystein proteases that have a fundamental role in apoptosis. In healthy cells, caspses exist as inactive precursors or caspase zymogens and will undergo aspartate-specific cleavage or proteolytic processing upon receiving an apoptotic signal (Jäger & Zwacka, 2010). These proteolytic products or active form of caspases will lead to activation of the apoptosis process (Olsson & Zhivotovsky, 2011). Caspases can be divided into two groups; initiator caspases and effector caspases. The initiator caspases are the caspases that get activated via recruitment to signaling complexes and it comprised of caspase-2, -8, -9 and -10 in mammals. The effector caspases which comprise of caspase-3, -6 and -7 can cleave several of the cellular substrates to produce biochemical effects associated with apoptosis. It also lacks of the ability to self-activate and hence require cleavage by activated initiator caspases to be an active form (Fulda & Debatin, 2006).

Generally, the Bcl-2 family of proteins comprises of pro-apoptotic and anti-apoptotic members which acts as a critical regulator of caspase activation during apoptosis (Kuwana & Newmeyer, 2003). Each member possesses at least one Bcl-2 Homology (BH) domain. The anti-apoptotic members include Bcl-2 and Bcl-xl which are characterized by the presence of BH1, BH2, BH3 and BH4 domains in their structure. The pro-apoptotic members of the Bcl-2 family include the Bax family of proteins such as Bax and Bak which possess BH1, BH2, BH3 domains and also the BH3 only proteins such as Bid and Bim. The pro-apoptotic members help cytochrome c
release while the anti-apoptotic members predominantly act on the Bax family of proteins to prevent release of cytochrome c from the mitochondria (Cory et al., 2003).

Figure 2.2(1): Apoptosis overviews. (Image obtained from Cell Signaling Technology, Inc, 2007)

2.2.2 Necrosis

Another form of cell death that has been actively studied is necrosis. Necrosis occurs when cells experienced a major insult, resulting in extensive failure of normal physiological pathways that are essential for the maintenance of cellular homeostasis. Necrotic cells is morphologically characterized by vacuolation of the cytoplasm, breakdown of the plasma membrane, swelling of organelles and induction of inflammation around the dying cell (Hanahan & Weinberg, 2011). A classic case of necrosis can be seen in conditions of ischemia that causes a severe depletion of oxygen, glucose, and other trophic factors which eventually induces the necrotic death of endothelial cells and some cells of the surrounding tissues (Kroemer et al., 2009).
This mode of cell death is often referred to as unscheduled cell death, implying that within multicellular organisms it is an unregulated process. It was suggested that activation of DNA damage response pathways induce necrotic cell death in cancer cells (Sun et al., 2006). It is believed that necrotic cell death is an alternative death mode for cells defect in apoptosis pathway. Alkalyting chemotherapy can induce an immediate metabolic crisis by depleting NAD$^+$ and ATP. Since most of cancer cells depend on glycolysis for ATP production, inhibition of aerobic glycolysis mediated metabolic crisis causes these cells to die via necrosis. In contrast, cancer cells treated with therapeutic stress can increase intracellular calcium concentration and ROS, then activates phospholipase A2 and subsequently leading to irreversible necrotic cell death. Figure 2.2(2) illustrates the effects of both stress (Amaravadi & Thompson, 2007).

**Figure 2.2(2):** The relationship between necrosis, apoptosis and autophagy cell deaths induce by therapeutic and metabolic stress. (Image obtained from Amaravadi & Thompson, 2007)
2.3 Cell cycle

2.3.1 Cell cycle overview

Cell growth and proliferation depend on highly regulated process which is known as cell cycle. It is a complex process and involves numerous regulatory proteins that direct the cell through a specific set of events leading to mitosis (Schafer, 1998). Basically, the main function of the cell cycle is to accurately duplicate DNA in the chromosomes and then segregate the copies into two daughter cells. Different forms of cyclins and cyclin-dependent kinases (Cdk) are identified as key roles for initiation the cell cycle events at appropriate times during the process (Jackson et al., 1995; Georgi et al., 2002).

Morphologically, the cell cycle can be subdivided into interphase and mitotic (M) phase which include prophase, metaphase, anaphase, and telophase (Mitchison & Salmon, 2001). Meanwhile G₁, S, and G₂ phases are the phases located in interphase. The G₁ and G₂ phases of the cycle are the “gaps” in the cell cycle placed between the two obvious landmarks, DNA synthesis and mitosis. During G₁ phase, a cell makes the decision to proceed, pause, or exit the cell cycle based on the received mitogenic and growth inhibitory signals. S phase is defined as the stage in which DNA synthesis occurs. Therefore, S phase cells have aneuploid DNA content between 2N and 4N. The G₂ phase is the second gap in the cell cycle in which the cell prepares for the process of division or M phase. In the M phase, in order for the cells to form two daughter cells, the replicated chromosomes are segregate into separate nuclei and undergo cytokinesis process. In addition to G₁, S, G₂, and M, the term G₀ is used to describe cells that have exited the cell cycle and become quiescent (Schafer, 1998; Mitchison & Salmon, 2001; DiPaola, 2002). Illustration of mammalian cell cycle is shown in figure 2.3.
2.3.2 Cell cycle checkpoints and Restriction point

Eukaryotic cells perform the fidelity of cell division by a mechanism known as cell cycle checkpoints. The mechanism provides the integrity of each cell cycle event by preventing initiation of the later event until the earlier event is completed. As an example, the completion of cellular DNA replication is a checkpoint that must be passed before the chromosome separation in mitosis can begin. Although the term checkpoint and the above example suggest a discrete point or time in the cell cycle, checkpoints are often more indistinct (Pardee, 1974; Hartwell & Weinert, 1989).

Most cells stop the cell cycle and enter a resting state called G₀ in a response to suboptimal growth conditions, such as, high cell density, serum deprivation, limitation of some nutrients and the presence of certain drugs. It is also shown that extracellular signals such as growth factors act primarily on cells in G₀ and G₁ phases. They stimulate cells from G₀ phase to enter the cell cycle and stimulate cells in G₁ phase to replicate DNA. However, once the DNA replication is started, growth factors have no control over the S phase progression. (Blagosklonny & Pardee, 2002; Foster et al., 2010).

Early in G₁ phase, the removal of growth factors will result in cells returning to G₀ phase. However, when cells reach a certain point in G₁ phase, they become committed to entering and completing the cell cycle. This important regulatory point in G₁ phase at which cells no longer require growth factors has been termed the Restriction point (Campisi & Pardee, 1984; Hartwell & Weinert, 1989; Assoian & Zhu, 1997). Therefore, this checkpoint is responsible for determining whether cells are irreversibly committed to initiate DNA synthesis and undergo cell division, or to enter a resting state. Arthur Pardee is the first person that described the restriction point and suggested the position of it to be anywhere between middle and late G₁ phase,
depending on the cell type (Pardee, 1974). Importantly, Pardee also found that the restriction point was defective in cancer cells, providing physiological relevance for the restriction point in cancer treatment.

Cell cycle arrest, also referred to as delay, is produced by a variety of factors that may be intrinsic (e.g. cell size) or extrinsic (nutrition factors) and may affect any checkpoint (Hilakivi-Clarke et al., 2004; Jorgensen & Tyers, 2004). Other factors, such as DNA damaging agents, can also trigger checkpoints that produce arrest in G₁ and G₂ phases of the cell cycle. Cells can also be arrested in S phase, which results in a prolonged S phase and slowed DNA synthesis. Arrest in G₁ allows repair before DNA replication, whereas arrest in G₂ allows repair before chromosome separation in mitosis (Mikhailov et al., 2002).

2.3.3 Cell cycle and cancer

Proliferation of normal cells occurs in response to developmental or other mitogenic signals whereas the proliferation of cancer cells proceeds essentially unchecked. For that reason, uncontrolled cell-cycle is considered as one of the fundamental aspects of cancer. There are direct link between the deregulation of cell-cycle control and cancer (Castedo et al., 2002). This assumption was first discovered when it was shown that the genes that encode Rb and p53, both of which empower cell-cycle progression, are frequently mutated or deleted in human cancers (Schafer, 1998). Low expression of p27 is frequently found in various human cancers. However, mutations or deletions in it were shown to be rare compared to Rb and p53 in cancer patients. Instead, a decrease in p27 protein stability contributes to its reduced level in cancer cells (Lidhar et al., 1999; Brown et al., 2004).
Although dysregulated cell cycle is frequently observed in cancer, accumulation of abnormal cancer cells was found even in the presence of only small numbers of cycling cells. The reason for this phenomenon is an apoptosis suppression, which has been recently recognized and accepted as another central event in the development and progression of cancer (Malumbres & Barbacid, 2009). Comparison of the mammalian cell cycle with human cell cycle is depicted in figure 2.3.

Figure 2.3: Comparison of the mammalian cell cycle with human cancer cell cycle. (Adapted from Malumbres & Barbacid, 2009)
2.4 Natural product as Anti-Cancer Agents

Natural products have been shown as potential agent in inducing cell death in cancer cells. Many natural products and synthetically modified natural product derivatives have been successfully developed for clinical use to treat human diseases in almost all therapeutic areas. Anti-tumor drugs derived from a natural product with semi-synthetic modification “ND” representing approximately 28.0% of all anti-cancer drug from 1940s to 2010 (Newman & Cragg, 2012). In fact, plant-derived compounds have played an important role in treatment of cancers, and some of the most promising and effective drugs have come up from plant sources like taxol®, camptothecin, combrestatin, vinblastine and vincristine. Recently, Polyphenon E®, one of natural product that was approved by FDA in 2007 for treatment of genital warts linked to human papilloma viruses (HPV) was found in recent studies to prevent bladder, prostate, and lung cancer (Graff, 2009; Newman & Cragg, 2012). Apart from these, many other plant-derived compounds are now in clinical trials as anticancer agents. Natural products have been the major sources of chemical diversity that drives pharmaceutical company to discover new insight of these substances as anti-cancer agent over the past century.

2.4.1 Botanical aspect of Meliaceae

Meliaceae, or the mahogany family, embrace over 1400 species in 51 genera of woody plants allocated in subtropical and tropical regions worldwide. Within the family, two major subfamilies were designated, Melioideae and Swietenioideae (Oliver-Bever, 1986). They have been recognized based on seed morphology (Harms, 1940), wood anatomy and more recently molecular data (Muellner et al., 2008). The name Cedreloideae has priority over Swietenioideae but is practically never used (Thorne & Reveal, 2007). Melioideae form a monophyletic group that includes 73% of
the genera and 87% of the species of the family (Muellner et al., 2008), and are characterized by naked buds, unwinged seeds, and fruits that are berries, drupes, nuts, or loculicidal capsules. Swietenioidae include 14 genera and only 51 species, most of which are used as timber worldwide. They are characterized primarily by their buds protected by scale-like leaves, septifragral capsules and winged seeds. Many species are deciduous, either in dry season or in winter, but some are evergreen. Mahogany is a very bulky canopy tree, sometimes reaching over 150 feet in height, with trunks sometimes exceeding six feet in diameter above a large basal buttress. It is generally open-crowned tree, with gray to brownish-red fissured bark. Considering its flowers, and fruit-structure, it is undoubtedly a climax family, more advanced than Rutaceae with which it is slightly related. The flowers are mostly free petals, but more especially by the androecium with filaments variously united into a tube which sometimes resembles a corolla (Benson, 1979; Mabberly & Pannell, 1989; Pannell, 1992).

**2.4.2 Chisocheton tomentosus properties**

*Chisocheton tomentosus* (Figure 2.4(1)) is a medium-sized tree that can grow up to 21 m. unbranched Corner’s Model or sparsely branched, often from near the base and 60 cm girth. The bole is sometimes weakly fluted or with small stilt roots. Its bark is blackish brown, smooth to weakly fissured, with conspicuous shield-shape, all young parts brown tomentose with irritant hairs. Its leaves are about 2 m long, imparipinnate-leaflets, rugose, shiny and glabrous above except for brown tomentose midrib, tomentose or tawny pubescent below where strongly reticulate with 12-30 veins on each side of midrib. Inflorescences to 1 m long, with flowers forming a terminal head in distal half. Flowers-hermaphrodite or unisexual on same tree; calyx 5-lobed; petals 5, free; filaments joined near base, terminated by a pair of thread-like appendages; anthers
10, hairy; disc small, united to base of filaments; ovary 5 (6)-locular; style head with 5 small stigmatic lobes. Fruits are small spherical with 7.5 cm diameter, sub-globular, golden brown velvety with irritant detachable hairs; seeds 3-5, to 4 cm long with white sarcotesta (Mabberly & Pannell, 1989)

![Figure 2.4(1): Chisocheton tomentosus fruit and leaves](image)

### 2.4.3 Chemical constituents of *Chisocheton* species

Compared with some of the well-studied genera in the Meliaceae family, such as *Azadirachta* and *aglaia* species, very little research has been done on the *Chisocheton* species. Earlier phytochemical studies on the bark extracts of *Chisocheton ceramicus* had identified four new limonoids (Mohamad *et al.*, 2008; Khalit Mohamad *et al.*, 2009), while no investigation has been found on *Chisocheton tomentosus*. Table 2.1 shows *Chisocheton* species, types of compound and compounds extracted from the plants.
Table 2.1: Occurrence of some selected chemical compounds in various species of *Chisocheton*

<table>
<thead>
<tr>
<th>No.</th>
<th><em>Chisocheton</em> species</th>
<th>Type of compounds</th>
<th>Compound</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>Chisocheton</em> Ceramicus</td>
<td>Limonoid</td>
<td>ceramicine A</td>
<td>(Mohamad <em>et al.</em>, 2008; Mohamad <em>et al.</em>, 2009)</td>
</tr>
<tr>
<td>2</td>
<td><em>Chisocheton</em> erythrocarpus</td>
<td>Limonoid</td>
<td>erythrocarpine A</td>
<td>(Awang <em>et al.</em>, 2007a)</td>
</tr>
<tr>
<td>3</td>
<td><em>Chisocheton</em> siamensis</td>
<td>Limonoid</td>
<td>dysobinin</td>
<td>(Maneerat <em>et al.</em>, 2008)</td>
</tr>
<tr>
<td>4</td>
<td><em>Chisocheton</em> macrophyllus</td>
<td>triterpenoid</td>
<td>moronic acid</td>
<td>(Inada <em>et al.</em>, 1993)</td>
</tr>
<tr>
<td>7</td>
<td><em>Chisocheton</em> penduliflorus</td>
<td>Sesquiterpenoi triterpenoid coumarin phenolic</td>
<td>allo-aromadendrane-10α,14-diol dammaradienone scoparone vanilinic acid</td>
<td>(Phongmaykin <em>et al.</em>, 2008b; Phongmaykin <em>et al.</em>, 2008a)</td>
</tr>
<tr>
<td>8</td>
<td><em>Chisocheton</em> weinlandii</td>
<td>Alkaloid</td>
<td>chisitine</td>
<td>(Tzouros <em>et al.</em>, 2004)</td>
</tr>
</tbody>
</table>

2.4.4 Properties and role of phytosterol in cancer

The plant-derived photochemicals known as plant sterols or phytosterols are the plant counterparts of cholesterol in mammals. Over 100 different phytosterols exist in nature, which belong to the triterpene family (Moreau *et al.*, 2002). Phytosterols play a structural as well as metabolic role in plant physiology and essential in the management of fluidity and permeability of plant cell membranes. Membrane functions such as simple diffusion, carrier-mediated diffusion, active transport and regulation of membrane-related proteins (e.g., enzymes, receptors and elements of signal
transduction) are also directed by phytosterols (Piironen et al., 2003). The most prevalent phytosterols in nature are β-sitosterol (SIT), campesterol and stigmasterol.

Figure 2.4(2) illustrates how similar the structures of these three phytosterols are in comparison to cholesterol. The difference occurs at C24 where the addition of a methyl group forms campesterol, an ethyl group forms SIT, and an ethyl group plus the addition of a double bond at C22 creates stigmasterol. Unrefined vegetable oils, cereals, vegetables, fruits, berries, beans/legumes, seeds and nuts all contain sizable amounts of SIT, campesterol, and stigmasterol (Piironen, et al., 2003). Phytosterols are structurally similar to cholesterol but slightly different at 24 carbon position with additional of ethyl group. It has more hydrophobic properties than cholesterol and poorly soluble in water and more soluble in oil phases. These properties increase affinity to micelles replace absorption of intestinal cholesterol into gut (Rubis, et al., 2008).

Figure 2.4(2): Structure of cholesterol and major phytosterol; β-Sitosterol, stimasterol and campesterol. (Adapted from Bradford & Awad, 2007)
Since plant sterols must be acquired from the diet and in vivo oxidation of phytosterol in plasma, they are not metabolized; their plasma concentrations depend on their intestinal absorption and biliary elimination rates. Besides being acquired using these methods, plant sterol in human serum can also be derived from the oxidation catalysed by UV light of skin phytosterols absorbed from cosmetic products (Tonello & Poli, 2006). Major form of phytosterols that exist in higher plant is β-sitosterol followed by campesterol and stigmasterol (Ju et al., 2004; Woyengo et al., 2009; Kurban et al., 2010; O'Callaghan et al., 2010; Otaegui-Arrazola et al., 2010). About 44 phytosterols have been identified to date (Paniagua-Perez et al., 2008). Recently in 2010, Kurban et al reported that the major phytosterol, β-sitsterol has a relatively large dipole moment and seem to be polar (hydrophilic) molecules (Kurban et al., 2010). A study done by Sudhamalla et al., 2010 found that β-sitosterol can bind to major carrier protein; human serum albumin protein mainly by hydrophobic interaction and hydrogen bond interaction. They come out with a hypothesis that elucidation of this molecular dynamics is important for understanding the stability of the protein-drug complex system in aqueous solution.

Most of the studies conducted so far measured anti-cancer effect of β-sitosterol using in vitro system where the compound was added directly to various cell lines of macrophage, cancer cells and animal models. It was shown that β-sitosterol was able to induce death in ACAT-competent macrophages (Bao et al., 2006). They also reported that macrophage cells death were via different mechanisms caused by subtle changes in sterol structure (Bao et al., 2006). A year later, Tabas, 2007 confirmed that an ethyl group on sterol side chain fundamentally alters the way cells respond in the mechanism of sterol-induced cell death in macrophage cells. Alappat et al., 2010 reported that combination of sitosterol with structurally similar molecules such as vitamin D promoted immune function in macrophage cells. They found that sitosterol can enhance
the immune modulatory action of vitamin D and postulated that this effect was as a result of sitosterol’s ability to influence the binding properties of vitamin D to its receptors.

