CHAPTER 1

INTRODUCTION

Cancer is a major health problem of global concern and is the second leading cause of death. Among a variety of cancers, colorectal cancer (CRC) is the third most prevalent malignancy in the world after lung and prostate/breast cancer. Colorectal cancer is estimated to account for 51690 new deaths to occur in the year of 2012 (Society, 2012). Colorectal cancer arises as a result of accumulation of genetic and epigenetic alterations that transform normal colonic epithelial cells into adenocarcinoma cells which are known as adeno-carcinoma sequence. The most prominent genes involved in these alterations include APC, K-ras, p53, c-myc, DNA mismatch repair genes, BRAF, PIK3CA and PTEN. Accumulation of these alterations promotes the outgrowth and results in clonal expansion of tumor cells (Fearon, 2011; Grady and Carethers, 2008).

Apoptosis or programmed cell death is essential in regulating tissue development and homeostasis which is characterized by a series of morphological and biochemical changes such as nuclear condensation, DNA fragmentation, membrane blebbing, phosphatidylserine externalization, loss of mitochondrial membrane potential and others (Fleischer et al., 2006; Wyllie, 1997). There are two focal apoptotic machineries which consist of death receptor or extrinsic pathway and mitochondrial or intrinsic pathway. For extrinsic pathway, it involves ligation or oligomerization of death ligand and death receptor (Yoon and Gores, 2002), whereas the mitochondrial-mediated pathway is activated by diverse stimuli such as growth factor withdrawal, DNA damage reagent, reactive oxygen species (ROS), UV and gamma radiation (Li-Weber, 2010; Pradelli et al., 2010). A loss of mitochondrial membrane potential results in translocation of proapoptotic Bax to mitochondria and the release of cytochrome c into cytosol. The extrinsic pathway leads to activation of caspase-8 whereas the intrinsic pathway leads to
activation of caspase-9, and followed by activation of the executioner caspase-3.
Numerous studies have reported that oxidative stress as a result of excessive production of reactive oxygen species (ROS) and depletion of glutathione level contribute to the initiation of apoptotic signaling (Ka et al., 2003; Tan et al., 1998).

Perturbation of apoptosis in cancer cells would eventually favor tumor progression (Whitfield, 2009). Hence, apoptosis is a relevant target for cancer therapies that can be exploited for the development of potential therapeutic drugs (Fesik, 2005). To date, there are a number of cancer therapies available and tailored to improve patient overall survival. However, all these therapies cause detrimental and adverse side effects. Therefore, developing high therapeutic efficacy and least palliative of plant-based anticancer agents has received great attention (Xie et al., 2011). Some of these anticancer agents possess the potential to trigger cell cycle arrest and/or cell demise by apoptotic or non-apoptotic mechanisms comprising senescence, necrosis, autophagy and mitotic catastrophe (Brown and Attardi, 2005; Okada and Mak, 2004).

There is an urgent need to develop new approaches based on natural products as chemotherapeutic agents, and to endow more effective and safer therapies. In recent times, it has been found that the prevention of diseases such as cancer, diabetes mellitus, inflammation and hypertension could be closely related with the ingestion of vegetables, fresh fruits and plants which contain rich horde of natural antioxidants (Abdelwahed et al., 2007). Rinds of the tropical fruits such as mangosteen, rambutan, mango, pomegranate and others are found to contain large variety of substances possessing antioxidant properties (Ling et al., 2010; Palanisamy et al., 2011; Seeram et al., 2005; Thitilertdecha et al., 2010), anti-hyperglycemic properties (Palanisamy et al., 2011) and anti-cancer properties (Larrosa et al., 2006; Moongkarndi et al., 2004; Seeram et al., 2005). Thus, the rind _N. ramboutan-ake_ (known commonly in Peninsular Malaysia as pulasan), which has shown strong antioxidant activities in our previous study (data not
shown) has become the target of interest in our current research. *N. ramboutan-ake* is a tropical fruit, in which the rind is normally discarded as waste after consumption. To the best of our knowledge, the effect of *N. ramboutan-ake* on human cancer cell lines has not been reported. Thus, the main objective of this study was to investigate the cytotoxicity, the apoptosis inducing effects of NRAF and elucidation of a possible apoptotic mechanism in HT-29 human colorectal adenocarcinoma cells.