In a study of different dimension, Rubis et al., 2010 observed that β-sitosterol can induce cytotoxic effect in multidrug resistant cancer cell. There are many pathways that were proposed for the phytosterol-mediated apoptosis process in cancer culture models. Elucidation of pathway works induced by phytosterol had been carried out by many researcher groups since a decade ago and since five years lately, a few papers had been published. Generally, they found that these compounds were targeting proteins that are involved in upstream and downstream signaling pathways which in turn empower it to drive apoptosis. The Ras/ERK and PI(3)K/Akt pathways stand out in this respect (Awad, et al., 2007; Moon, et al., 2007; Moon, et al., 2008; Park, et al., 2008). At the same time, it also influences cell division and cell fate (Moon, et al., 2008; Chien, et al., 2010; Hsu, et al., 2011).

2.4.5 Phytosterol oxides in culture and in vivo

Oxysterols are oxidized derivatives of cholesterol and phytosterols. Due to the presence of an unsaturated ring structure (Ryan, et al., 2005) and a double bond between C5 and C6, sterols can undergo oxidative processes, resulting in the formation of oxysterols or sterol oxidation products (SOPs). However Koschutnig et al., 2009 showed that the oxidation process could also be caused by physiological condition such as heating and long-time storage.

In healthy human, the amounts of different type of oxysterols appear in various levels. Since cholesterol oxidation products (COPs) in in vitro were five times more cytotoxic than phytosterol oxidation products (POPs), the uptake of POP is essentially important for normal and cancer cells regression. Oxyphytosterols differ from the
parent sterol only in a few structural changes. Commonly steroid ring oxidation products are hydroxy- (OH-), keto (=O), expoxy and triol-derivatives (Hovenkamp, et al., 2008). The most abundant POPs in human plasma are β-epoxysitostanol and sitostanetriol, while campestanetriol, a-epoxysitostanol, 7-ketositosterol and 7β-hydroxsitosterol concentrations were lower (Tonello & Poli, 2006; Otaegui-Arrazola, et al., 2010).

Recently various studies were carried out to investigate the effects of oxysterol in various cancer cell lines, namely, Caco-2 cell and U937 cells. However other cell lines used were HepG2, MCF-7, Ishikawa and stomach cells (Newill et al., 2007; Koschutnig, et al., 2009). Newill et al., 2007 found that oxidation of stigmasterol products with noncytotoxic concentration of 10.0 μM are able to decrease proliferation of cells induced by 17β-estradiol (E2). They also found that this inhibition effect depend on estrogen receptor status. Their findings showed that oxy-stigmasterol have affinity to bind to ERβ compared with ERα. Meanwhile, O’Callaghan et al., 2010 reported that these oxidized derivative stigmasterol could induce apoptosis in U937 human monocytic cell line.

In HepG2 cell lines, sterol oxides especially β-sitosterol oxides can induce cytotoxicity. Interestingly, different groups found distinctly different concentrations that induce cytotoxic effect. Koschutnig et al., 2009 found with low sample concentration of 30.0 μM of β-sitosterol oxides were able to start killing cells. In contrary, Ryan et al., 2005 found toxicity with concentrations higher than 60.0 μM.

So far there are no investigations on the effect of oxyphytosterol on Ca Ski cells. Wang et al., 2006 showed that microtubular inhibitory effect was induced as a result of β-sitosterol treatment on SiHa cells growth. They treated the SiHa cells with β-sitosterol for 5 days and observed abnormal microtubular network using confocal microscopy. FACS analysis of β-sitosterol-treated SiHa showed increasing
accumulation of cells in S phase. Moreover, the proportion of polymerization of microtubule reduced in a time dependent manner after β-sitosterol treatment measured by immunoblotting analysis. Hence, they concluded the proliferation inhibition by β-sitosterol in SiHa cells were mediated by inhibition of the microtubular polymerization.

In another study initiated by Li et al., 2010 investigated the apoptosis induction potential of PE, a novel-lipid soluble extract from Pinellia pedatisecta Schott and molecular mechanism of its action in Ca Ski and HeLa cells. Their results indicated that HeLa and Ca Ski cells underwent apoptosis after 24 h treatment with PE. PE treatment increased expression of caspase-8, caspase-3, p53, p21 and Bax mRNA and protein levels while decreased Bcl-2 mRNA as well as protein level. Although they used the extract, they hypothesized that β-sitosterol contained in Pinellia pedatisecta is responsible for induction of cytotoxicity and apoptosis on Ca Ski and HeLa cells and they concluded that PE functioned as tumor suppressor. However further work in evaluating β-sitosterol effect is needed to confirm that β-sitosterol is the anticancer agent in Pinellia pedatisecta. Till today, no work has been carried out to evaluate the effect of β-sitosterol or its oxides toward oral squamous cancer cell. Although this type of cancer is common in developing countries and intake of sitosterol is highest in eastern contries, it is interesting to see if there is a correlation of these phytosterols on oral squamous cancer cells.

Nevertheless, a recent pattern of research by molecular investigators is to consider the side or protection effects of natural product on normal cells. Rubis et al., 2008 looked into the beneficial or harmful influence of phytosterols on human cells. They discussed the cytotoxic effects of β-sitosterol and its epoxy-derivative toward human abdominal aorta endothelia cells HAAE-2. They found that β-sitosterol was more cytotoxic than its epoxy-derivative. Their findings also showed that β-sitosterol can induce apoptosis in this cell line by increasing caspase-3 activity and formation of
fragmented DNA. However, all this effect was not observed in cells treated with its epoxy derivative. They concluded that the oxy-derivative sterol is less cytotoxic toward normal cells compared to \( \beta \)-sitosterol.

2.5 Bcl-2 Family Proteins

2.5.1 Bcl-2 Family Proteins

The Bcl-2 proteins are a family of proteins that possess either pro- or anti-apoptotic properties. The first member of the family to be discovered was Bcl-2 (Reed, 2008). In attempting to determine the order of events that occurred during the development of B-cell lymphomas and leukemias in adults, recombinant DNA technology was used to investigate the chromosomal rearrangements. The 14;18 translocation was found in a patient-derived follicular lymphoma cell line. Characterization of this translocation showed that the breakpoint cluster region-2, Bcl-2 gene was now arranged in close proximity to the heavy chain immunoglobulin enhancer. This resulted in constitutive overexpression of Bcl-2 leading to clonal expansion of the B cells that contained this translocation (Pegoraro et al., 1984). Accordingly, Bcl-2 was considered as a putative oncogene. Since the discovery of Bcl-2, many more members of this family have been identified. Bcl-2 is essential to the process of apoptosis because it suppresses the initiation of the cell death process by binding to and sequestering BH3-only proteins thereby protecting the integrity of the mitochondrial membrane and inhibiting the apoptotic cascade.

2.5.2 Anti-apoptotic Protein

The Bcl-2 family shares homology in four domains referred to as the Bcl-2 homology (BH) domains. BH1, BH2, and BH3 facilitate the binding between Bcl-2 proteins. Figure 2.5(1) shows functional subgroup of Bcl-2 family. In Bcl-2, Bcl-xL, Bfl-1/A1 or Mcl-1, the BH1 and BH2 domains form a ‘hydrophobic pocket’ on the
surface of the protein. The BH3 domains of the pro-apoptotic proteins bind into the pocket created. A BH4 domain is found only in the anti-apoptotic Bcl-2 proteins and is thought to be essential for survival. It has been implicated in providing protection through the activation of survival signaling mechanisms (Adams & Cory, 1998; Cory, et al., 2003).

Localization of the anti-apoptotic Bcl-2 family proteins varies. Their hydrophobic carboxy-terminal domain helps target them to the cytoplasmic face of three intracellular membranes: the outer mitochondrial membrane, the endoplasmic reticulum (ER) membrane and the nuclear envelope. They function mainly at the mitochondria to prevent the activation of Bax and Bak and ultimately apoptosis from occurring. More specifically, Bcl-2 has been shown to be localized to both the mitochondria and to the (ER) (Hacki et al., 2000); association with the mitochondria is involved in sequestering BH3-only proteins while association with the ER is involved in the stress response and Ca2+ release (Bassik et al., 2004).

Recent evidence suggests that Bcl-2 can be converted from an anti-apoptotic protein to a pro-apoptotic protein following the binding of Nur77 (Kolluri et al., 2008). Nur77 is an orphan member of the steroid/thyroid/retinoid nuclear receptor superfamily (Lin et al., 2004; Han et al., 2006). The protein can move from the nucleus to the mitochondria and then interacts with Bcl-2 protein within its N-terminal loop region between BH domains 3 and 4, resulting in a conformational change. The conformational change ultimately converts Bcl-2 from a pro-survival protein to an anti-survival protein (Thompson & Winoto, 2008). It was suggested that translocation of Nur77 from the nucleus to cytoplasm is regulated by activation of JNK and inhibition of Akt (Han, et al., 2006). Currently, the attempts are being made in activating Nur77 from the nucleus to the mitochondria in cells overexpressing Bcl-2 where the goal is to make Bcl-2 proteins responsible for cell death rather than a survival advantage.
Figure 2.5(1): The Bcl-2 family. Three subfamilies are indicated: The Bcl-2 cohort promotes cell survival, whereas the Bax and BH3-only cohort facilitate apoptosis. (Adapted from Kuwana & Newmeyer, 2003)

2.5.3 Pro-apoptotic Protein

Pro-apoptotic Bcl-2 proteins may have BH1-3 domains or a BH3 domain only. Bax and Bak contain all three BH domains. Bak resides on the mitochondrial membrane and Bax resides as a monomer in the cytosol. In response to chemotherapeutic agents, Bax changes conformation, integrates into the outer mitochondrial membrane where Bax and Bak homo-oligomerize create pores through which cytochrome c is released leading to caspase activation and ultimately apoptosis (Mikhailov et al., 2003). The concurrent deletion of both Bak and Bax dramatically impairs apoptosis in many tissues and accordingly either Bak or Bax are essential for apoptosis (Upreti et al., 2008; Gillissen et al., 2010).
The remainder of the pro-apoptotic proteins are referred to as ‘BH3-only’ proteins and cannot function alone to cause cytochrome c release. These proteins are activated through many mechanisms including increased transcription, protein stabilization, and post-translational modification (Happo et al., 2012). More specifically, Bmf and Bim have both been shown to be bound to actin or dynein motor complexes within microtubules and upon microtubule disruption they are activated and released (Pinon et al., 2009); PUMA and Noxa are induced in response to p53 (Shibue et al., 2006); Bid is activated by proteolysis to tBid upon engagement of the death receptors with ligand (Yi et al., 2003; Kantari & Walczak, 2011); Bad is activated by dephosphorylation in response to growth factor withdrawal (Tsang et al., 2008). The BH3-only proteins act upstream of Bax and Bak, and require either Bax or Bak to elicit apoptosis.

Currently there are two models proposed on how activation of Bax and Bak occur. It was termed as direct and indirect activation (Figure 2.5(2)). The direct activation model proposes that only certain BH3-only proteins such as Bim and Bid can bind Bax and Bak directly and promote their activation (van Delft & Huang, 2006; Chipuk & Green, 2008; Cui et al., 2008; Dewson & Kluck, 2009). The remaining BH3-only proteins, called sensitizers such as PUMA, Noxa, Bad and other cannot bind to and directly activate Bax and Bak (Chipuk & Green, 2008) but instead they bind to the anti-apoptotic proteins thus displacing the activators that allow them to activate Bax and Bak (Basañez & Hardwick, 2008; Cui, et al., 2008; Tait & Green, 2010). According to the indirect activation model, Bax and Bak must be bound by anti-apoptotic proteins to prevent their activation (Adams & Cory, 2007; Uren et al., 2007; Brunelle & Letai, 2009; Leslie, 2009). It is suggested that all of the BH3-only proteins...
can target and then neutralize the anti-apoptotic proteins, allowing for Bax and Bak activation. However, the mechanism by which Bax and Bak become activated or ‘primed’ in the indirect model is still controversial.

Figure 2.5(2): Model of (a) direct and (b) indirect activation of Bax/Bak. (Image obtained from Adam & Cory, 2007)
2.6 Caspase Family of Protease Enzymes

2.6.1 Caspase Family Members Overview

Proteases are one wide class of human proteolytic enzymes found in mammalian which are extensively researched by scientists for its tumor suppression potential. Intracellular proteases have a wide range of protease including cysteine-dependent aspartate-specific proteases (caspases) which tightly regulated cascade of proteolytic activities that result in apoptosis process. The human degradome demonstrates at least 569 protease members produced by human cells and 145 of its represent cysteine proteases (Lopez-Otin & Matrisian, 2007). Since first discovered two decades ago, studies conducted had shown caspases to play central functions in apoptotic process and inflammatory signaling pathways. Generally, these proteins had been considered as key mediators in inducing apoptotic effects especially in cancer cell in vivo and in vitro (Kumar, 2006).

Currently, the researchers had found 14 different types of caspases and two-thirds of it, is involved in cell death process. These proteins can be group further into two categorize, first known as large prodomains that consist of caspase-1, -2, -4, -5, -8, -9, -10, -11, -12, -13 and -14 and second as short prodomain that consist of caspase-3,-6 and -7. Figure 2.6(1) illustrates the structures of caspases that exist in the human mammalian cells. Short prodomain proteins are believed not to change back to its initial forms when cells are committed to apoptosis after its activation. Activation of these proteins showed complex ways in normal cells (Lavrik et al., 2005; Pop & Salvesen, 2009)
Caspase-8 is a cysteine protease that initiates apoptotic cell death in response to cell surface receptor activation. It is also referred as the initiator extrinsically-triggered apoptosis and play important role in cellular activation and differentiation downstream of a variety of cell surface receptor (Kantari & Walczak, 2011; Oberst & Green, 2011; van Raam & Salvesen, 2012). Genomic studies have shown that the caspase-8 gene maps to chromosome 2q33. It is a 55 kDa protein of 480 amino acids that comprise of two death-effector-domains (DED) in its prodomain at the N- and a C-terminal catalytic protease domain. Two isoforms of caspase-8 had been identified which are caspase-8a and caspase-8b that are concomitantly expressed and both signal to cell death (Fulda, 2009). It has been biochemically demonstrated that activation of procaspase-8 occurs by dimerization (Fuentes-Prior & Salvesen, 2004; Pop & Salvesen, 2009).
A study reported that effector caspase which is caspase-6 regulates activation of caspase-8 in cytosol (Cowling & Downward, 2002). Flice-like inhibitory protein (FLIP) was identified as an inhibitor for deactivation of this caspase (Oberst & Green, 2011). It was reported that caspase-8 is down-regulated in several cancer cell lines due to different mechanisms of action (Philchenkov et al., 2004; Olsson & Zhivotovsky, 2011). An example is in the breast cancer cell line such as MCF-7 cells, Wu et al., 2010 proved that CpG site promoter methylation could lead to this phenomenon.

Caspase-9 is the initiator caspase of the post-mitochondrial intrinsic pathway (Kurokawa & Kornbluth, 2009). The activation of this caspase is achieved via cleavage at PEPD315kA and DQLD330kA site in the linker region. The cleavage at PEPD315kA is essential for inhibition of caspase-9 by X-linked IAP (XIAP) whereas cleavage at DQLD330kA will remove the inhibitory effect of XIAP and subsequently promotes apoptosis. Moreover caspase-3 was found to activate the proteolytic potential of caspase-9 by removing the neoeptope exosite required for caspase-9 inhibition (Timmer & Salvesen, 2006; Pop & Salvesen, 2009). Mutation or loss of CASP9 heterozygosity is not frequently observed in human cancers (Olsson & Zhivotovsky, 2011). It is suggested that majority of caspase activity is modulated by phosphorylation of variety of kinases or phosphatases. Studies demonstrated phosphorylation on caspase-9 prodomain at threonin 125 by extracellular regulating kinase (ERK) suppresses caspase-9 activation (Martin et al., 2008; Kurokawa & Kornbluth, 2009).

Caspase-3 is the most widespread effector caspases that has been extensively investigated since 15 years ago. Caspase-3 is produced as an inactive 32-kDa pro-enzyme which is cleaved at an aspartate residue to yield a 12-kDa and two 17-kDa active fragments. This active form then target a wide range of cellular substrates including structural proteins and DNA repairs enzymes (O’Donovan et al., 2003). DNA fragmentation, chromatin condensation and nuclear disruption are characteristics of
apoptotic nuclear changes resulting from translocation of active caspase-3 into the nuclease. Luo et al., 2010 found that caspase-3 harbors a crm-1-independent nuclear export signal (NES) in it small subunit. They proved that the p3-mediated specific cleavage activity of active caspase-3 abrogates the function of the NES thus facilitating the nuclear entry of this caspases. This caspase shares considerable structural homology with caspase-7 but it exhibits differential activity towards multiple substrate proteins, including Bid, XIAP, gelsolin, caspase-6 and cochaperone p23. It is also indicated that caspase-3 has its own role without playing nonredundant role with others caspases within the cell death machinery (Kothakota et al., 1997; Putt et al., 2006; Walsh et al., 2008).

Currently very little work has been carried out on the activation of caspase-6 (Lu & Chen, 2011). A study conducted by Klaiman et al., 2009 proposed a mechanism of how these proteins could be activated either in vivo or in culture. Over-expression of pro-caspase-6 which lacked the “safety catch” can generate active subunits of caspase-6. This process is known as self-activation and is not inhibited by IAP families. Activation is done by cleavage at the D23, D179 and D193 residue of heterotetramer of two p20 and two p10 subunits to produce the active form of caspase-6. They also found that treated caspase-6 transfected-HEKT cells with staurosporin reduced level of pro-caspase-3 while not influencing the level of active caspase-3, indicating that subunit turn over occurred rapidly in treated culture (Lamkanfi et al., 2006).

2.6.2 The Caspase Pathway

The complexity of caspase pathway has been discussed by many since three decades ago. Many of the early studies reported on this matter have been reviewed by Slee et al., 1999. They proposed four routes or phase to caspase activation within the cell death programme which are initiation, commitment, amplification and demolition.
Amplification or activation of downstream caspases demonstrated that activating caspase-9 is the most important event in propagating the death signal by activating other caspases. The use of the dATP activated cell lysate model facilitated to understanding the order of caspases in cells undergoing apoptosis by intrinsic pathway. The model shows active caspase-9 simultaneously activates caspase-3 and caspase-7, and then active caspase-3 which drive the activation of caspase-2 and caspase-6. Removal of caspase-6 from this system, lead to blockage in the activation of caspase-8 and caspase-10 and this observation suggest that caspase-6 is essential in the caspase pathway. The schematic representation of the pathway proposed by Slee et al., 1999 is illustrated in Figure 2.6(2) with exception of the two double arrows (Lavrik et al., 2005).