The specific objectives of this study were:

1) To investigate the *in vitro* cytotoxic activity of the crude extract and fractions of *Nepheium ramboutan-ake* rind against selected cancer cell lines (HT-29, HCT-116, Ca Ski and MDA-MB231) using MTT assay.

2) To investigate the apoptosis inducing effects of *N. ramboutan-ake* assessed by Hoescht 33342 and Propidium Iodide (PI) staining for detection of morphological changes and flow cytometry analysis for Annexin-V and JC-1 binding.

3) To evaluate the modulation of intracellular oxidative homeostasis exerted by aqueous fraction through the detection of the intracellular glutathione (GSH) level and intracellular reactive oxygen species (ROS) level.

4) To elucidate the apoptotic mechanisms of the aqueous fraction through the expression of Bax and Bcl-2 protein and caspase activities (caspase-3/7, -8 and caspase-9).
CHAPTER 2
LITERATURE REVIEW

2.1 Cancer

The literature often defines cancer simply as deregulated proliferation of abnormal cells or inability of cells to undergo apoptotic cell death (Steller, 1995; Thompson, 1995). Cancer is a major health problem in all over the world and is the second leading cause of death after heart disease (Society, 2012). Cancer cells are formed as a consequence of the loss of normal cell regulatory mechanisms that control growth and multiplication. Various factors such as internal factors (inherited mutations, hormones, mutations and immune conditions) and external factors (diet, tobacco, radiation and environmental) are able to cause cancer (Anand et al., 2008). All these factors especially dietary habits, smoking, alcohol consumption profoundly affect and trigger the development of cancer through mutation or interfere with normal cell differentiation. The process of cancer development can be divided into multiple steps. Transformation starts with DNA damage and mutations in the initiation phase which is irreversibly altered. This is followed by growth of transformed cells in the promotion stage, leading to malignant growth and invasion in the progression stage (Pan et al., 2011). Further progression steps occur upon clonal expansion of the initial cells and accumulation of a sufficient number of mutations and epigenetic alterations to acquire growth stimulus-independency and resistance to growth inhibitors and apoptosis, ultimately leading to an unlimited replicative potential. The acquisition of the ability to invade the surrounding tissue defines the malignant character of cancer cells, while the process through which cells can migrate to distal organs and acquire the potential to form metastasis represents the attainment of a full malignant cancerous phenotype (Xie et al., 2011).
2.2 Colorectal cancer and carcinogenesis

The colon comprises of the majority of the large intestine and the last few inches of the rectum. Colorectal cancer is a disease in which cancer cells evolve from the glandular tissues of the colon or rectum. Approximately 90% of colorectal cancers are derived from benign adenomatous lesions which are estimated to take 5–15 years to evolve into invasive cancer. The colorectal cancer commonly spreads from the colon or the rectum to other parts of the body such as the liver or the lungs. Colorectal cancer results in significant morbidity and mortality in worldwide. On a global scale, it is the third most common malignant neoplasm among various cancers in adult men and women according to the statistics from American Cancer Society (Society, 2012). The development of colorectal cancer begins as a noncancerous polyp which is a growth tissue in the lining of the colon or rectum. These polyps develop into a cancerous cell known as adenomas (Stewart et al., 2006).

Contributory agents and mechanisms in CRC include dietary and lifestyle factors and inherited and somatic mutations. Among the most significant dietary and lifestyle risk factors for CRC appear to be a diet rich in unsaturated fats and red meat, total energy intake, excessive alcohol consumption, and reduced physical activity (Huxley et al., 2009; Potter, 1999; Slattery, 2000).