Recently, latest update of the ordering signaling of caspase action was elucidated by Inoue et al., 2009. Since apoptosis can be activated by the intrinsic pathway which is mainly regulated by the initiator and effector caspase, targeting these caspases with small interfering RNA and caspase inhibitor as well as using caspase deficient cells helped to confirm the precise ordering of caspases in intact cells. The dATP activated cell lysate model shows caspase-9 directly processes and activates caspase-3 and caspase-7, and then active caspase-3, processes caspase-2 and caspase-6, and subsequently the activated caspase-6 processes caspase-8 and caspase-10. Further study conducted using siRNA and caspase inhibitor demonstrated that caspase-7 can also directly process and activate caspase-2 and caspase-6, and then active caspase-6 process caspase-8 and cleavage of caspase-6 substrates, including lamin A/C. Figure 2.6(2) illustrates the hierarchical ordering of caspase in cells inducing mitochondrial-dependent apoptosis pathway.
2.6.3 IAP Family proteins

Proteolysis is an irreversible event. The inhibitor of apoptosis, IAP family is a type of proteins that have two functions in cells: firstly, it can degrade the caspases via the ubiquitin-mediated proteosome pathway and; second it can inhibit enzymatic activity of caspases (McCarthy & Dixit, 1998; Suzuki et al., 2001; Gyrd-Hansen & Meier, 2010). Baculovirus Inhibitor of Apoptosis Repeat motif or BIR which contain of 1, 2, or 3 repeats of a conserved 65 residue sequence of IAP are responsible for these two functions to occur in cells (Takahashi et al., 1998). However recent studies revealed other domains in IAPs such as RING (Really Interesting New Gene) domains which were shown to have E3 ubiquitin-ligase activity (Suzuki, et al., 2001; Schile et al., 2008). Recent study by Damgaard & Gyrd-Hansen, 2011 reported that IAPs are also involved in regulation of inflammation and innate immunity.

Figure 2.6(2): Schematic representation of hierarchical ordering of caspases in cells undergoing apoptosis activated by the intrinsic pathway. The double arrows represent the new pathways of activation proposed by Inoue et al. (Image obtained from Inoue et al. 2009)
In recent years, it has been reported that IAPs are targets of two major pathways, the intrinsic and extrinsic pathway of apoptosis. IAPs also influence a third minor pathway in which granzyme B directly activates caspase-3. Currently, eight human IAP family members have been classified based on the presence of one to three BIR domains. It has been grouped into three classes comprising of class one such as XIAP, cIAP1, cIAP2, ILP2, and MLIAP. Meanwhile, NAIP and survivin represent class two and class three respectively (LaCasse, et al., 1998; Schimmer, 2004; Wei et al., 2008).

Most of the studies on the mammalian IAPs is X-linked IAP (XIAP) and has been shown to potently inhibit the enzymatic activity of caspases at both the initiation and execution phases of apoptosis which are caspase-9 and caspases-3/-7 respectively, with the majority revealing a tightly association of IAPs and cancer. For example, XIAP levels are elevated in many cancer cell lines, and several reports have shown that suppression of XIAP protein levels can sensitize cells to chemotherapeutic drugs (Eckelman et al., 2006).

2.6.4 Role of caspase in cell cycle modulation

Caspase can contribute to not only apoptosis but also non-apoptotic phenomena in normal and cancer cells. Many reports have shown the involvement of caspase to cell cycle regulation. Advance techniques such as knockdown by RNAi or/and short hairpin RNA, suppress apoptosis by caspase inhibitor, and in vivo model confirmed the role of caspase in modulation of this mechanism.

In B cells, caspase-3 could mediate cleavage of CDK inhibitor p21^{Cip/Waf1} at the C terminal proliferation cell nuclear antigen (PCNA)-binding site to abolish interaction of CDK inhibitor p21^{Cip/Waf1} with PCNA and caused arrest of cell progression in late G\textsubscript{i}/S phase transition. This result is consistent with the role of caspase-mediated cleavage of CDK inhibitor p27^{Kip1} in induction of cell cycle progression of proliferating...
lymphoid cells. In caspase-3 knock-out mice, hyperproliferation of B cells were detected and this phenomenon confirmed that caspase-3 act as negative regulator of B-cell cycling. In contrast, active caspase-3 could be found in nuclei of dividing cells in the proliferative regions of rat forebrain suggest a function of caspase-3 in the modulation of cell growth. These results clearly indicated that caspase cleavage mechanism is one of the responsible mechanisms in regulating cell cycle progression (Lamkanfi, et al., 2006). Further study conducted by Brown et al., 2007 showed that caspase inhibition could also block cell death and induce cell cycle arrest in cytokine-deprived B-cells cell line.

Phosphorylation of caspase-9 by ERK1/2 and CDK1/cyclin B1 in G₁ phase and G₂/M respectively are proposed to modulate cell growth. The phosphorylation process prevents activation of caspase-9 and its ability to activate caspase-3 downstream \textit{in vitro}. Allan and Clarke, 2007 found caspase-9 is phosphorylated at Thr125 periodically during the cell cycle. They used microtubule poisons, nocodazole and taxol for treatment of cancer cell lines to observe the fraction of arrested cells in mitosis. This observation was accompanied with phosphorylation of caspase-9 at Thr125 and suggested the involvement of caspase 9 in cell cycle arrest. Further results confirmed that caspase-9 is only phosphorylated by CDK1/cyclin B1 in cell arrested in G₂/M phase (mitotic cells). Moreover the blockage of phosphorylation of caspase-9 by mutant increase incidence of apoptosis in siRNA transfected-U2OS cell line after treated with nocodazole. Majority of these cells also showed positive for active caspase-3. They finally concluded that slow caspase-9 dephosphorylation is the responsible mechanism for initiation of apoptosis from the arrested mitotic state.

Li et al., 2007 reported the cell cycle dependency of caspase activation in Fas-induced apoptosis in leukemia cells. They investigated expression of two caspases which are caspase-8 and caspase-3. Their results showed cells expressing the active
form of caspase-3 were positive for cyclin E and negative for cyclin A/B1 whereas expression of active form of caspase-8 were negative for cyclin A/B1/E. They also confirmed that active form of caspase-3 and caspase-8 cells were expressed in early G₁ phase by FACS analysis of Ki-67 status in these cells. Further work indicated that the active form of caspase-8 were produced in early or middle of G1 phase prior to the activation of caspase-3 in late G1 and early S phase of cell cycle. The results were validated by immunoblotting analysis of p27Kip1 protein which demonstrated activation of both caspases in p27Kip1 negative cells in late G₁ phase. However, when these cells were pre-treated with phorbol 12-myristate 13-acetate (PMA) followed by anti-Fas antibody, cell cycle arrest were observed and more striking results showed activation of these two proteins were blocked. Hence, they postulated that the dependency of cell cycle on caspase activation might be influenced by the speed of caspase activation.

As mentioned above, it is well accepted that caspase-8, caspase-9 and caspase-3 were crucial in regulation of cell cycle. However, in recent years, the role of other caspases such caspase-7 and caspase-2 have also been shown to modulate this process. Hashimoto et al., 2008 showed that caspase-7 may be involved in cell cycle progression at the mitosis phase. They directly targeted caspase-7 in HepG2 with small interfering RNAs (siRNAs) and short hairpin RNA (shRNA) to prevent cell proliferation through the cell cycle arrest at mitosis. Inhibition of caspase activities induces mitotic arrest. Three years later, Hashimoto et al., 2011 reported that inhibition of caspases resulted in the inhibition of cell proliferation via cell cycle arrest at G₂/M and G₁ phase. FACS analysis of peptide caspase inhibitor-treated HeLa cells indicated caspase-8 might contribute to the cell cycle regulation through activation of caspase-3 and/or caspase-7. It is confirmed that caspase-9 does not appear to function as an upstream regulator of caspase-3 and/or 7 during the cell cycle progression.
Taghiyev et al., 2011 showed the role of caspase-2 in cell cycle regulation. It was found that caspase-2 activity is important for proliferation by cell of androgen-dependent prostate cancer cell line. Importantly, this is the first group to identify the role caspase-2 in modulation of prostate cancer cells. Caspase-2 formed complexes with the cell cycle regulator proteins cyclin D3, CDK, and p21/Cip 1. They also demonstrated that AR transactivation in prostate cancer is regulated by caspase-2.

In conclusion, mechanisms such as caspase cleavage, caspase activation as well as caspase inhibition are depicted to play an important role in modulation of cell cycle either in normal cells or cancer cells. It also showed the majority of CDK inhibitors like p21, p27 and p17 are targeted by these mechanisms in determining the fate of cell growth. However, there remain controversies as to which phases this mechanism take place. The caspase regulation occured in G\textsubscript{1} phase was postulated by most rather than in mitotic or G\textsubscript{2}/M phases. Current knowledge provided the involvement of majority of caspases in cell growth and differentiation. Most importantly molecules that can target and regulate this type of protein are considered as good candidates in regulation of both apoptosis and cell cycle progression pathways (Fulda, 2010).

2.7  Signal Transduction and Apoptosis

2.7.1  Extracellular Signal-regulated Kinase (ERK)

Mitogen-activated protein kinases (MAPKs) play key roles in the cellular response to extracellular stimuli. Figure 2.7 shows three major mammalian MAPK subgroups that have been identified: extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK12/3), and p38 kinase (p38α/β/γ/δ) (Dhillon et al., 2007; Roberts & Der, 2007). Two isoforms of ERK are ERK2 and ERK1 which are also known as p42 and p44MAPK, respectively, and officially named MAPK 1 and 3 were discovered 27 years ago. (Cagnol & Chambard, 2010; Martin & Pognonec, 2010). The ERK pathway is activated by growth factor-stimulated cell surface receptor.
Specifically, MEK1/2 phosphorylate and activate the ERK1/2. The active forms then phosphorylate and regulate the activity of substrate found in nucleus and cytoplasm. (Roberts & Der, 2007; Martin & Pognonec, 2010). The ERK pathway is commonly associated with proliferation and survival of cells. Growth factors and mitogens signal through the ERK pathway in order to regulate gene expression and prevent apoptosis (Balmanno & Cook, 2008; Ramos, 2008).

ERK pathway has been shown to be up-regulated in variety of cancers (Vicent et al., 2004; Judd et al., 2012). Its effects on the regulation of apoptosis occur through the modulation of post-translational modifications of apoptotic regulatory proteins such as Bim, Mcl-1 and caspase 9 (Ley et al., 2003; Ding et al., 2008; Martin, et al., 2008). Phosphorylation of Bim resulted in its dissociation from anti-apoptotic Bcl-2 proteins and its subsequent degradation; the anti-apoptotic Bcl-2 proteins are left to prevent Bax activation and thereby prevent apoptosis (Mérino et al., 2009). The ERK cascade can lead to the phosphorylation and inactivation of caspase 9, again protecting cells from apoptosis. Small-molecule inhibitors of the ERK pathway have been developed as an attempt to induce apoptosis in cells that have become addicted to this pathway for survival and have the ability to resist apoptosis induced by currently used cancer chemotherapeutic agents.

As noted, ERK activation is necessary for cell proliferation. The activation is important for G1 progression and the synthesis of autocrine growth factors. Sustained activation of ERK1/2 is required for continued expression of cyclin D1 in G1 phase. Inhibition of ERK pathway by pharmacological approach was reported to delay the G2/M progression of cells. However, ERK1/2 signaling is not required in late G2 for timely entry into or exit from mitosis in both normal and transformed cells (Shinohara et al., 2006). The magnitude of ERK1/2 signal plays a key role in determining the final cellular outcome of this pathway. High intensity of ERK1/2 signaling induced the
expression of the CDK inhibitors p21, p16 and p15 in certain cell lines which can also contribute to cell cycle arrest. However, induction of cell cycle arrest by hyperactivation of ERK1/2 was also observed in cell lines and immortalized primary cells (Meloche & Pouyssegur, 2007)

Some cell lines model showed the ability to use the Fas pathway for both apoptosis and proliferation (Desbarats et al., 2003; Li et al., 2008). ERK pathway has been proposed to play a key role in regulation of Fas (CD95) expression. Peli et al., 1999 and Kazama & Yonehara, 2000 reported that Ras can directly regulate the transcription of Fas. Research conducted by Li et al., 2003 studied the apoptosis mediated by interferon α (IFNα) effects on Fas expression and ERK function in mouse ASZ001 basal cell carcinoma (BCC) cell line. Their results showed that IFNα mediated apoptosis in BBC cells and inhibited wild-type mitogen-activated ERK phosphorylation but not activated mitogen-activated MEK. The authors also showed that IFNα induced Fas expression was as a result of interfering with MEK function. Therefore these two events were reported as strong events for IFNα-based potential BCC therapy.

Chang et al., 2004 investigated the molecular mechanism of ZD1839-induced G1 cell cycle arrest and apoptosis in human lung adenocarcinoma A549 cells. They proved that apoptosis induction is accompanied by a marked increase of Fas protein expression and caspase-2, -3 and -8. Immunoblotting analyses of several proteins involve in cell cycle regulation depicted ZD1839 treatment to directly or indirectly regulate these proteins followed by G1 cell cycle arrest. Involvement of ERK-MAPK pathway was confirmed by observation on inhibition of the activated ERK proteins after ZD1839 treatment. Schematic diagram (Figure 2.7) of the G1-phase arresting and apoptotic signaling pathway proposed the involvement of inactivation of ERK-MAPK pathway and up-regulation of Fas protein respectively induced by ZD1839 in A549 cells.
Further in a recent study by Liu et al., 2009 reported the function of ERK inactivation in inducing up-regulation of Fas and FasL-mediated cell death in Taiwan Cobra phospholipase A2 (PA2)-treated U937 cells. Their result indicated that U937 cells underwent apoptosis after treatment with PLA2. Immunoblotting analysis showed increase of Fas and FasL protein expression is elicited by ROS-mediated p38α MAPK activation and ERK inactivation.

Various natural products have been shown to regulate ERK-MAPK pathway (Sarkar et al., 2009). Triterpenoid such as β-sitosterol found in natural products was hypothesized to play a key role in regulation of this crucial pathway. Moreover, in recent years, more of the possible regulatory properties of β-sitosterol on ERK/MAPK pathway was revealed (Awad et al., 2003; Bao et al., 2006; Moon et al., 2007; Tabas, 2007; Moon et al., 2008; Woyengo et al., 2009; Baskar et al., 2010). β-sitosterol was also reported to influence Fas expression in cancer cells. Since transcription of this protein is directly regulated by Ras-MAPK, regulation of its expression might involve β-sitosterol-MAPK status. It was suggested that inhibition of MEK, a molecule downstream of Ras might be due to a small molecule which also inhibited ERK phosphorylation. (Bao et al., 2006; Awad et al., 2007; Park et al., 2008).

Targeting ERK-MAPK pathway and Fas proteins respectively appeared to be an effective route in inducing apoptosis and cell cycle arrest in cancer cells. Importantly, this pathway has an effect on the regulation of pro and anti apoptotic proteins in cancer cells. Since the ERK-MAPK pathway is believed to regulate G1 phase in cell cycle progression and down-regulation of its expression was found in various cancer cells halted apoptosis, targeting it by natural compounds may have important implication in cancer progression. Currently, no work has been carried out to investigate the effects of phytosterol oxides on regulation of these two proteins.
Figure 2.7: Schematic overview of MAPK pathway. (Image obtained from Dhillon et al., 2007)
CHAPTER 3
MATERIALS AND METHODS

3.1 7α-hydroxy-β-sitosterol Natural Compound

3.1.1 Plant Material

Dried bark of *Chisocheton tomentosus* was collected from Mersing, Johor, Malaysia. The sample was identified by Mr. Teo from the Department of Chemistry, Faculty of Science, University of Malaya. A voucher specimen KL-4251 was deposited in the Department of Chemistry Herbarium, University of Malaya.

3.1.2 Extraction and Purification of CT1 compound from *Chisocheton tomentosus*

Dried ground bark of *Chisocheton tomentosus* (3.5 kg) was defatted with hexane and extracted with dichloromethane. Dichloromethane was removed by evaporating and 10g of extracts were subjected to a silica gel column (hexane-dichloromethane, 95:5). Polarity of the mobile phase was increased with acetone and finally with acetone/methanol to yield 24 fractions. One of the fractions was further separated on a silica gel column with acetone/dichloromethane/hexane (25:25:50) to give five other fractions. The third fraction in the solvent was allowed to evaporate to yield colorless crystals of 7α-hydroxy-β-sitosterol. Structural elucidation was established through several spectroscopic methods; UV, IR, MS (GCMS), 1D (1H-NMR, 13C-NMR, and DEPT), 2D-NMR (COSY, HMQC and HMBC), as well as X-ray reflection technique.
3.1.4 Preparation of Stock and Working Solution

CT1 (20 mg), obtained from Ibrahim Najmuldeen, PhD candidate (Department of Chemistry, University of Malaya) was dissolved in DMSO to make a stock solution of 50 mM. Stock solution was stored at -20°C and further diluted in DMSO to prepared 10 mM working solution used for cell treatment.

3.2 Cell lines

3.2.1 Reagents

Dulbecco’s modified Eagle’s medium (DMEM) and Roswell Park Memorial Institute-1640 (RPMI-1640) were purchased from Thermo Scientific (IL, USA). Trypsin and fetal bovine serum (FBS) were purchased from Sigma Aldrich (Kansas, USA). Mammary epithelial growth media (MEGM) and all antibiotics were purchased from Lonza Inc. (MD, USA). Dimethyl-2-thiazolyl-2,5-diphenyl-2H-tetrazolium bromide (MTT) reagent, annexin V-fluorescein isothiocyanate (FITC) apoptosis detection kit, propidium iodide (PI), RNase and Suicide Track™ DNA ladder isolation kit were purchased from Calbiochem (CA, USA). Primary antibodies caspase 9, caspase 3, caspase 6, caspase 8, XIAP, Bcl-2, Bax, Bim, Fas-L, p42/44 and β-actin were obtained from Cell Signaling (MA, USA).

3.2.2 Cell culture

A total of five cancer and one normal cell lines were used in this study, which are summarized in the Table 3.1. For routine maintenance, MCF-7 cells were cultured in RPMI-1640, while HSC-4, HSC-2, Ca Ski and HepG2 cells were cultured in DMEM with both media types supplemented with 10% (v/v) FBS 100 U/ml penicillin and 100 mg/ml streptomycin. HMEC cells were cultured in serum free (MEGM). Cells were grown as monolayers at 37°C in humidified atmosphere with 5% CO₂ and 95% air.
Table 3.1: Type of cancer and normal cell lines with the indication of sources and various culture media used for cultivation

<table>
<thead>
<tr>
<th>Cancer Cell Line</th>
<th>Type</th>
<th>Source</th>
<th>Culture Media</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca Ski</td>
<td>Human Epidermoid Cervical Carcinoma</td>
<td>Faculty of Medicine, University of Malaya</td>
<td>DMEM</td>
</tr>
<tr>
<td>HepG2</td>
<td>Human Hepatocyte Liver Carcinoma Cells</td>
<td>Faculty of Medicine, University of Malaya</td>
<td>DMEM</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Human Breast Adenocarcinoma Cells</td>
<td>CARIF</td>
<td>RPMI1640</td>
</tr>
<tr>
<td>HSC-2</td>
<td>Human Oral Squamous Carcinoma Cells</td>
<td>CARIF</td>
<td>DMEM</td>
</tr>
<tr>
<td>HSC-4</td>
<td>Human Oral Squamous Carcinoma Cells</td>
<td>CARIF</td>
<td>DMEM</td>
</tr>
<tr>
<td>HMEC</td>
<td>Human Mammary Epithelial Cells</td>
<td>Lonza, USA</td>
<td>MEGM</td>
</tr>
</tbody>
</table>

3.2.3 Cell sub-culture

All cell lines were split every three to four days, or when 80-90% surface confluence was attained. Used media was aspirated using a vacuum pump connected to a Pasteur pipette and discarded. Cells were washed twice with 2 ml of 1X PBS to remove any residual serum that could inactive trypsin activity. The PBS was removed and 1 ml of 0.25% (v/v) Trypsin-EDTA solution was added. Cells were then incubated at 37°C for 10 mins to completely detach cells from the surface of T-25cm² flask (Nunc, Denmark). Media (2 ml) containing 10% (v/v) FBS was added to inactive trypsin activity, and pipette into a 15 ml centrifuge tube. Neutralized cells were then centrifuged at 500 x g for 10 mins, and the supernatant was discarded. The cell pellet was re-suspended in fresh media containing 10% (v/v) FBS, 100 U/ml penicillin and 100 ug/ml streptomycin, and split into three or four flask for further usage.
3.2.4 Cell counting

A dye exclusion viability assay using haemacytometer was used to determine the number of cells present in a specific population. Monolayer of cells were detached by trypsinization, centrifuged and resuspended in media. Approximately 20 µl of cell suspension was mixed well with 20 µl of 0.04% (v/v) trypan blue (Merck, Germany) dye solution. After 3 mins, 10 µl of the solution was then transferred to a haemacytometer counting chamber, and spread evenly by capillary action. Using an inverted fluorescence microscope (Nikon, Japan) at 10X magnification, the numbers of unstained viable cells in each of the four square grid corners were counted and the average numbers of cells were obtained. Each square grid represent a 0.1 mm$^3$ or 10$^{-4}$ ml volume, and the concentration of cells were determined as shown below (Equation 3.1) with a dilution factor of two. To determine the percentage of cell viability, both dead (stained) and viable (unstained) cells were counted separately, and calculated as shown below (Equation 3.2). The desired concentration of cell suspension (number of cells/ml) was calculated and prepared for cell plating. The haemacytometer slide and glass cover slip was immediately rinsed and cleaned with 70% (v/v) ethanol (Merck, Germany) between samples and after use.

\[
C = \frac{(N/V)}{D} \quad \text{(Equation 3.1)}
\]

\[
\text{% Viability} = \frac{(NV/ND + ND)}{NV} \times 100\% \quad \text{(Equation 3.2)}
\]

Where:

- \(C\) = Cell concentration
- \(N\) = Average number of cells counted
- \(V\) = Volume counted (ml)
- \(D\) = Dilution factor
- \(NV\) = Total number of viable cells
- \(ND\) = Total number of Dead cells
3.3 Cytotoxicity Assays

3.3.1 MTT Assay

MTT reagents were prepared by adding 50 mg of MTT to 10 ml of 1x PBS (MediaTech, USA). The reagent was vortexed to ensure that the MTT powder was completely dissolved. MTT working solutions were stored in the dark at room temperature (25°C) and at 4°C in the dark for long storage. The final concentration of MTT working solution used in the MTT cell viability assay was 5 mg/ml. The cytotoxic effects of CT1 on all cell lines were determined by measuring MTT dye uptake and metabolism. Briefly, both cancer and normal cells were washed twice with 1X PBS (MediaTech, USA). 1X PBS solution was aspirated and cells were detached with 0.25% (v/v) trypsin (SAFC Biosciences, USA)-EDTA (Gibco, USA) solution. Cell pellets were obtained by centrifuging at 500 x g for 10 mins and re-suspended in media. Determination of viable cell count was done using trypan blue dye exclusion method according to section 3.2.4 to give the desired cell density of 1.0 x 10^5 cells/ml. A total of 1.0 x 10^4 cells were plated in triplicates at 100 µl/well in a 96 flat bottomed well plate and incubated for 24 h at 37°C to allow adherence to the well surface. CT1 treatment were done at various ascending concentrations (10, 20, 40, 60, 80 and 100 µM), and at various incubation periods (6 h, 12h, 18h and 24 h). Wells containing solvent control (DMSO) were prepared to ensure that cytotoxicity was not solvent induced. Wells containing cells at descending concentrations (10,000 cells, 5,000 cells, 2,500 cells, 1250 cells and 0 cells) via a serial dilution was used to construct standard curves for quantification purposes. After incubation of CT1, 20 µl of 5 mg/ml MTT reagent (Calbiochem, USA) was added into each well and mixed by gently tapping the plate. The plate was incubated for 1 h in the dark at 37°C until a purple formazon precipitate was clearly visible. The media was then aspirated, and 200 µl of DMSO
(Merck, Germany) was then added to all wells to dissolve the purple formazan precipitate. The plate was left to stand for 15 mins on shaker in the dark to allow complete colour stabilization of the formazan compound. Absorbance values of each well were measured at 570 nm wavelength with a 650 nm reference wavelength. All measurements were conducted using the Tecan Sunrise microtitre plate reader (Tecan, Switzerland) and quantified using the Magellan Version 6.3 (Tecan, Switzerland) software.

3.3.2 LIVE DEAD Viability/Cytotoxicity Assay

Assessment of cell viability after treatment with CT1 was accomplished using the LIVEDEAD Viability/Cytotoxicity Kit for Mammalian Cells (Molecular Probes, Invitrogen, USA) according to manufacturer’s protocol. Cells were cultured as monolayer on cover slip placed in 6-well cell culture plates for 6 h and 12 h. Upon complete attachment, 1.0 x 10⁵ cell/well were treated with CT1 at IC₅₀ concentration for 12 h. Harvesting of cells as aspirating the used media and washing of cells twice with 1X PBS (MediaTech, USA) to remove traces of serum containing esterases, which can interfere with the assay through hydrolyzation of the calcein-AM fluorescence dye. Staining of viable versus non-viable cells was done using a dual fluorescence staining system consisting of calcein-AM (Molecular Probes, Invitrogen, USA) which emits green fluorescence when cleaved by intracellular esterase, and ethidium homodimer (EthD) (Molecular Probes, Invitrogen, USA) which emits red fluorescence upon entering non-viable cells and binding to nucleic acid. Both dyes were mixed together and 150 µl of calcein-AM (2 µM) and EthD (4 µM) was added evenly onto the 22 mm cover slip containing treated cells. All cover slips were incubated for 45 mins at room temperature in the dark to ensure complete formation of fluorescence products within cells. Following incubation, excess dyes were washed off with 1X PBS (MediaTech, USA) and cover slips gently mounted on microscope slides. All cover slips were sealed.
with fixogum to prevent evaporation and allowed to dry at room temperature for 10 mins. Excitation and emission wavelengths of both fluoresceins were set at 494/517 nm for calcien-AM and 528/617 nm for EthD respectively. Visualization of samples were done using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Japan) under 100X magnification with separate pass filter for viewing of both stains.

3.4 Migration Assay

3.4.1 Wound Healing Assay

Wound healing assay was used to examine the anti-migration effects of CT1 on cancer cells. Cells were grown on 6-well plates for 24 h followed by treatment with mitomycin-c for 2 h. Media was removed and equal size wounds were created with a pipette tip. Cells were washed twice with 1X PBS and serum free media was added. Each group was pre-incubated with mitomycin c for 2 h followed by incubation with media only, DMSO and CT1 for 24 h. Images were captured using a Nikon Eclipse TS-100 fluorescence microscope (Nikon, Tokyo, Japan) under 100X magnification and analyzed using T-Scratch software v7.8.

3.5 Flow Cytometry-based Apoptosis Assays

3.5.1 Fixation of cancer cells

Cancer cells were treated with CT1 and incubated for 12 and 24 h at IC₅₀ concentration for 24 h before harvesting. After treatment, used media containing detached cells were collected in 15 ml tubes. The remaining adherent cells were trypsinized, neutralized by media containing 10.0% (v/v) FBS and centrifuged at 1,000 x g for 5 mins. Supernatants was discarded and cell pellets were rinsed with 1 ml of 1X ice-cold PBS (MediaTech, USA) and centrifuged at 1,000 x g for 5 mins. All cells were re-suspended and diluted in 1 ml of 1X ice-cold PBS (MediaTech, USA) and 3.0
ml of 70% ethanol were added with drop wise into for fixation. The fixed cells were stored at 4°C overnight and -20°C for several weeks prior the analysis.

3.5.2 Cell Cycle Analysis

Fixed cancer cells were centrifuged at 1,000 x g for 5 mins and washed twice with 5 ml of 1x ice-cold PBS to remove the presence of ethanol. The supernatant was discarded and the cell pellet was re-suspended gently. Staining of DNA content was done by adding 1 ml of propidium iodide (Calbiochem, USA) solution (50 µg/ml) to the cell pellet, and kept in the dark at 4°C. To remove RNA, 50 µl RNase A (Calbiochem, USA) solution (10 mg/ml) was added to each tube, and incubated for 30 mins at 37°C in the dark. All samples were stored away from light until analyzed by a flow cytometer. Cell cycle experiments were carried out using a BD FACSCanto 11™ flow cytometer (Becton Dickenson and Co., CA, USA) and analyzed using BD FASCDiva v4 Software (Becton Dickenson and Co., CA, USA).

3.5.3 Annexin-V FITC and PI staining

Detection and differentiation of various apoptosis stages were conducted using the Annexin V-FITC Apoptosis Detection Kit (Calbiochem, USA). Tumour cell lines were treated with P1 CT1 for 24 and 48 hrs at IC_{50} values before harvesting. After treatment, used media containing detached cells were collected in 15 ml tubes. The remaining adherent cells were trypsinized, neutralized by media containing 10% (v/v) FBS and centrifuged at 1,000 x g for 5 mins. Supernantants discarded and cell pellets were rinse with 1 ml 1X ice-cold PBS and centrifuged at 1,000 x g for 5 mins. All cells were re-suspended and diluted in 6 ml of media to a final concentration of 1.0 x 10^5 cell/ml. Aliquots containing 500 µl of media (5.0 x 10^5 cells/ml) were transferred into microcentrifuge tubes, and 10 µl of media binding reagent (Calbiochem, USA) was added. Exposure of externalized phosphatidyl serine (PS) was detected by adding 1.25
μl of FITC-conjugated Annexin-V anticoagulant (200 μg/ml) (Calbiochem, USA) into each tube, and incubated for 15 mins at room temperature in the dark. All tubes were then centrifuged at 500 x g for 5 mins and the supernantant was discarded. The pellet was then re-suspended in 500 μl of 1X ice cold binding buffer (Calbiochem,USA), followed by the addition of 10 μl of PI (30 μg/ml) (Calbiochem, USA). All tubes were kept on ice in the dark until analyzed by a flow cytometer. All assays were carried out using the BD FACSCanto 11™ flow cytometer (Becton Dickenson, USA).

3.5.4 Data analysis using FASCDiva software

Total populations of 1.0 x 10⁴ cells were counted for each sample and a four quadrant dot plot of untreated and treated samples were created using FSC-parameter and SSC-parameter based on gated populations. Sole FITC signals were detected at a 518 nm wavelength, while sole PI signals were detected at a 620 nm wavelength. Combination of both FITC and PI signals were detected at a 488 nm wavelength. Quantification and visualization of early-apoptosis, late-apoptosis and necrotic cell populations were defined by quadrants boundaries that were created based on single dye-signal controls of each tumor cell line.
3.6 DNA Fragmentation Assay

3.6.1 DNA Extraction

Confirmation of apoptosis-mediated cell death induced by CT1 was conducted using the Suicide Track DNA Ladder Isolation Kit (Calbiochem, USA). Cancer cell lines were grown on 60 mm petri dished until 80% confluence, followed by treatment with CT1 at IC$_{50}$ concentration for 24 h. After treatment, both detached and attached cells were collected after washing with 1X PBS and trypsinization. Cell pellets were obtained and resuspended in 55 µl of solution #1 (Calbiochem, USA) containing cell lysis buffer and nuclease inhibitor, for every 1.0 x 10$^6$ cells harvested. Subsequently, 20 µl of solution #2 (Calbiochem, USA) containing RNase was added followed by incubation at 37°C to degrade all forms of cellular RNA. After the breakdown of total RNA was complete, 25 µl of Solution #3 (Calbiochem, USA) was added and gently mixed to separate DNA from the cell lysate, and was allowed to incubate overnight at 50°C. Following incubation, 500 µl of resuspension buffer (Calbiochem, USA) was added to each tube and mixed by pipetting. In order to increase the visualization of the DNA pellet, 2 µl of a fluorescence dye, Pellet Paint Co-Precipitant (Calbiochem, USA) and 60 µl of sodium acetate (3M, pH 5.2) (Calbiochem, USA) was added to the mixture. A total of 662 µl of isopropanol was added into each tube and centrifuge at 16,000 x g for 5 mins. The supernatant was aspirated, leaving behind a pink DNA pellet which can be visualized under UV illumination. The DNA pellet was then washed twice with 500 µl of 70% (v/v) ethanol and 100% (v/v) ethanol respectively, with the pelleting of total DNA via centrifugation at 16,000 x g for 5 mins between each washing step. After washing, all DNA pellets were air-dried for 15 mins resuspended in 50 µl of resuspension buffer (Calbiochem, USA) and stored at -20°C for further use. A
positive control consisting of HL-60 apoptotic cells treated with actinomycin D (Calbiochem, USA) was extracted in the same manner as mentioned above.

3.6.2 Quantification of DNA

Purity assessment and quantification of total extracted DNA samples were conducted via spectrophotometry. A total of 5 µl DNA sample was diluted with 495 µl of distilled water to make a final volume of 500 µl, with a dilution factor of 100. All samples were added into 10 mm x 10 mm disposable UV cuvettes (Eppendorf AG, Germany), with distilled water as blank controls. DNA quantification was based on absorbance values at 260 nm wavelength, with a conversion factor of 50. Absorbance ratios for A_{260}/A_{280} and A_{260}/A_{230} were obtained to assess the purity of DNA samples against protein and solvent contamination respectively. All spectrophotometric readings were obtained using a Bio-Photometer (Eppendorf AG, Germany) and normalized with resuspension buffer (Calbiochem, SD, USA) to ensure consistency in sample concentration before being analyzed by agarose gel electrophoresis.

3.6.3 Agarose gel Electrophoresis

Visualization of apoptotic total DNA from cells treated with CT1 was done via agarose gel electrophoresis. Firstly, 1.5% (w/v) agarose gels were prepared by adding 0.6 g of agarose, low electroendosmosis (EEO) (Amresco, USA) to 50 mL of 1X Tris base, acetic acid and EDTA (TAE) buffer, prepared from 10X TAE liquid concentrate (Amresco, USA). The mixture was heated for approximately 3 mins using a microwave oven (Panasonic, Malaysia) and poured into a balanced 5.5 x 12.0 cm gel casting tray (BayGen, China) with an attached 8-well comb (BayGen, China) with 1.5 cm spacing. This mixture was allowed to solidify for approximately 45 mins at room temperature (27°C). The casting tray containing the solidified gel was then submerged in 1X TAE buffer inside an electrophoresis chamber (BayGen, China) after removal of the comb. A
total of 10 uL of DNA sample was mixed with 2 uL of 6X loading dye [10 nM Tris-HCl (pH 7.6), 0.03% (w/v) bromophenol blue, 0.03% (v/v) xylene cyanol, 60% (v/v) glycerol, 60 mM EDTA] (Calbiochem, USA) before loading. DNA molecular weight markers used was 5 µl of the 100 bp DNA ladder (Ready-to-use) (Calbiochem, USA). The agarose gel was electrophoresed for 1 h, at 120 V and 80 mA. When the electrophoresis was completed, the gel was stained in 0.2 µg/L of ethidium bromide solution (Promega, USA) for 20 mins and de-stained in distilled water for 5 mins. All gels were viewed and photographed under UV transillumination in a ChemiImager TM 4400 (Alpha Innotech, USA) at 302 nm wavelength.