Colorectal cancers develop from normal colonic epithelium to hyper proliferative epithelium and then further on into adenoma, carcinoma and eventually metastasis, invasion and angiogenesis through accumulation of genetic and epigenetic alterations according to the model of Fearon and Vogelstein (Fearon, 2011; Willett, 1995). The process of development of colorectal cancer from normal colonic epithelium to carcinoma through adenoma as intermediate is often known as adenoma-carcinoma sequence. The sequential process of gene mutations and epigenetic alterations is
contributed to initiation and progression of benign adenomas to malignant adenocarcinoma because these mutations affect signaling pathways that regulate hallmark behaviors of cancer. Genetic alterations include gene mutations and amplification whereas epigenetic alterations include aberrant DNA methylation and chromatin modifications. Vital genes involved in this process include adenomatous polyposis coli (APC), K-ras, CTNNB1, Tp53, TGFBR2, mismatch repair (MMR) genes and others (Figure 2.1) (Fearon, 2011; Grady and Carethers, 2008; Grady and Markowitz, 2002).

Figure 2.1: The development of colorectal cancer.

Accumulation of genetic and epigenetic alterations causes genomic instability to occur. There are two types of genomic instability which are chromosomal instability and microsatellite instability that emerge prior to the progression to malignancy but after adenoma formation. For chromosomal instability, it results in chromosome translocations or alterations in number to occur such as aneuploidy and chromosomal gain. However, in some cases, the tumors display microsatellite instability as a result of inactivation of mutation mismatch repair system which its roles are to detect and repair
the basepair mismatches and the occurrence of frameshift mutations in microsatellite throughout the genome (Lao and Grady, 2011).

The epigenetic mechanisms have a role in cancer development that comprise of four main categories which are DNA methylation of cytosine bases in CG-rich sequences (CpG islands); post-translational covalent histone modification (proteins that form the nucleosomes) and non-covalent mechanisms such as incorporation of histone variants; nucleosome positioning and non-coding RNAs including microRNAs (miRNAs) (Sharma et al., 2010). For instance, the anomalous hypermethylation of genes appear to be usual molecular mechanism for silencing tumor suppressor genes and as a result of cancer formation through the transcriptional repression of these genes (Lao et al., 2011). These accumulations of genomic and epigenetic instability will contribute to the development of colorectal cancer. As a consequence, these will trigger alterations in the signaling pathway by inducing apoptosis and eventually progresses through either inactivation of tumor suppressor genes or activation of oncogenes.

2.3 Treatments for colorectal cancers

Over decades, the standard cancer therapy approaches available to improve patient prognosis include chemotherapy, radiotherapy and surgery (polypectomy, resection and colorectomy). Surgery often is the most preferable approach for colorectal cancer. Conversely, most of the post surgery patients with colorectal cancer oblige adjuvant therapy. Depending on the grade of cancer, patients may also receive chemotherapy before (neoadjuvant) or after treatment (adjuvant). In the late 1990’s, a six month course of chemotherapy for instance, 5-fluoruracil (5-FU) / folinic acid (FA), become a standard adjuvant therapy to the patients that underwent surgery which is also beneficial to the patients’ overall survival (Dube et al., 1997; Haller et al., 2005). Until recently, a
finding reported that oral capecitabine adjuvant therapy is more effective than the intravenous 5-FU/FA (Twelves et al., 2012).

Oxaliplatin (Eloxatin) interferes with DNA synthesis, and is commonly used to treat advanced colorectal cancer. Oxaliplatin is often co-treated with 5-FU and folinic acid which is referred to as FOLFOX. In addition, combinations of the agent irinotecan (Campto) with 5-FU and FA (known as FOLFIRI) are tailored for the first line treatment of colorectal cancer or alone as second line therapy (Cao et al., 2006; Goldberg et al., 2007). Irinotecan is a topoisomerase I inhibitor and prevents DNA decatenation or ‘unwinding’. The type I and type II topoisomerases (topo I, topo II) are enzymes required for altering DNA topography and play a particularly important role in the cell cycle (Nitiss, 2009). The topo I and topo II enzymes induce temporary single-strand breaks, and double-strand breaks respectively to alter DNA structure. Irinotecan can form stable complexes with DNA and topo I, and during DNA replication these stable complexes can obstruct advancing replication forks, resulting in double-strand breaks and inhibition of DNA replication (Liu et al., 2000). Irinotecan and topotecan which are used in the treatment of colon cancer as well as cervical, ovarian and small cell lung cancer are synthetic analogues of the natural agent camptothecin. Despite all the sophisticated strategies, these treatments come with severe side effects, escalating toxic effects and occurrence of resistance constraint their effectiveness (Alcindor and Beauger, 2011; Goldberg et al., 2004).
2.4 Modes of cell death