3.7 Protein Expression Analysis

3.7.1 Extraction of cytoplasmic and nuclear protein

CT1 treated MCF-7 cells were harvested and total protein was extracted using the NE-PER® nuclear and cytoplasmic extraction kit (Pierce, IL, USA) according to manufacturer’s protocol. Nuclear and cytoplasmic proteins were extracted from MCF-7 cells treated with IC\textsubscript{50} values of CT1 for 24 h. Following treatment, the used media containing detached cells were aspirated and collected in 15 ml centrifuge tubes. The remaining cells were then detached with 0.25% (v/v) trypsin-EDTA solution and pooled with cells from the used media. The mixture of both attached and detached cell populations were then obtained by centrifuge at 500 x g for 5 mins after discarding the supernatant. Extractions of both nuclear and cytoplasmic fractions from whole cell lysates were performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit according to the manufacturer’s protocol. Briefly, the cell pellet was re-suspended in 200 µl of ice cold CER 1 (Pierce, USA) containing a cocktail of 1X protease and phosphatase inhibitors (Pierce, USA), and vortexed vigorously for 15 sec. Cells lysates were incubated on ice for 10 mins to allow complete lysis of cells, followed by the
addition of 11 µl of CER 11 reagent (Pierce, USA). Lysates were then vortexted for 10 sec, incubated on ice for 2 mins and vortexed again for 10 sec. The cytoplasmic fraction was obtained from the supernantant after centrifuge at 4°C at 16,000 x g for 5 mins using the Sorvall Legend Micro 17R (Thermo, USA) refrigereated centrifuge. The pellet containing intact nuclei was re-suspended with 100 µl of ice cold NER (Pierce, USA) containing a cocktail of 1X protease and phosphatase inhibitor (Pierce, USA), followed by vortexing for 15 sec. The sample was then incubated on ice for 10 mins and vortexed again for 15 sec every 10 mins for a total duration of 40 mins. Samples were centrifuged at 16,000 x g for 10 mins at 4°C and the supernatant containing the nuclear extract was transferred to a new pre-chilled tube. Protein concentrations of all nuclear and cytoplasmic samples were quantified using the Quick Start Bradford Protein Assay Kit 2 (Bio-Rad, USA).

3.7.2 Protein Quantification

Quick Start Bradford Protein Assay Kit 2 was used to determine concentration of proteins extracted from MCF-7 cells. A total of 10 µl of each sample and standard reagents were added into 10 mm x 10 mm cuvettes. A 100X dilution was done by adding 990 µl of 1x Bradford dye reagent (Bio-Rad, USA). Samples were gently mixed by pipetting and incubated at room temperature for 10 mins to allow the formation of homogenous mixture. A spectrophotometer (Eppendorf AG, Germany) was used to measure absorbance at 595 nm wavelength of each sample. Correlation between absorbance readings and protein concentration was achieved by plotting a standard curve using Bovine Serum Albumin (BSA) standards (Bio-Rad, USA) at concentrations of 0.25 mg/ml to 2 mg/ml. Both cytoplasmic and nuclear protein concentration were normalized with dH2O to a final concentration of 2.5 mg/ml and 2 mg/ml respectively. A total of 20 µl of normalized cytoplasmic protein samples were mixed by pipetting with 5 µl of 5x lane marker reducing sample buffer (Thermofisher, USA) containing
0.3 M Tris-HCl, 5% (v/v) SDS, 50% (glycerol), 100 mM DTT and a pink tracking dye. All samples were then boiled in a water bath (Memmert, Germany) at 95°C for 5 mins and allowed to cool to room temperature. Cooled samples were then centrifuge at 12,000 x g for 2 mins before a total of 20 µl of each protein sample was loaded into each well for SDS-PAGE.

### 3.7.3 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gels (SDS-PAGE) were prepared to fractionate extracted cytoplasmic and nuclear proteins from untreated and CT1 treated cancer cells according to size. To separate protein ranging in size between 14 to 70-kDa, a 12% (w/v) resolving gel and a 4% (w/v) stacking gel was prepared according to Appendix 2. All reagents were mixed in the order list in Appendix 2, with TEMED and freshly prepared APS being added last to initiate gel polymerization. Two mini-gels with a dimension of 18 cm x 16 cm x 1 mm were prepared each time by clipping four glass plates (BioRad, CA, USA) together on casting tray (BioRad, CA, USA). The resolving gel was loaded until 80% of the glass plate was filled and allowed to polymerize for 45 mins. A layer of 0.1% (v/v) SDS (Promega, USA) was added over the resolving gel during the polymerization process to prevent oxidization and dehydration of the resolving gel. After complete polymerization, the 0.1% (v/v) SDS solution was blotted out using Kim-wipes (Kimberly-Clark, Canada), and the 4% (w/v) stacking gel was loaded until 100% of the glass plate was filled. A 10-well gel comb with 1 mm thickness was inserted into the stacking gel to prepare the wells, and allowed to polymerize for another 45 mins. After polymerization was complete, the gels were transferred to a Mini-PROTEAN 3 Cell gel tank (BioRad, CA, USA), and gel combs were gently removed. The inner portion of the gel tank was filled with 1X Tris-Glysine-SDS (TGS) running buffer (BioRad, CA, USA), while the outer portion was filled to about 50% of tanks’ depth with 1X TGS running buffer. Running buffer was
pipette into each well to remove any traces of unpolymerized gel before sample loading. A total of 20 μl of denatured protein sample and 5 μl of Spectra Multicolor Broad Range Protein Ladder (Fermentas, Canada) were loaded into each well (Protein ladder sizes are listed in Appendix 1. Gel electrophoresis was performed by running the gel at 100 V with free flowing current for 15 mins using Power Supply-Power Pac (Bio-Rad, USA) to allow the samples to align before entering the resolving gel, followed by 120 V with free flowing current for 70 mins to resolve the protein samples.

3.7.4 Western Blotting

Upon completion of electrophoresis, stacking gels were removed from resolving gels using Kim-wipes. Resolving gels containing separated proteins, 0.2 μm pore size nitrocellulose membrane (BioRad, CA, USA) and extra-thick blotting papers (BioRad, CA, USA) were soaked and equilibrated in 1X TGS transfer buffer with 20% (v/v) methanol (Merck, Germany) for 10 mins. A transfer sandwich was then prepared and placed in TransBlotter-SD Semi Dry Transfer Cell (Bio-Rad, USA). A blotting roller (Milipore, USA) was used to remove presence of air bubbles between each layer of transfer sandwich. Transfer of proteins to membrane was done at 50 mA with free flowing voltage for 90 mins using MP-2AP Power Supply (Major Science, Taiwan). The efficiency of protein transfer was monitored by staining the membrane with 0.1% (w/v) Ponceau S (Sigma, USA) in 5% (v/v) acetic acid (Merck, Germany) for 5 mins while shaking. The blotted resolving gel was stained with BluePrint Stain (Amresco, USA) for 30 mins and de-stained in dH2O until the gel background was clear to access the amount of non-transferred proteins. Upon checking the efficiency of transferred protein, the membrane was then de-stained twice in 5% (v/v) acetic acid solution for 5 mins, followed by two washed in dH2O while shaking on Reciprocal Shaker MS-RC (Major Science, Taiwan) for 5 mins. After washing was completed, membranes were blocked for 1 h while shaking at 25°C in a blocking buffer consisting of 2% (w/v) BSA,
0.5% (w/v) skim milk powder (Merck, Germany), 1X Tris-buffered saline (TBS) buffer and 0.05% (v/v) Tween-20 (Promega, USA) to prevent non-specific background binding of the primary and secondary antibodies. Blocked membranes were incubated in primary antibodies at appropriate dilution (1:1000) in 10 ml of blocking buffer overnight at 4°C. Optimal antibody concentrations were determined according to manufacturer’s protocol and summarized in table 3.2. Following primary antibody incubation, membranes were washed three times for 5 mins each with 1xTBS-T buffer while shaking at 25°C. Bound primary antibodies were probed with secondary antibodies conjugated to horse radish peroxidase (HRP) using a 1:1000 dilution rate (Table 3.1) for 1 hr while shaking at 25°C. Membranes were then washed three times with 1X TBS-T buffer for 5 mins each time, followed by a single wash 1X TBS buffer 5 mins. Membranes were then blotted dry from excess wash buffers with Kim-wipes. Detection of bound antibodies were conducted by adding 1 ml of Super-Signal West Pico chemiluminescence substrate (Thermo Scientific, USA) and 1 ml of H$_2$O$_2$ enhancer solution which reacts with HRP conjugated to secondary antibodies. Membranes were incubated for 5 mins at 25°C in the dark to allow formation of chemiluminescence detection signals and excess substrate on the membrane were then blotted away and syringe wrapped. Intensities of chemiluminescence signals from bound antibodies were detected using x-ray films within the next 60 mins.
**Table 3.2:** Summary of type, source and optimized dilution for primary and secondary antibodies used in western blotting experiments

<table>
<thead>
<tr>
<th>Antibodies (Type)</th>
<th>Company (Source)</th>
<th>Optimal Antibody Dilution</th>
<th>Blocking Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>Bax</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>Bcl-2</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>Bim</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>ERK 1/2</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>FasL</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>XIAP</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>Caspase 9</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) NFSDM</td>
</tr>
<tr>
<td>Caspase 8</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) BSA</td>
</tr>
<tr>
<td>Caspase 3</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) NFSDM</td>
</tr>
<tr>
<td>Caspase 6</td>
<td>Cell Signaling</td>
<td>1:1000</td>
<td>5.0% (w/v) NFSDM</td>
</tr>
</tbody>
</table>
3.7.5 X-Ray Film Detection

Chemiluminescence stained membrane were placed in a medical x-ray cassette with Kodak Green 400 Screens (Kodak, USA) in order to prevent exposure to light. Chemiluminescence signals were exposed in a dark room onto a green x-ray film (Kodak, USA) for 10 sec to 60 sec. Exposed films were developed in 1X developer solution (Kodak, USA) for 1 min and washed in dH₂O before fixing the film in 1X fixing solution (Kodak, USA). Fixed films were finally rinsed under running tap water to remove fixing chemicals and dried. Documentation of films was done using a CanonScan LiDE600 scanner (Canon, Vietnam). Relative intensities of bands were quantified using the ImageJ v1.43 analysis software (NIH, MD, USA).

3.8 Statistical analysis

All results were expressed as mean ± SEM obtained from three independent experiments. Statistical significances between various groups were determined using one-way ANOVA with a p-value ≤ 0.05 threshold.
CHAPTER 4
RESULTS

4.1 Characterization of 7α-Hydroxy-β-sitosterol (CT1)

The sample was characterized by Dr. Ibrahim Najmuldeen from the Department of Chemistry, Faculty of Science, University of Malaya. Different methods were used in order to confirm it molecular structure.

4.1.1 Ultraviolet–visible spectroscopy and Infrared spectroscopy

The compound was isolated as a colorless crystal mp 138-140°C. The UV spectrum showed absorption at λ 302nm and 254nm, the IR spectrum indicated the presence of hydroxyl group by the absorption band at 3430 cm⁻¹, the mass spectrum revealed a molecular ion peaks at m/z 429. Corresponding molecular formula is C_{29}H_{50}O_{2}.

4.1.2 Nuclear magnetic resonance spectroscopy

The $^1$H NMR spectrum (Table 4.1, Figure 4.11) of CT1 showed six methyl groups resonated as singlets, doublets and triplet in the region of δ_{H} 0.65-0.95. A characteristic doublet for H-6 methine proton appeared at δ_{H} 5.55 indicating the presence of double bond functionality between C-5 and C-6, two downfield signals at 3.81 and 3.54 were assigned to the H-7 and H-3 respectively, indicating to the presence of oxygen functionality at their carbons.
### Table 4.1: 1D ($^1$H and $^{13}$C) and 2D (HMQC, and HMBC) NMR spectral data of CT1

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta_{H}$ (int.; mult.; J(Hz))</th>
<th>$\delta_{C}$</th>
<th>HMQC</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>1.80 (1H, m)</td>
<td>37.08</td>
<td>H$_a$-C1</td>
<td>1,3,5,10</td>
</tr>
<tr>
<td>1b</td>
<td>1.01 (1H, m)</td>
<td></td>
<td>H$_b$-C1</td>
<td>1,3,5,10</td>
</tr>
<tr>
<td>2a</td>
<td>1.80 (1H, m)</td>
<td>31.3</td>
<td>H$_a$-C2</td>
<td>1, 3</td>
</tr>
<tr>
<td>2b</td>
<td>1.47 (1H, m)</td>
<td></td>
<td>H$_b$-C2</td>
<td>1, 3</td>
</tr>
<tr>
<td>3</td>
<td>3.54 (1H, m)</td>
<td>71.3</td>
<td>H-C3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>2.29 (2H, d, 5)</td>
<td>42.0</td>
<td>H$_2$-C4</td>
<td>2,5,6,10</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>146.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.55 (1H, d, 5.0)</td>
<td>123.8</td>
<td>H-C6</td>
<td>4,7,8,10</td>
</tr>
<tr>
<td>7</td>
<td>3.81 (1H, br. s)</td>
<td>65.4</td>
<td>H-C7</td>
<td>5,6,9</td>
</tr>
<tr>
<td>8</td>
<td>1.43 (1H, m)</td>
<td>37.5</td>
<td>H-C8</td>
<td>14,10,4,13,9</td>
</tr>
<tr>
<td>9</td>
<td>1.15 (1H, m)</td>
<td>42.3</td>
<td>H-C9</td>
<td>8,10,11,12,19</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>37.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>1.49 (2H, m)</td>
<td>20.7</td>
<td>H$_2$-C11</td>
<td>9,10,12,13</td>
</tr>
<tr>
<td>12a</td>
<td>1.97 (1H, m)</td>
<td>39.2</td>
<td>H$_a$-C12</td>
<td>9,13,14</td>
</tr>
<tr>
<td>12b</td>
<td>1.12 (1H, m)</td>
<td></td>
<td>H$_b$-C12</td>
<td>9,13,14</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>42.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1.41 (1H, m)</td>
<td>49.4</td>
<td>H-C14</td>
<td>15,16,17,18</td>
</tr>
<tr>
<td>15a</td>
<td>1.66 (1H, m)</td>
<td>24.3</td>
<td>H$_a$-C15</td>
<td></td>
</tr>
<tr>
<td>15b</td>
<td>1.08 (1H, m)</td>
<td></td>
<td>H$_a$-C15</td>
<td></td>
</tr>
<tr>
<td>16a</td>
<td>1.83 (1H, m)</td>
<td>28.3</td>
<td>H$_a$-C16</td>
<td>13,20,21</td>
</tr>
<tr>
<td>16b</td>
<td>1.22 (1H, m)</td>
<td></td>
<td>H$_a$-C16</td>
<td>13,20,21</td>
</tr>
<tr>
<td>17</td>
<td>1.14 (1H, m)</td>
<td>55.7</td>
<td>H-C17</td>
<td>18, 21, 15,16</td>
</tr>
<tr>
<td>18</td>
<td>0.65 (3H, s)</td>
<td>11.7</td>
<td>H$_3$-C18</td>
<td>12,13, 14, 17</td>
</tr>
<tr>
<td>19</td>
<td>0.95 (3H, s)</td>
<td>19.2</td>
<td>H$_3$-C19</td>
<td>1, 2, 9, 10,11</td>
</tr>
<tr>
<td>20</td>
<td>1.33 (1H, m)</td>
<td>36.1</td>
<td>H-C20</td>
<td>17, 18, 23, 24</td>
</tr>
<tr>
<td>21</td>
<td>0.89 (3H, d, 6.4)</td>
<td>18.3</td>
<td>H$_3$-C21</td>
<td>17, 20, 22</td>
</tr>
<tr>
<td>22</td>
<td>2.24 (2H, m)</td>
<td>33.8</td>
<td>H$_2$-C22</td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>1.22 (2H, m)</td>
<td>29.8</td>
<td>H$_2$-C23</td>
<td>22, 24, 25</td>
</tr>
<tr>
<td>24</td>
<td>0.93 (1H, m)</td>
<td>49.4</td>
<td>H-C24</td>
<td>29, 26, 27</td>
</tr>
<tr>
<td>25</td>
<td>1.64 (1H, m)</td>
<td>29.0</td>
<td>H-C25</td>
<td>24, 26, 27, 28</td>
</tr>
<tr>
<td>26</td>
<td>0.81 (3H, m)</td>
<td>19.9</td>
<td>H$_3$-C26</td>
<td>24, 28</td>
</tr>
<tr>
<td>27</td>
<td>0.77 (3H, m)</td>
<td>18.9</td>
<td>H$_3$-C27</td>
<td>24, 25, 26</td>
</tr>
<tr>
<td>28</td>
<td>1.22 (2H, m)</td>
<td>23.1</td>
<td>H$_2$-C28</td>
<td>23,24,25,27,29</td>
</tr>
<tr>
<td>29</td>
<td>0.83 (3H, m)</td>
<td>12.1</td>
<td>H$_3$-C29</td>
<td>23, 24, 25, 28</td>
</tr>
</tbody>
</table>
The broad-band decoupled $^{13}$C- NMR spectrum (Table 4.1, Figure 4.12) of CT1 displayed twenty nine carbon atoms in the molecule, the DEPT spectra exhibited six methyl, ten methylene and ten methine carbons, while the remaining three carbons were quaternary as deduced from broad–band spectrum. No signals was observed beyond $\delta_C$ 146.3 and, therefore, it was concluded that no ketonic function in this molecule in comparison with the next compound. The downfield signals at $\delta_C$ 146.3 was attributed to olefinic quaternary carbon, the C-6 olefinic methine carbon appeared at $\delta_C$ 123.8, two more signals for oxygen-bearing carbons at $\delta_C$ 71.3 and 65.4 were ascribed to C-3 and C-7 respectively.

Figure 4.11: $^1$H-NMR spectrum of CT1
4.1.3 Correlation spectroscopy (COSY)

The COSY spectrum (Figure 4.13) indicated the presence of two major spin systems, spin system “a” and spin system “b”, spin system “a” started with the couplings of C-3 proton with the C-4 methylene protons, meanwhile, spin system “b” the olefinic proton of C-6 showed vicinal connectivity with C-7 methine proton.
4.1.4 Heteronuclear multiple-bond correlation spectroscopy (HMBC)

All protons of methyl, methylene, \( \text{sp}^3 \) methine, and \( \text{sp}^2 \) methine were approved by HMQC (Table 4.1, Appendix 7). The HMBC spectrum of CT1 (Table 4.1, Appendix 8) showed long range correlation of C-19 proton with quaternary carbon of C-10, similarly C-18 methyl protons showed HMBC connectivity with quaternary C-13, so they show that the methyl group (19 and 18) should be attached directly with C-10 and C-13 respectively, and the long chain substituent should be attached in the position of C-17 according to their correlations by HMBC.
4.1.5 Gas Chromatography–Mass Spectrometry and X-ray crystallography

On the basis of UV, IR, GC-MS (Figure 4.14), $^1$H/$^13$C NMR, and comparing with literature review, it was concluded that the molecule should be 7-hydroxy-β-sitosterol. This was further supported by X-Ray reflection technique (Figure 4.15).

![Figure 4.14: GC-MS of CT1](image1)

![Figure 4.15: X-Ray structure of CT1](image2)
4.2 Cytotoxicity Assay

4.2.1 CT1 induces cytotoxic effect on various cancer cell lines

The MTT cell viability assay was used to examine the cytotoxicity effects of CT1 on the proliferation on five cancer and one HMEC normal cell lines. The results indicated that CT1 induces cell line selective-cytotoxicity in a dose and time dependent manner over a 100 µM treatment regime and 24 h of exposure (Table 4.2). Minimal cytotoxic effects were observed on HMEC cells, where approximately 17.6 ± 4.2% killing was observed under highest CT1 concentration treatment conditions (80 µM).

The sensitivity of the cancer cell line can be observed in descending order: MCF-7, HSC-4, HepG2, Ca Ski and HSC-2, with highest levels of cytotoxicity being observed in human breast cancer, MCF-7 cells. Table 4.2 shows approximately 78.7 ± 3.1% killing in MCF-7 cells with the lowest IC50 value recorded at 16.0 ± 3.6 µM among all cell lines tested. Meanwhile 74.5 ± 3.6% and 59.4 ± 3.2% killing with IC50 values of 25.0 ± 3.3 µM and 19.5 ± 2.6 µM were detected in HepG2 and HSC-4 cells respectively. No significant cytotoxic effects (high IC50 values) were observed on Ca Ski and HSC-2 cell lines suggesting that CT1’s action was selective among various cancer types. Thus, both cell lines were excluded from following assay. Viability of cells treated with DMSO without CT1 were insignificantly affected (≤2.0 %) thereby ruling out the involvement of solvent-induced cytotoxicity.

Both time and dose dependent assays (Figure 4.21 and 4.22) supported the need to further investigate the apoptotic effects of CT1 and its potential as an anti-tumor drug for the treatment of MCF-7, HepG2 and HSC-4 cells. All of the following experiments were carried out based on IC50 values as obtained from our present MTT data and are summarized in Table 4.2.
Figure 4.21: Comparison of total relative cell viability (%) between various cancer cell lines and normal cell line (HMEC) after treatment with CT1 at different concentrations (0 to 100 μM) at 24 h post-treatment time, indicating dose-dependent cytotoxicity. Results were expressed as total percentage of viable cells. Each value is the mean ±SEM of three replicates.

Figure 4.22: Comparison of total relative cell viability (%) between various cancer cell lines and normal cell line (HMEC) after treatment with 100 μM of CT1 at different post-treatment time, indicating time-dependent cytotoxicity. Results were expressed as total percentage of viable cells. Each value is the mean ±SEM of three replicates.
Table 4.2: Summary of IC₅₀ values and total cell viability of CT1 treated cancer cell lines and HMEC cells as obtained from MTT cell viability assays after 24 h exposure. All data are presented as mean ± SEM after deduction of DMSO solvent induced cytotoxicity of three independent experiments.

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Time (h)</th>
<th>IC₅₀ (µM) †</th>
<th>Cell viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human mammary epithelial cells</td>
<td>24</td>
<td>n.d</td>
<td>82.4 ± 4.2</td>
</tr>
<tr>
<td>(HMEC)</td>
<td>48</td>
<td>n.d</td>
<td>80.3 ± 5.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>n.d</td>
<td>55.5 ± 5.5</td>
</tr>
<tr>
<td>Human breast adenocarcinoma</td>
<td>12</td>
<td>38.2 ± 3.2</td>
<td>36.5 ± 1.9</td>
</tr>
<tr>
<td>(MCF-7)</td>
<td>18</td>
<td>28.6 ± 4.1</td>
<td>29.3 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>16.0 ± 3.6</td>
<td>21.3 ± 3.1</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>30.3 ± 2.4</td>
<td>48.6 ± 1.7</td>
</tr>
<tr>
<td>Human oral squamous cell carcinoma (HSC-4^COX2)</td>
<td>12</td>
<td>28.8 ± 5.1</td>
<td>48.1 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>24.2 ± 3.1</td>
<td>43.2 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>19.5 ± 2.6</td>
<td>40.6 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>76.8 ± 5.2</td>
<td>35.9 ± 8.4</td>
</tr>
<tr>
<td>Human hepatocyte carcinoma (HepG2)</td>
<td>12</td>
<td>74.0 ± 4.3</td>
<td>32.5 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>75.0 ± 5.1</td>
<td>26.8 ± 7.2</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>25.0 ± 3.3</td>
<td>25.2 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>n.d</td>
<td>94.6 ± 4.2</td>
</tr>
<tr>
<td>Human cervical carcinoma (Ca Ski)</td>
<td>12</td>
<td>91.9 ± 5.2</td>
<td>49.0 ± 6.4</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>92.6 ± 3.2</td>
<td>48.8 ± 5.7</td>
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<tr>
<td></td>
<td>24</td>
<td>84.3 ± 4.4</td>
<td>39.3 ± 4.1</td>
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<tr>
<td>Human oral squamous cell carcinoma (HSC-2^COX2)</td>
<td>12</td>
<td>n.d</td>
<td>83.4 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>88.4 ± 2.3</td>
<td>77.9 ± 2.7</td>
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<tr>
<td></td>
<td>24</td>
<td>90.3 ± 3.8</td>
<td>65.9 ± 3.5</td>
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</table>

† n.d denotes that total cell viability was maintained > 50% viability at maximum incubation time and CT1 concentration.
4.2.2 Confirmation of cytotoxicity effect of CT1

Since MTT assay can provide an underestimation of the anti-proliferative effect of certain natural product (P. Wang et al., 2010), the need for careful valuation of the cytotoxic effect of CT1 by other methods is crucial for carry out downstream assays. To further illustrate the cytotoxic inducing effect induced by CT1 treatment, we utilize a Live/Dead cytotoxic viability assay to objectively confirm cell cytotoxicity.

Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, determined by the enzymatic conversion of the virtually non-fluorescent cell-permeating calcein AM to the intensely fluorescent calcein. The polyanionic dye calcein is well retained within live cells, producing an intense uniform green fluorescence in live cells (ex/em ~495/~515 nm). Ethidium homodimer-1 (EthD-1) enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells (ex/em ~495–635 nm).

As shown in Figure 4.23, all cancer cell lines tested showed a time-dependent death response after treatment with respect IC50 values. This observation is quite consistent with the previous results obtained from MTT assay. This assay indicate that upon CT1 exposure with 12 h IC50 values, there is an increase in cell death after 12 h in MCF-7, HepG2 and HSC-4 cells, compared to the HMEC cells. MCF-7 cells showed significant cell death among cell lines evaluated induced by CT1 where approximately 50.35 ± 3.86 % of killing was observed. Meanwhile 53.44 ± 2.17 % and 37.27 ± 0.67 % killing were observed in HSC-4 and HepG2 cells respectively.
**Figure 4.23:** Live/Dead viability/cytotoxicity assay depicting the cytotoxic effects of CT1 in cancer cell lines with minimal cytotoxic effects on human mammary epithelial cells normal control cells (A) Fluorescence microscope images of viable cells stained with acetomethoxy derivate of calcein (green) and non-viable cells stained with ethidium homodimer 1 (red). (B) Percentage of viable cells as calculated under a fluorescence microscope. A total of four random quadrants were selected from each triplicate for quantification. All data were presented as mean ± SEM.
4.3 Apoptosis determination

4.3.1 CT1 induces apoptosis-mediated cell death

We next determined whether CT1 cytotoxic effects were mediated through apoptosis or necrosis using double fluorescence staining of annexin V-FITC/PI flow cytometry assay and DNA fragmentation assay. An increase in cellular staining with FITC-conjugated annexin-V serves as an early marker for apoptosis while staining with PI indicates loss of cell membrane integrity.

Treatment of cancer cells was found to induce apoptotic cell death by observing a shift in viable cell population from early to late stage of apoptosis, followed by secondary necrosis. Figure 4.31a shows the percentage of MCF-7 viable cells decreased from 91.2% to 45.1%, while total percentage of apoptotic MCF-7 cells was 46.6% after 24 h. Minimal percentage (4.8%) of apoptotic population shifts were observed in HMEC cells after highest IC$_{50}$ of CT1 treatments (Figure 4.31b).

Meanwhile figure 4.32a shows total percentage of apoptotic HSC-4 cells was 34.8% after treated with CT1 for 24 h. HepG2 death cells increased from 8.0% to 26.5%, where 19.6% of apoptotic cells were detected after 24 h (Figure 4.31b).
Figure 4.31: CT1 potentiates apoptosis mediated cell death in MCF-7 human breast cancer cells. (A) MCF-7 cells and HMEC cells were treated with CT1 at IC$_{50}$ concentrations for 24 h. Dot plots are a representative of 1.0 x 10$^4$ cells of three replicates with percentage of cells indicated in each quadrant (B) Percentage of annexin V-FITC staining cells as obtained from FACSDiva acquisition and analysis software. All data were presented as mean ± SEM.
Figure 4.32: CT1 induces apoptosis mediated cell death in HSC-4 human oral and HepG2 human liver cancer cells. HSC-4 cells and HepG2 cells were treated with CT1 at IC₅₀ concentrations for 24 h. Dot plots are a representative of 1.0 x 10⁴ cells of three replicates with percentage of cells indicated in each quadrant. (B) Percentage of annexin V-FITC staining cells as obtained from FACSDiva acquisition and analysis software. All data were presented as mean ± SEM.
4.3.2 Confirmation of CT1’s apoptosis-inducing effects

One of the major hallmarks of apoptosis-mediated cell death is the occurrence of chromatin condense and laddering. DNA extracted from CT1-treated cells also showed DNA laddering with approximately 180 bp to 200 bp intervals as the result of endonuclease action at sites between nucleosomes, thus confirming the occurrence of apoptosis.

Figure 4.33a demonstrated fragmentation of MCF-7 genomic DNA after 24 h of CT1 treatment. Similar fragmentation pattern could be observed in HSC-4 and HepG2 cells treated with CT1 after 24 h exposure (Figure 4.33b and c). However, fragmentations of genomic DNA in 12 h incubation with CT1 were not detected. There are two possible reasons that may explain this observation, firstly is that concentration of CT1 not good enough to induce this enzymatic reaction in all cancer cells and second possible reason is this process dependent on time. In contrast, treatment of CT1 in HMEC normal cell did not induces any form of laddering. Genomic DNA from HMEC remained intact even after 24 h treatment (4.33d). A positive control was also demonstrated in each figure, consisting of leukocyte cell (HL-60) undergoing apoptosis upon treatment with antinomycin D, provided by the kit manufacture.
Figure 4.33: Confirmation of apoptosis mediated cell death through observation of a 200 to 250 bp DNA laddering using the DNA fragmentation assay. (A) MCF-7 (B) HepG2 (C) HSC-4 and (D) HMEC cells were treated with CT1 for 12 h and 24 h followed by analysis of extracted DNA on 1.0% (w/v) agarose gel electrophoresis. +ve: positive control HL-60 cells undergoing apoptosis cells upon treatment with antinomycin D. M: 100 bp DNA size marker.
4.4 Cell cycle arrest

4.4.1 Induction of cell cycle arrest by CT1

The effects of CT1 on cell cycle progression using flow cytometry PI-based staining showed an increase in the population of MCF-7 sub-G₁ phase from 11.1% to 55.0% after 24 h incubation. The increase was consistent with a reduction in the G₀/G₁ phase from 70.8% to 35.0% after 24 h indicating a potential cell cycle arrest during the G₀/G₁ phase as there were minimal changes in both S and G₂/M phases after 24 h of treatment (Fig. 4.41a and b).

Cell cycle profiles for HMEC normal cell controls were found to be consistent after 12 h and 24 h exposure. No significant sign of cell cycle arrest were observed. The populations of HMEC cells in each cell cycle phase were maintained in a consistent manner even after 24 h of exposure.

Surprisingly, different cell cycle pattern in both HSC-4 and HepG2 cancer cell upon treatment with CT1 were observed. The population of both cells in each cell cycle phase was maintained after 12 and 24 h treatment. No detection of cells in Sub-G₁ in both cancer cells tested. Therefore, it can be concluded that CT1 induces potential G₀/G₁ phase arrest in MCF-7 cells, while did not affected cell cycle regulation of HSC-4 and HepG2. CT1 also demonstrated did not cause any significant changes to normal HMEC cell cycle pattern.
Figure 4.41: Cell cycle distribution of MCF-7 and HMEC cells using flow cytometry after staining with propidium iodide (PI) for 12 h and 24 h. I: Sub-G₁; II: G₀/G₁; III: S; IV: G₂/M.
Figure 4.42: Cell cycle distribution of HSC-4 and HepG2 cells using flow cytometry after staining with propidium iodide (PI) for 12 h and 24 h. I: Sub-G₁; II: G₀/G₁; III: S; IV: G₂/M.
4.5 Wound healing Assay

4.5.1 Induction of anti-migration effects of CT1

Wound healing assays also showed that HSC-4 cells treated with CT1 migrated at a slower rate compared to MCF-7 cells, indicating that CT1 was more effective in preventing cellular migration in HSC-4 oral cancer cells. Anti-migration effects on HepG2 was found to be absent with treated cells showing comparable migration rates as untreated cells (Figure 4.5a and 4.5b).

<table>
<thead>
<tr>
<th></th>
<th>Untreated</th>
<th>Treated DMSO</th>
<th>Treated CT1</th>
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<tr>
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<td><img src="image8.png" alt="Image" /></td>
<td><img src="image9.png" alt="Image" /></td>
</tr>
</tbody>
</table>
4.5 **Figure 4.5**: (A) Wound healing assay displaying the anti-migration effects of CT1 on HSC-4 cells, with minimal effects on MCF-7 cells and not at all on HepG2. All cells were treated with mitomycin c to halt proliferation, followed by CT1 at IC$_{50}$ concentrations for 12 h. (B) Wound edge images of each independent triplicate were captured and measured at 24 h post-treatment using T-scratch software, and percentage of migration is indicated as mean ± SEM.

4.6 **Western Blotting Analysis**

4.6.1 **CT1 reduces ERK1/2, Bcl-2 and Bim, while increasing FasL protein levels**

Western blot analysis of CT1 treated MCF-7 cells were carried out to observe the effects on ERK1/2, FasL and Bcl-2 family of apoptotic proteins. Results showed that protein levels of both ERK1 and ERK2 declined over 24 h, while the level of the FasL increased dramatically by almost five-folds over 24 h compared to untreated cells (Fig. 4.6a and 4.6b). Protein levels of the pro-apoptotic Bim and Bcl-2 also decreased after CT1 treatment and were completely absent 12 h post-treatment, favoring the induction of apoptosis (Fig. 4.6a and 4.6b). Furthermore, protein levels of pro-apoptotic Bax were found to be slightly elevated after 24 h, with consistent XIAP protein levels. Due to the dimerization nature of Bcl-2 family proteins, the ratio between Bax/Bcl-2 protein levels were measured and indicated a 9.7 fold increase at 12 h, which further increased to 26.6 folds higher compared to control cells at 24 h (Fig. 4.6a and 4.6b).
Figure 4.6: Observation on the effects of CT1 treatment on MCF-7 protein level using Western blot over 24 h. (A) CT1 was found to decrease ERK1/2 and anti-apoptotic Bcl-2 and Bim protein level, while increasing FasL protein levels. XIAP and pro-apoptotic Bax protein were unaffected following CT1 exposure. β-actin was used as a normalization control for all experiments. (B) Quantification of protein band intensities were determined by densitometry analysis and normalized to β-actin using the ImageJ v1.43 software. All results were presented as mean normalized intensity ±SEM of three replicate experiments.

4.6.2 CT1 induces intrinsic caspase-mediated apoptosis in MCF-7 cells

The involvement of FasL and Bcl-2 family members implied that induction of apoptosis by CT1 was mediated via both the mitochondrial and death receptor pathway. Therefore, we also assessed the protein levels of various procaspases through Western blot analysis. Our data demonstrated that CT1 induced the reduction of procaspase-3, -6, and -9 levels leading to an increase in active effector caspase forms (Fig. 4.7a and 4.7b). Reduction in the protein levels of procaspase-8 was found to be transient over the first 6 h with protein levels starting to increase 12 h post-treatment onwards, suggesting that the extrinsic death receptor pathway was used to augment the apoptotic signals arising from the intrinsic pathway.
Figure 4.7: Activation of caspase upon CT1 treatment in MCF-7 cells. (A) Western blot analysis on protein level of various procaspases upon CT1 treatment. MCF-7 cells were treated with 16 μmol/l of CT1 for 6 h, 12 and 24 h respectively. Western blot of cell extract were probed using the indicated procaspases antibodies and β-actin as a normalization control (B) Normalization on band intensities between procaspases and β-actin was determined by densitometry using ImageJ v1.43 software and result were presented as a mean normalized intensity ±SEM of three independent experiments.
CHAPTER 5
DISCUSSION

The aim of the present study was to evaluate the anti-cancer effects and molecular mechanisms of the pure compound 7α-hydroxy-β-sitosterol (CT1) isolated from bark of Chisocheton tomentosus in cancer cell lines.

Natural products have shown various effects as potential agents for treatment of a vast majority of human diseases. These products are able to influence the regulation of several diseases such cancer (Newman & Cragg, 2012), neurodegenerative disorder (da Rocha et al., 2011), autoimmune disease (Tao & Lipsky, 2000), cardiovascular disease (Shukla et al., 2010) and diabetes mellitus (Hung et al., 2012). Natural compounds with low molecular weight or known as small molecule and are commonly referred as natural products are crucial for high activity on a biological target. The products target proteins with a high number of protein-protein functional interactions and thus facilitate disruption of essential biological pathways, resulting in competitor death (Dančík et al., 2010).

These potentially biologically active compounds also can promote different physiological condition by targeting various genes (Walsh & Fischbach, 2010), proteins (Bykov et al., 2002), enzymes (Jedinák et al., 2006) and biochemical pathways (Ancuceanu & Istudor, 2004) in normal and abnormal mammalian cells. Generally, the compounds can be categorized based on their chemical structure such as flavonoids, lignans, terpenoids, phenols and others (Koch et al., 2005). In contrast, compounds with higher molecular weights are absorbed by cells less efficiently and have reduced accessibility towards targeted sites. Besides, it is impossible to escape via the kidney and will persist in blood circulation for long periods (Seymour et al., 1995). Understanding the mechanisms of these active compounds may lead to production of a
potent drug in the future. This current study involves a pure compound from *Chisocheton tomentosus* plant which is a member of the Maliaceae family, and was characterized as a phytosterol oxide (Najmuldeen *et al.*, 2008).