Cell death can occur in many forms encompassing apoptosis, necrosis, autophagy and other modes of cellular demise. Decades ago, cell death field has drawn great attention and exploitation of these led to insightful impacts in aging, cancer, degenerative diseases and development (Fulda et al., 2010; Lockshin and Zakeri, 2001). The most prevalent machinery to execute cancer cell death is through the induction of apoptosis. Consequently, there is an emerging interest to utilize chemotherapeutic natural derived compounds in the treatment of cancers through induction of apoptosis pathway. These naturally derived compounds have been widely reported to mediate diverse machineries against cancer which comprise of cell cycle progression arrest, immune response, induction of apoptosis and DNA damage.

2.5 Apoptosis and cancer

The term apoptosis (coined by Kerr, Wyllie and Currie from the Greek word meaning “dropping off”, as in leaves dropping from a tree is now applied to explain a type of cell death (Kerr et al., 1972). Apoptosis or programmed cell death is pivotal in the regulation of homeostasis and tissue development, conjointly it is also implicated in a wide array of pathologies and currently known to be one of the essence of multi-step carcinogenesis and therapy resistance (Garber, 2005; Sanmartin et al., 2005). It is responsible in removing redundant or abnormal cells and maintain constant amount of cells. The amalgamation of a series of molecules which comprise of signal molecules, receptors, enzymes and gene regulating proteins are required for the initiation and execution of apoptosis. Among them, the caspase activation signaling pathway play a crucial role at the center of the apoptosis machinery which is regulated by a diverse of molecules including the inhibitor of apoptosis protein, Bcl-2 family proteins and calpain,
Caspases are responsible directly or indirectly for the morphological and biochemical events that characterized as classical apoptosis (Reed et al., 2000). Several cellular pathways culminate in the activation of caspases and apoptosis. Biochemical events that involved in apoptosis is characterized by alterations of mitochondrial membrane potential, DNA fragmentation, externalization of phosphatidylserine and caspase activation whereas for morphological events include chromosomal condensation, cell shrinkage and membrane blebbing. All this morphological and biochemical events will occur via the activation of a cell-intrinsic suicide mechanism (Fleischer et al., 2006; Wyllie, 1997). This cell death process can be categorized into four phases including initiation, execution, disintegration, and elimination. In the beginning, cell shrinkage occurs followed by chromatin condensation (pyknosis) in the nucleus and membrane blebbing at the cell membrane. Morphologically, other organelles remain intact. Subsequently, nucleus disintegrates into fragments (karyorrhexis) which appear in the late stage of apoptosis (Wong and Kadir, 2012). Finally, apoptotic bodies arise as membrane-bound particles. Cells eventually disintegrate into a phase of no return. Phagocytic cells or macrophages in the neighborhood rapidly engulf the apoptotic bodies. Thereby, release of the intracellular debris of the apoptotic bodies trigger inhibition on the inflammatory reactions (Elmore, 2007; Majno and Joris, 1995).

Accumulation of genetic alteration during carcinogenesis is due to impairment of normal growth inhibition by increased cell growth and by inhibition of apoptosis, resulting in clonal expansion of tumor cells (Kelloff et al., 2000). Therefore, the equilibrium between cell growth and apoptosis in colonic mucosa is utmost important for the development and maintenance of normal cells and cancer cells. The perturbation in the balance between cell proliferation and apoptosis in colonic mucosa has pathologic consequences and results an escape from the normal homeostasis of cell number
towards the progression of cancer cells (Hao et al., 1998; Whitfield, 2009). Inhibition of proliferation and increase in apoptosis of these aberrant cells are the key mechanisms to prevent colon cancer. Above and beyond, apoptosis also presents as a window that can be exploited for the development of potential therapeutic drugs. Apoptotic machineries might be significantly altered in cancer cells with respect to normal cells, and these differences might present a therapeutic window that can be exploited for the development of useful anticancer drugs. Moreover, cancers that possess alterations in proteins involved in cell death signaling are often resistant to chemotherapy and are more difficult to treat using chemotherapeutic agents that primarily work by inducing apoptosis (Fesik, 2005).