As mentioned above, the discovery of natural products is important to boost the researches in pharmacology and industrial activities that subsequently will benefit humankind. Thus, finding the compounds with molecular weights as low as possible (small molecules) with potential biological activities is desirable to achieve the goal. The well-known example of natural product that acts as a potent drug in cancer chemotherapy is paclitaxel or Taxol® that was isolated from the bark of *Taxus brevifolia*. The molecular weight and structure was confirmed and published after five years of its discovery (Wani, *et al.*, 1971).

There are a number of different approaches to assess the cytotoxicity of natural products in cells. These include epidemiology, human clinical, animal and *in vitro* studies (Devlin *et al.*, 2005). The present study used the *in vitro* approach for evaluating the effects of CT1 on cytotoxicity and apoptosis mechanism. The advantages of this approach are easy to carry out, relatively inexpensive, simple system and useful to provide mechanistic information (Venkataramanan *et al.*, 2006). However, there are limitations of this method. The major limitation is that it does not represent the exact process *in vivo*. In addition, it does not account for poor bioavailability of the active compound *in vivo*.

Since CT1 is a rare natural phytosterol oxide compound and the mechanism of its action is not well understood, the *in vitro* method is the best approach to study its effects (Hoffman *et al.*, 1984; Jia & Liu, 2007). In this study, the cells were incubated with different concentration of CT1 for different interval time up until 24 h which would provide an understanding on how cancer cells will respond to external stimuli.
Since the molecular weight of CT1 is 430 g mol\(^{-1}\), it is considered a small molecule and a good candidate to be evaluated. It is believed that compounds with highest IC\(_{50}\) values are not suitable as a drug because the difficulty to react effectively \textit{in vivo} (Vogler, \textit{et al.}, 2008). However, getting the precise information on the mode of action of compounds can serve as useful data that helps generate more potent drugs which kill or disable cancer cells without causing excessive damage to normal cells (Rubis, \textit{et al.}, 2008). In this study, MTT and Live Dead assays were carried out with three main objectives, firstly, in determining which cell lines were able to demonstrated significant cytotoxic activity after treatment of CT1, secondly, to investigate whether the cytotoxic activity of CT1 were dose or time dependent, and thirdly, to determine the IC\(_{50}\) values for various cell lines which is important in subsequent downstream assays.

Although very limited reports showed cytotoxic effects of CT1 in cancer cells, some of the results have shown CT1 to be directly or indirectly involved in the apoptosis pathway modulation. The work by Koschutnig \textit{et al.} strengthened the point that CT1 could induce cell cytotoxicity in liver cancer cells. The authors suggested that CT1 induced cell death mediated lipid peroxidation since no detection of early apoptotic cells measured by annexin V-PE and 7 ADD dual staining (Koschutnig, \textit{et al.}, 2009). To my knowledge, this is the second study on CT1 following that by Koschutnig \textit{et al.}, 2009.

Phytosterol oxides have shown to induce cytotoxicity in various types of cancer cells (Ryan, \textit{et al.}, 2005). HepG2 cell line was selected out of five cell lines investigated in this present study. Since phytosterol oxides can distribute through different tissues, mainly in the liver (Otaegui-Arrazola, \textit{et al.}, 2010), manipulation of this cell line is the best way for toxicity evaluation (Koschutnig, \textit{et al.}, 2009). On HepG2 cells, our cytotoxicity results were consistent with previous studies. By subjecting 25 µM of CT1 for 24 h, approximately 50 % cell viability has decreased.
However, this concentration was much lower compared to previous reports. Previous concentrations range between 30 - 45 µM to achieve the same percentage of cell viability caused by CT1 (Ryan, et al., 2005; Koschutnig, et al., 2009). In contrast to Koschutnig and Ryan that found CT1 to only have cytotoxic effects without any apoptosis potential (Ryan, et al., 2005; Koschutnig, et al., 2009), this study provided evidence for both cytotoxic and apoptosis-mediated cell death induced by CT1.

Recently, extensive investigation has been carried out on the effects of phytosterols in human breast cancers (Awad et al., 2001, 2003; Muti et al., 2003; Awad, et al., 2007, 2008; Park et al., 2008), however only one study reported that β-sitosterol could give moderate side effects on human normal cell and less effect was observed for its oxide derivatives (Rubis, et al., 2008).

The effects of new crystal form of the CT1 compound on MCF-7 cell line were investigated. Viability of MCF-7 cells decreased to 50% at 24 h following treatment with 16.0 µM of CT1. However, the effect of CT1 appeared faster (24 h) than pure β-sitosterol which can only be observed after 48 or 72 h. This result confirmed that treatment at 24 h is important for CT1 treated MCF-7 cells. Optimum time is crucial for most anti-cancer drug since the stability of protein-drug complex will disrupted if persist too long in aqueous solution (Sudhamalla, et al., 2010).

Second, the cytotoxicity effect of CT1 on oral cancer cells was investigated. Taking into account the oral cancer cases (Zainal & Nor Saleha, 2011) and food-containing phytosterol consumption among Malaysian (Ng et al., 1991), it was important to investigate the relationship between these types of cancer cells with CTI. Our findings showed that CT1 can induce cytotoxic in HSC-4 squamous oral cancer cell but not in HSC-2 cells. These two cell lines differ in cyclooxygenase-2 activity (COX-2) whereby HSC-2 cells express a higher COX-2 activity (Akita et al., 2004). A
report by Prieto showed that β-sitosterol failed to inhibit cyclooxygenase-1 activity in leukocytes cells (Prieto, 2006). This led us to make an assumption that activity level of COX-2 in cancer cell is crucial for induction of cytotoxicity by CT1. Half of cytotoxic effect was achieved when HSC-4 cells were treated with 19 µM CT1 while no effects were observed on HSC-2 cells with CT1 concentrations ranging between 5-100 µM.

Recent studies on exploring the effects of natural products on cervical cancer (Zou et al., 2005; Siddiqui et al., 2011), showed proliferation of cancer cells can be inhibited. Since the extracts contain a majority of chemical constituents such as β-sitosterol, alkaloid and fatty acids, one of these constituents, or a combination of a few constituents are suggested to be responsible for the cytotoxic activity detected on the cancer cells (Li, et al., 2010; Srivastava et al., 2011). In order to evaluate the cytotoxic effect of CT1 alone in cervical cancer, we introduce this compound onto Ca Ski cell lines. Surprisingly, only minimal cytotoxic effects were observed when the cells were treated with CT1 concentrations ranging between 5-100 µM.

Lastly, since previous studies on the biological and safety aspects of oxyphytosterol are unclear (Newill, et al., 2007; Otaegui-Arrazola, et al., 2010) and cytotoxic analysis of β-sitosterol and its epoxy derivative on endothelia cells proved that β-sitosterol was more toxic than its epoxy derivative (Rubis, et al., 2008), in the present study, CT1 was tested on human mammary epithelial cells, HMEC. MTT cytotoxicity assays were performed on the normal cells because of the need to compare the effect of CT1 on a similar cytological cell type of the various cancer cells, which are of epithelial origin. By introducing the highest concentration of CT1, a statistically 19.7% of death cells were observed on HMEC cells at 48 h. This effect is significantly lower than was seen in cancer cells at similar doses. This result supports the safety profile of CT1, like other phytochemicals which showed relatively low toxicity against normal cells (Engberg et al., 1998).
Results of this study clearly indicate the potential benefits of phytosterol oxides, specifically CT1 in the prevention of breast adenocarcinoma. Cytotoxicity data has demonstrated that IC$_{50}$ values of CT1 after 24 h of incubation on all cancer cell lines tested were within the range of 16 µM to 32 µM, which is comparable to most commercialized phytocompounds (Elmore et al., 2005). These results have also demonstrated that the treatment of CT1 in tumor cells resulted in a dose- and time-dependent inhibition of cell proliferation and its cytotoxic degree is selectively different among tumor cells. MTT assay clearly indicated that the rate of killing between cell lines varied, thus the attainment of various IC$_{50}$ values. It is suggested that variations in CT1 toxicity in different cancer types may be attributed to genetic variability of cell lines (O'Shea et al., 2011). The intracellular balance between tumor suppressor genes was also likely to play a role in creating a diversified microenvironment influencing the outcome on how each cell type react toward an-anti cancer drug. Other reason is the aggressiveness of each cancer genotype towards CT1 required to achieve IC$_{50}$ level in respective cancer types.

The ability of cancer cells to migrate into different site of tissues is one of the critical factors contributing to progression of cancer. This process fundamentally involves angiogenesis, granulation tissues formation and re-epithelialization (Gottrup et al., 2000; Hanahan & Weinberg, 2000, 2011). Majority of natural products that are categorized under anti-neoplastic drugs, have the ability to block migration of cancer cells (Sagar et al., 2006; Lecomte et al., 2011). Wound healing assay is a relatively inexpensive assay, easy to carry out and offer the opportunities to investigate cellular migratory responses (Carretero et al., 2007; Liang et al., 2007; Oberringer et al., 2007).
MCF-7, HSC-4 and HepG2 cells were pre-treated with mytomycin c before treatment with CT1 to inhibit proliferation of cells (Carretero, et al., 2007). Mytomycin c is a reliable DNA synthesis inhibitor used to inhibit proliferation of cells on closing of wound edges (Gottrup, et al., 2000). Surprisingly, CT1 did not inhibit MCF-7 cell migration, but instead, promoted migration slightly when compared with DMSO-treated cells. A study by Awad et al., 2001 reported that β-sitosterol significantly promoted migration of the MDA-MB-231 cells. Additionally, a paper published two years later showed that oxysterol or sterol oxide from osteoblast medium has been found to positively regulate migration of MCF-7 cells (Silva et al., 2003). These previous results showed a correlation with our present results, which indicated that after 24 hours incubation with CT1, MCF-7 cells was able to migrate in closing of wound edges.

In evaluating the inhibitory effects of CT1 toward HSC-4 cell line, it was inhibited after 24 h incubation. A recent study demonstrated one of the secreted proteins known as connective tissues growth factor (CTGF) has the ability to reduce the migration of oral cancer cells (Chuang et al., 2011). Meanwhile, Chien et al., 2011 reported that expression of full length CTGF in MCF-7 cells can reduce cell proliferation and increase migration of cells. The effect of CT1 in term of the migration process in this study has been consistent with past results reported by Chuang et al., 2011 and Chien et al., 2011. These viewpoints lead to the assumption that CT1 may act directly with CTGF in order to promote and inhibit migration of MCF-7 and HSC-4 cells respectively. From the result obtained, it can be concluded that CT1 inhibited the migration of HSC-4 cell line and promoted the migration of MCF-7 cell line. Further molecular works such as western blots are needed to confirm the involvement of CTGF role in this process.
As stated previously, elucidation mode of cell death by pure compound are crucial steps in development of the compound. One of the main features of cancer is the dysregulation of apoptosis (Hanahan & Weinberg, 2011). Apoptosis process is a mode of cell death which is important for cells to maintain its homeostasis (Kerr, et al., 1972). Majority of anti-cancer drugs must have the potential to induce this process following treatment. Hence, to further understand the underlying mode of cell death behind this treatment response, we decided to look at the apoptosis process, where two types of assay were conducted on MCF-7 cells, HSC-4 cells and HepG2 cells; annexin V-FITC and DNA fragmentation assay. Translocation of phospholipid phosphatidylinerine proteins from the inner leaflet to the outer leaflet of the plasma membrane is an indicator for the early stage of apoptosis (Fadok et al., 1992). However, these proteins were not only detected on cells undergoing apoptotic morphological changes but were also found on necrotic cells. Identification of appropriate markers is an important step to reduce false positive in detection of apoptotic cells in vitro since apoptosis and necrosis occur simultaneously in many pathological condition (Hirt & Leist, 2003; Kirkland et al., 2007).

Since annexin V can bind to phosphatidyl serine protein when cells undergo apoptotic process, probes of annexin V conjugated with FITC were used to measure apoptotic cells quantitatively by flow cytometry (Vermes et al., 1995; Zhang et al., 1997). The advances in flow cytometry techniques have boosted research in understanding modes of cell death (Bedner et al., 1999). By staining cells with a combination of FITC-conjugated annexin V and PI it is possible to detect nonapoptotic live cells (FITC-negative/PI-negative), early apoptotic cells (FITC-positive, PI-negative), and late apoptotic or necrotic cells (FITC-positive, PI-positive) (Koopman et al., 1994; van Engeland et al., 1998). Through flow cytometric methods employing annexin V-FITC and PI dual staining, it was clearly shown that following treatment of
cancer cell lines with CT1, the population of viable cancerous cells shifted toward apoptosis. The current findings demonstrated that CT1 treatment showed good percentages in inducing apoptosis in the three cell line analyzed. Increasing of annexin-V staining after incubation with CT1 represented those certain types of cells undergoing mode of cell death by apoptosis process.

At the molecular level, small fragments of DNA cells could be a marker for apoptotic cells (Facchinetti et al., 1991). However, morphological evidence depicting apoptosis is not always followed by DNA fragmentation. Cohen et al., 1992 found that in the presence of zinc (Zn\(^{2+}\)), dexamethasone could induce apoptosis process by forming a number of apoptotic features except DNA laddering. This observation was also supported by Falcieri et al., 1993 which showed that human acute lymphoblastic leukemia cell line, MOLT-4 cells did not display internucleosome DNA fragmentation after treatment with staurosporine although morphological changes typical of apoptosis was detected. Although these two findings proved that DNA fragmentation is not a compulsory process in apoptotic cells, however, many studies used this assay to determine and confirmed the occurrence of apoptosis in cancer cells.

Hasima et al., 2010 demonstrated that 1’S-1′-acetoxyeugenol acetate (AEA), an analogue of 1’S-1′-acetoxychavicol acetate (ACA), isolated from the Malaysian ethnomedicinal plant *Alpinia conchigera* Griff (Zingiberaceae) could induce apoptosis mediated cell death in MCF-7 cells. Confirmation of mode of cell death was carried by observation of DNA laddering using DNA fragmentation. A year later, the same group had published a paper discussing the role of Erythrocarpine A, a new liminoid compound isolated from *Chisocheton erythrocarpus* Hiern was able to induce apoptosis in oral squamous carcinoma cell (HSC-4). This work had used the DNA fragmentation assay for confirmation of the apoptosis process in this oral cell line (Nagoor, et al.,
Recently, Samarakoon et al., 2012 have reported that decoction induce the fragmentation of DNA in HepG2 cells, leading to increase rates of apoptosis.

At the moment, measurement of DNA fragmentation by conventional method or agarose gel is still considered as a reliable method to determine the mechanism of cell death especially with respect to apoptosis. Although there are advance assays in determining different mechanism of cell death such as Terminal deoxynucleotidyl dUTP nick end labeling (TUNEL) assay. Kraupp et al., 1995 had reported previously that this assay could not discriminate among apoptosis, necrosis and autolysis cell death. Based on this report, in the present study, CT1 was evaluated for its apoptotic effect on MCF-7, HepG2, HSC-4 and HMEC cells by the conventional DNA fragmentation assay. Indeed, formation of DNA laddering was found to be associated with CT1 treatment in all three cancer cell lines tested. The result obtained from this assay show a consistency with previous result from our annexin V-FITC/PI staining assay.

Cell cycle regulation is important for cell growth and proliferation. One of the main factors inducing unlimited growth of cancer cells is as a consequent of defect in cell cycle and apoptotic machinery. DNA content analysis is the best approach to determine the arrest of cell cycle (Darzynkiewicz et al., 1992). This study also demonstrated the ability of CT1 to induce cell cycle arrest following treatment. Changes in PI-stained DNA intensities have show that CT1 inhibited cell cycle progression effects at the G0/G1 phase only in MCF-7 cells and not the HMEC normal cells. On the other hand, CT1 was found not to induce cell cycle arrest in HSC-4 and HepG2 cells. The effect of CT1 on the cell cycle has never been investigated in the past, and is reported in this study for the first time.
MCF-7 breast cancer cells were chosen as the cell line of the study of western blotting due to it consistent results seen on annexin V-FITC assay, DNA fragmentation and cell cycle analysis. To explore further possible molecular mechanisms, we investigated the impact of CT1 on the expression of Fas-Ligand protein. A recent study reported that nanoparticles such as quantum dots could induced cell death via up-regulation of Fas receptor together with lipid peroxidation. Induction of these mechanisms led to activation of the caspases cascade and eventually inducing apoptosis (Choi et al., 2007). Since CT1 could enhanced level of lipid peroxidation (Koschutnig et al., 2009) in cancer cells, we measured the level of Fas-Ligand proteins in MCF-7 cells. Results showed that treatment of MCF-7 cells with CT1 for 6, 12 and 24 h increased level of these proteins. This is consistent with the treatment of β-sitosterol in breast cancer that depicted involvement of Fas regulation in inducing cell death (Awad et al., 2007). Future work is required to further understand the relationship between mechanisms of lipid peroxidation and increased levels of Fas ligand proteins in breast cancer cells since Awad et al., 2007 showed level of Fas receptor proteins only in neuroblastoma cells.

Members of the Bcl-2 family have been identified as key regulators of apoptosis, and are further divided into pro-apoptotic and anti-apoptotic members (Petros et al., 2004). Indeed, in order to induce apoptosis, the majority of Bcl-2 family proteins must directly or indirectly play a role in this process. These proteins include pro-survival, pro-death and BH3-only protein. Once a cell receives a death stimuli, BH3-only protein can be activated and bind to pro-survival protein. The binding leads pro-death proteins such as Bak and Bax to form oligomers on mitochondrial membrane. Then Bcl-2 proteins often form heterodimer complexes with Bax proteins, which results in the release of cytochrome c from the mitochondria and subsequent induction of cell death (Hoetelmans et al., 2000). Hence, an increase in the ratio of Bax/Bcl-2 is
considered as one of the major markers of pre-apoptosis. Several natural compounds that have been shown to influence the Bax/Bcl-2 ratio in cancer cells are (-)-epigallocatechin-3-gallate (EGCG) and resveratrol (Hsuuw & Chan, 2007; Androutsopoulos et al., 2011).

In the present study, antibodies against Bcl-2 anti-apoptotic and Bax pro-apoptotic proteins were used to investigate the effect of CT1 on Bcl-2 family protein. The reason we selected Bcl-2 pro-survival protein to further investigate the expression of anti-apoptotic protein in CT1-treated MCF-7 cells was because of the ability of this protein to bind with anti-survival protein Bax. It was reported that these two proteins can form heterodimer and finally determine the cell fate (Conus et al., 2000; Ding et al., 2010). Compared to other anti-apoptotic protein such as Bcl-xL, this protein does not bind to Bax or Bak in cytosolic cell despite being able to block apoptosis. Moreover, results from previous studies (Nadler et al., 2008; Oakes et al., 2012) showed that Bcl-2 is highly expressed in breast cancer patients and therefore was identified as a good marker candidate to be evaluated in our study.

Western blot analysis showed there was a slight increase in Bax protein expression after CT1 treatment in MCF-7 cells. More importantly, the expression levels of Bcl-2 protein were concurrently down-regulated after CT1 treatment; hence the ratio of pro-apoptotic proteins to the anti-apoptotic proteins was altered in favor of apoptosis. Thus, the results suggest that an increased of Bax/Bcl-2 observed in this study may be one of the critical mechanisms through which CT1 induces apoptosis in MCF-7 cells.

As mentioned previously, BH3-only protein can act as a key regulator to initiate cell death. However, the exact mechanism induced by this protein remains unclear. Since this protein can bind to pro-survival Bcl-2, further investigation might be an
initial step in understanding the relationship between pro-survival and pro-apoptotic proteins in inducing apoptosis. Interestingly, result from Merino et al. (2009) proved that small splice variant of Bim, (BimS) can also bind transiently to pro-apoptotic Bax to induce maximal apoptosis activity. Figure 5.0 illustrates the model for initiation of apoptosis by Bim.

Figure 5.0: Model for the initiation of apoptosis by Bim (A) In the absence of Bim, Bax is kept in check by both subsets of its prosurvival relatives (“Bcl” represents Bcl-2, Bcl-xL, and Bcl-w; “Mcl” represents Mcl-1 and A1; Willis et al., 2007). (B) WT Bim is proposed to also bind transiently to Bax, giving maximal activity. (Adapted from Merino et al., 2009)

Western blot analysis shows that CT1 was able to reduce protein level of cytosolic Bim after 12 h. This finding is contrary with other previous work that showed decreasing of Bim protein is responsible for growth promotion in cancer cells. The reasonable explanation of this situation is based on the assumption that cytosolic Bim binds tightly to pro-survival Bcl-2 on the surfaces of mitochondria and nuclei (Puthalakath et al., 1999). Since current protein extraction protocol collected only cytoplasmic and not nuclei proteins, this might explain why level of Bim in present study differed compared to previous literatures (O’Connor et al., 1998). Further investigation is needed to validate the biological function of these splice variant of Bim.
as other splice variants were expressed transiently during apoptosis. In reference to this, results of present study suggest that CT1 effectively induces apoptosis in MCF-7 cells through dysregulation of the Bax/Bcl-2 ratio via modulation of Bim protein level, which in turn results in the cleavage of procaspase-9, -3 and -6 into active effector caspases.

Extracellular signal-regulated kinase (ERK1/2) which are expressed ubiquitously in mammalian cells, are multifunctional serine/threonine kinases that phosphorylate a vast array of substrates localized in all cellular compartments (Ley, et al., 2003; Balmanno & Cook, 2008). Degradation of Bim is believed to be caused by activation of ERK1/2 protein. The activation can promote degradation of this protein via the proteasome pathway. This finding was supported by Ley et al., 2003 as the activation of Erk1/2 pathway is necessary and sufficient to promote phosphorylation that leads to degradation of this protein. Besides, serum withdrawal can also promote the expression and dephosphorylation of Bim protein. Since current result showed that Bim level decreased after 12 h treatment, the need to investigate the protein level of ERK1/2 is necessary. This result will lead me to understand the role of Bim in CT1-mediated apoptosis. Furthermore, all treatments were done in media supplement with 10.0% of fetal bovine serum, thereby ruling out the serum deprivation-induced cell death.

The activation of the mitogen-activated protein kinase (MAPK/ERK) cascade contributes towards proliferation of breast cancer cells which can be prevented by using agents that prevent ERK1/2 activation (Yuste et al., 2005). In normal cells, sustained activation of ERK1/2 promotes G1 to S phase progression and inhibits antiproliferative genes, while its hypoactivation by MEK inhibitors can induce cells to undergo cell cycle arrest (Fiddes et al., 1998; Meloche & Pouyssegur, 2007). The current study showed that CT1 was able to inhibit cell cycle progression at the G0/G1
phase after 24 h, which corresponded to reduced protein levels of ERK1/2 as shown in Western blot analysis. It can be concluded that CT1 could act either as agent in directly preventing or promoting specific pathways in order to prevent Erk1/2 activation. Further investigations are required to fully understand the role of this compound on Erk1/2 activation process such as in silico analysis.

Symes et al., 2008 found that expression of inhibitor of apoptosis proteins (IAP) in several primary prostate cancer cells did not correlate with susceptibility towards FasL-mediated cell death. Their work also proved that treatment using a known chemotherapy drugs could enhanced Fas expression and subsequently led to increased cell killing. In the present study, treatment with CT1 was able to induce FasL expression in MCF-7 cells, which translated into a 1.0-7.0-fold enhancement of protein level by western blot analysis. On the other hand, the expression of XIAP was found not to correlate with susceptibility to CT1/FasL-mediated cell killing.

Activation of pro-caspases proteins could be induced by several ways. This activation process could be caused by serum deprivation (Schamberger et al., 2005), over-expression of their pro-caspases (Klaiman, et al., 2009), cleavage of pro domain by upstream caspases (Meergans et al., 2000; Fuentes-Prior & Salvesen, 2004) and homodimerization process (Oberst & Green, 2011; van Raam & Salvesen, 2012). However, the cleavage of the zymogen is not always an obligatory requirement for caspase activation (Kumar, 2006). Also, activation of a majority of the caspases is not the sole contributing factor of apoptosis (Klaiman, et al., 2009; Huang, et al., 2011; van Raam & Salvesen, 2012). In MCF-7, activation of caspase 3 only occurs by removal of their pro-domain by upstream heterogenous caspase, activated caspase 9. Meanwhile, activation of caspase 6 in Hek293T could be carried out by over-expression their effector pro-caspase 6 and serum deprivation. However, the effector pro-caspase 6 is not activated by over-expression in MCF-7 cell line. It is suggested that MCF-7 cells
can produce significant level of active caspase 6 within 24h of serum deprivation, however, its activation is not accompanied with cell death, mitochondrial dysfunction and apoptosis as assessed by MTT assay and sub-G₁ DNA content respectively (Klaiman, et al., 2009).

Western blot analysis also showed that treatment of CT1 not only activates apoptosis effector proteins such as caspase-3 and caspase-6, which is evident through a decrease in procaspase-3 and procaspase-6 protein levels, but also activates caspase-8. This strongly suggests that signals favoring the induction of apoptosis does not solely originate from the mitochondrial pathway, but may also be augmented by the extrinsic death receptor pathway, specifically through Fas-mediated mechanisms (Awad, et al., 2007). This is further supported in studies involving the caspases cascades where activated caspase 6 can directly process the transient activation of caspase 8 through an amplification loop to enhance apoptotic signals (Cowling & Downward, 2002; Inoue et al., 2009). In consistent with this, an initial reduction in procaspase-8 levels at 6 h was observed, followed by a subsequent increase over the next 12 h. Although the expression level of cytochrome c was not measured in the present study, there is literature report indicating that isomer of CT1, was able to induce the release of cytochrome c in the cytosol of colon cancer cells (Roussi, et al., 2007).

Suzuki et al., 2001 and Takahashi et al., 1998 showed that active-forms of caspase-3 protein level in Fas-mediated apoptotic cells was specifically decreased in the presence of wild type XIAP. Decreased of this protein expression was suggested as a result of proteasomal degradation of XIAP’s E3 activity. They also provided evidence that XIAP binds to the active-form caspase-3 protein rather than procaspase-3 protein to initiate ubiquitin-proteosome pathway.
In addition, observation of active-form caspase 3 protein level in strauroporin-treated HEK293T cells by Klaiman et al., 2009 showed that the subunit is rapidly degraded as the lower levels of pro-caspase3 were not accompanied with an increase in active form of caspase-3. Since production of active caspase by removal of pro-caspase domain will expose IBMs motif, and the linker region BIR1 and BIR2 of XIAP to strengthen the binding into the active site of catalytic pocket and allowing IBMs to finally inhibit the caspase activation. This is in concordance to present western blot analysis that showed the level of XIAP remains consistent over 24 h, while the level of Fas ligand increased over 24 h compared to untreated cells. Hence, it can be hypothesized that undetectable active-form caspase-3 in the present study was caused by ubiquitin-protein ligase activity of XIAP.

The present study also showed that CT1 is able to induce cell growth arrest in the G₁ phase of the cell cycle via the specific inhibition of ERK1/2 pathway followed by apoptosis via changes of the ratio of the apoptotic related protein Bax/Bcl-2. Based on this information, it was also concluded that activation of the caspase cascade as a result of CT1 treatment increased the Fas ligand proteins level.

This study describes the drug development process beginning from cytotoxicity pre-screening assay to apoptotic assays, and finally provided evidence that CT1 sensitizes breast cancer cells through the regulation of pro-apoptotic and anti-apoptotic proteins as seen from western blotting analysis. Thus, it is suggested that CT1 possesses potential anti-proliferative, pro-apoptotic and anti-metastatic towards cancer cells. The understanding on how CT1 potentiates apoptosis as shown in this study is crucial in further understanding the mechanism underlying carcinogenesis. This knowledge will provide the basis for newly targeted therapies, hence giving cancer researcher the better insight for future chemotherapeutic approaches.
CHAPTER 6
CONCLUSION

The aim of the present study was to determine whether CT1, a rare naturally occurring compound derived from *Chisocheton tomentosus* plant could be used as a potential chemo-preventive agent in the treatment and prevention of cancers such as breast, liver and oral cancer. To the best of my knowledge this is the first study that has examined the effect of hydroxy-derivative phytosterol extracted from *Chisocheton tomentosus* family on the cell death function of normal cells and various types of cancer cells. In addition, this study revealed a novel molecular target of CT1 in breast cancer.

Results demonstrated that CT1 induces cytotoxic effect on MCF-7, HepG2 and HSC-4 cancer cells in-vitro via decreasing their proliferation rate measured by MTT and Live Dead assays. In contrast, CT1 showed minimal cytotoxic effect on the normal cell line, HMEC as well as the cervical Ca Ski and oral HSC-2 cancer cell lines. These data suggest a selective cytotoxic effect of CT1 on cancer cells and its protection effect against normal cells. Apoptosis process has been suggested to contribute to cytotoxicity of CT1. Flow cytometry analysis using annexin V conjugate with FITC demonstrated that cells treated with CT1 increased annexin V-binding cell sub-population in all the cancer cell lines tested. However, HMEC cells showed only a slight annexin V-binding cell sub-population before and after CT1 treatment which suggested that these cells are not significantly affected by CT1 treatment. Further finding demonstrated that treatment of CT1 enabled the activation of endonuclease-mediated nucleosome excision leading to the observation of DNA laddering of about 180-200 base pair on agarose gel of the three cancer cell lines tested.
Since apoptosis depends on the balance of cell growth and proliferation which are regulated mainly by cell cycle events, investigation on the effect of CT1 on cell cycle arrest using flow cytometry analysis showed that CT1 treatment triggered the checkpoint that produced arrest in G₀/G₁ phases in MCF-7 cells. On the contrary, CT1 did not show any significant arrest in HepG2 and HSC-4 cell lines. Meanwhile, cell cycle profile for HMEC cells control was found to be consistent for both exposure times. CT1 treatment also induced HSC-4 cells to migrate at slower rate compared to MCF-7 cells. On the other hand, CT1 treatment does not have any anti-migration effects on HepG2 cells which depicted comparable migration rates as untreated cells. This finding provides a rational to further investigate the migratory protein for better understanding of the anti-cancer mechanism action of this compound.

Bcl-2 family and caspases proteins are showed to play a crucial role in modulating apoptosis in cancer cells. Western blot results demonstrated that CT1 was found to inhibit the proliferation of MCF-7 human breast cancer cells through dysregulation of Bax/Bcl-2 ratio and the induction of G₀/G₁ cell cycle arrest via inactivation of ERK1/2. In addition treatment of CT1 induced the reduction of procaspase-3, -6, and -9 levels, leading to an increase in active effector caspase forms. Reduction in the protein levels of procaspase-8 was found to be transient, suggesting the involvement of both caspase mediated intrinsic and extrinsic pathways.

These result demonstrated the cytotoxic and apoptotic ability of CT1 in cancer cells. More importantly, the present study showed that this compound does not exhibit an adverse effect on the normal cells, HMEC. Further elucidations on other apoptotic targets coupled with in vivo studies are required for further development of this phytosterol and subsequently clinical trials involving patients with especially breast carcinoma.
REFERENCES


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cells during late G2 for timely entry into or exit from mitosis. *Mol Biol Cell, 17*(12), 5227-5240.


APPENDICES

Appendix 1: Molecular Markers

i. Protein Ladder – Spectra Multicolor Broad Range Protein Ladder (Fermentas, Canada)

ii. DNA Molecular Weight Marker – AM41 DNA Fragmentation Kit (Calbiochem, USA)
Appendix 2: List of reagents for SDS-PAGE.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Stacking gel</th>
<th>Resolving gel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.0%</td>
<td>12.0%</td>
</tr>
<tr>
<td>40.0% Acrylamide</td>
<td>500.0 μl</td>
<td>4.5 ml</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5M Tris-HCl (pH 6.8)</td>
<td>1.26 ml</td>
<td>-</td>
</tr>
<tr>
<td>1.5M Tris-HCl (pH8.8)</td>
<td>-</td>
<td>3.75 ml</td>
</tr>
<tr>
<td>10.0% (w/v) SDS</td>
<td>50.0 μl</td>
<td>150.0 μl</td>
</tr>
<tr>
<td>Distilled H₂O</td>
<td>3.18 ml</td>
<td>6.25 ml</td>
</tr>
<tr>
<td>N, N, N’, N’-Tetramethyl-ethylenediamine (TEMED)</td>
<td>5.0 μl</td>
<td>7.5 μl</td>
</tr>
<tr>
<td>10.0% (w/v) Ammonium Persulfate (APS)</td>
<td>25.0 μl</td>
<td>75.0 μl</td>
</tr>
<tr>
<td>1.0% Bromophenol Blue</td>
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<td>-</td>
</tr>
<tr>
<td>Total volume</td>
<td>5.0 ml</td>
<td>15.0 ml</td>
</tr>
</tbody>
</table>
Appendix 3: LIVE DEAD Viability/Cytotoxicity Assay (CT1 Data)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Viable Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>HMEC</td>
<td>97.8 ± 0.87</td>
</tr>
<tr>
<td>MCF-7</td>
<td>95.6 ± 1.32</td>
</tr>
<tr>
<td>HSC-4</td>
<td>98.2 ± 1.89</td>
</tr>
<tr>
<td>HepG2</td>
<td>96.2 ± 0.54</td>
</tr>
</tbody>
</table>

Appendix 4: Wound Healing Assay (CT1 Data)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>% Migrated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>HSC-4</td>
<td>59.8 ± 2.7%</td>
</tr>
<tr>
<td>MCF-7</td>
<td>50.3 ± 5.0%</td>
</tr>
<tr>
<td>HepG2</td>
<td>12.23 ± 0.7%</td>
</tr>
</tbody>
</table>
### Appendix 5: Annexin-V Apoptosis Assay (CT1 Data)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Quadrant</th>
<th>% Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>HMEC</td>
<td>Viable</td>
<td>92.6±2.3</td>
</tr>
<tr>
<td></td>
<td>Early apoptotic</td>
<td>1.25±1.1</td>
</tr>
<tr>
<td></td>
<td>Late apoptotic</td>
<td>2.9±0.7</td>
</tr>
<tr>
<td></td>
<td>Non-viable necrotic</td>
<td>3.15±0.6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Viable</td>
<td>9.12±1.4</td>
</tr>
<tr>
<td></td>
<td>Early apoptotic</td>
<td>3.2±0.4</td>
</tr>
<tr>
<td></td>
<td>Late apoptotic</td>
<td>5.1±0.9</td>
</tr>
<tr>
<td></td>
<td>Non-viable necrotic</td>
<td>0.5±0</td>
</tr>
<tr>
<td>HSC-4</td>
<td>Viable</td>
<td>91.7±1.83</td>
</tr>
<tr>
<td></td>
<td>Early apoptotic</td>
<td>1.75±0.77</td>
</tr>
<tr>
<td></td>
<td>Late apoptotic</td>
<td>5.9±0.56</td>
</tr>
<tr>
<td></td>
<td>Non-viable necrotic</td>
<td>0.65±0.49</td>
</tr>
<tr>
<td>HepG2</td>
<td>Viable</td>
<td>92±2.12</td>
</tr>
<tr>
<td></td>
<td>Early apoptotic</td>
<td>1.1±0.28</td>
</tr>
<tr>
<td></td>
<td>Late apoptotic</td>
<td>4.8±0.84</td>
</tr>
<tr>
<td></td>
<td>Non-viable necrotic</td>
<td>2.1±0.14</td>
</tr>
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**Appendix 6: Cell cycle Analysis (CT1 Data)**

<table>
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<tr>
<th>Cell lines</th>
<th>Cell Cycle Phases</th>
<th>% Cells</th>
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</thead>
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<tr>
<td></td>
<td>0 h</td>
<td>12 h</td>
</tr>
<tr>
<td>HMEC</td>
<td>Sub G1</td>
<td>3.75±0.25</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>78.75±1.25</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>7.6±0.9</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>9.9±0.6</td>
</tr>
<tr>
<td>MCF-7</td>
<td>Sub G1</td>
<td>11.1±0.9</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>70.8±1.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>6.15±1.05</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>11.95±1.05</td>
</tr>
<tr>
<td>HepG2</td>
<td>Sub G1</td>
<td>0.2±0.25</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>72.81±1.2</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>24.8±1</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>2.42±0.45</td>
</tr>
<tr>
<td>HSC-4</td>
<td>Sub G1</td>
<td>0.6±0</td>
</tr>
<tr>
<td></td>
<td>G0/G1</td>
<td>73.4±2.25</td>
</tr>
<tr>
<td></td>
<td>S</td>
<td>24.0±2.25</td>
</tr>
<tr>
<td></td>
<td>G2/M</td>
<td>2.0±0</td>
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</table>
Appendix 7: HMQC spectrum of CT1
Appendix 8: HMBC spectrum of CT1