As one of the most challenging tasks concerning cancer is to induce apoptosis in malignant cells, researchers increasingly focus on natural products to modulate apoptotic signaling pathways (Reuter et al., 2008). Decades ago, numerous researches have established a pledging future for apoptosis based cancer therapies (Fischer et al., 2007). In this scenario of discovery of small molecule modulators of apoptosis, considerable effort is being aimed at improving the prototypic drugs and replacing them with small molecule organic compounds, which could set the stage for future therapeutics (Fischer and Schulze-Osthoff, 2005). For example, curcumin, a natural compound isolated from the plant *Curcuma longa*, has chemopreventive properties, which are mainly due to its ability to arrest cell cycle and to induce apoptosis. However, curcumin is still ongoing in the phase II clinical trial to develop as a drug (Carroll et al., 2011).
2.6 Apoptotic pathways

Apoptosis is a highly regulated mechanism by which cells undergo cell death through a series of activation. Briefly, there are two focal apoptosis machineries which are the extrinsic and the intrinsic pathways (Ziegler and Kung, 2008). Both intrinsic and extrinsic pathway initiate the process leading to cellular destruction, characterized by DNA fragmentation, loss of mitochondrial membrane potential and release of apoptotic substrates (Fleischer et al., 2006).

2.6.1 Extrinsic pathway

The extrinsic pathway is potentiated by the oligomerization between specific ligands (CD95-L/ APO-1-L/ Fas-L) and surface receptors, such as CD95/Fas/Apo1, tumor necrosis factor receptor 1 (TNFR1), TNF receptor 2 (TNFR2) and death receptors 3–6 (DR3–6), which are directly coupled to the cell death machinery (Degterev et al., 2003; Klein et al., 2005; Peter and Krammer, 2003). Binding to the receptor induces receptor oligomerization by ligands, followed by the binding of Fas-associated death domain (FADD) adapter protein and cause FADD aggregation and the appearance of death effector domain (DED). Procaspase 8 interacts with the exposed DED of FADD adapter protein through the DEDs and results in the formation of massive molecule complex known as death-induced signaling complex This programmed cell death is mediated through either intrinsic or extrinsic pathway where both initiate the process leading to cellular destruction, characterized by DNA fragmentation, loss of mitochondrial membrane potential and release of apoptotic substrates (Fleischer et al., 2006). or initiator apoptosome. The augmentation of regional concentration of zymogens is postulated to be the grounds for caspase activation which means as high concentration of procaspase-8 will be auto activated into caspase-8. Further on, a diverse of
downstream pathways is activated. One of the pathways is through the activation of caspase-8 which then directly recruits effectors caspases-3 and -7 (Klein et al., 2005). In contrast, another pathway indirectly activates caspase-3 through truncating Bid (a pro-apoptotic Bcl-2 family member) into active tBid. tBid will execute the downstream activation mitochondria signaling which lead to the release of cytochrome c and induction of apoptosis as shown in Figure 2.2 (Wei et al., 2000).
Figure 2.2: Schematic diagram illustrated key molecular events which are death receptor-mediated procaspases- activation pathway. Adapted from Krakstad and Chekenya, 2010.
2.6.2 Intrinsic pathway

The intrinsic pathway can be indirectly executed through the caspases either by activating the cell death receptor pathway or by exposing to a plethora of stimuli such as cell detachment, DNA damage, hypoxia, cellular distress and cytotoxic drugs, which act intracellularly (Degterev et al., 2003; Reuter et al., 2008). Activation of the intrinsic pathway occurs through the mitochondria which is the target of all these stimuli and acts as the key regulator of the Bcl-2 family members to propagate the apoptotic signal (Danial et al., 2004). Bcl-2, Bcl-w and Bcl-xL are anti-apoptotic Bcl-2 proteins, while pro-apoptotic proteins comprise of Bax, Bad, Bim and Bid (Debatin, 2004; O’Neill et al., 2004). An excess of pro-apoptotic over anti-apoptotic signals induces mitochondrial outer membrane permeabilization (MOMP), which prompts the opening of mitochondria permeability transition pores and the release of a set of proteins including Smac/Diablo, cytochrome c, Omi/HtrA2, AIF and endonuclease G from the mitochondrial intermembrane space to the cytosol. These apoptogenic proteins in the cytosol then execute the cell death. For instance, in the presence of cytochrome c, it binds to the terminal region of adaptor molecule apoptotic protease-activating factor 1 (Apaf-1) and dATP, which then recruits the initiator procaspase-9 to create a protein complex known as apoptosome, eventually stimulates the executioner caspase-3 and caspase-7 (Green, 2005). Subsequently, caspase-3 cleaves apoptotic substrates to trigger cellular and biochemical events of apoptosis. Besides, procaspase-9 will be subsequently activated by caspase-3 and a positive feedback regulation mechanism is triggered. Smac/DIABLO and Omi/HtrA2 complete the caspase cascade activation via deactivating the inhibitor of apoptosis proteins. Smac binds to the inhibitor of apoptosis proteins and deactivates them, inhibiting the inhibitor of apoptosis proteins from halting the apoptosis. Besides, the key substrates which are cleaved by caspases are PARP, lamin, fodrin and histone leading to the characteristic morphological alterations.
Caspase-activated deoxyribonuclease which is a magnesium-dependent endonuclease can be activated by caspases. Caspase-activated deoxyribonuclease is essential for DNA degradation that is involved in apoptosis pathway. Caspase-activated deoxyribonuclease resides in the nucleus which complexes with inhibitor of caspase-activated deoxyribonuclease in normal cells. In apoptosis, caspase-3 will cleave inhibitor of caspase-activated deoxyribonuclease and release caspase-activated deoxyribonuclease from the complex which eventually leads to DNA fragmentation (Enari et al., 1998). Lamin A and fodrin are vital elements of nuclear and cytosolic skeleton with respectively. Caspase cleaved lamin will give rise to the chromatin condensation and the disintegration of the nuclear membrane. In contrast, appearance of apoptotic body formation is resultant of the cleavage of fodrin by caspase (Fan et al., 2005). Finally, the cell undergoes apoptosis through a series of changes (Figure 2.3).
Figure 2.3: Extrinsic and intrinsic pathways which in turn initiate caspases activation are illustrated. Adapted from Circu and Aw, 2012.
2.7 Redox homeostasis and cancer

Redox homeostasis is fundamental to cell survival. However, redox alterations play a pivotal role which emerges as one of the ways in combating cancer. Reduction/oxidation in short is termed as redox. Oxidation depicts the loss of electrons whereas reduction depicts the gain of electrons (Rigas and Sun, 2008). Redox homeostasis is regulated by the balance between free radicals production rate and antioxidant homeostasis.

2.7.1 Oxidative stress

Oxidative stress is referred as an excess of reactive oxygen species generated as the consequence of an imbalance between production of free radicals which is known as pro-oxidant and biochemical elimination or defense mechanism which is known as antioxidant homeostasis (Barnham et al., 2004; Reuter et al., 2010). Oxidative stress has been implicated in a wide array of neurodegenerative disorders including Alzheimer’s disease and Parkinson’s disease as well as a host of pathologies such as cancer, diabetes mellitus, arthritis, cardiovascular, post-ischemic perfusion injury autoimmune disorders and chronic inflammatory diseases (Rigas et al., 2008).

The source of energy ATP depends greatly on the redox chemistry, as it is maneuvered by the alteration of free energy which closely related to electron or hydrogen transfers (Frein et al., 2005). Under normal condition, redox state is maintained by the equilibrium between ROS production rate and efficient elimination mechanism by antioxidants. ROS are free radicals, other unpaired electrons molecules and oxidants such as $O_2^{-}$ and $H_2O_2$. In contrast, antioxidants such as reduced glutathione (GSH), glutathione peroxidase, superoxide dismutase or dietary-derived antioxidant, vitamin E, vitamin C play key roles to combat the excess formation of free radicals (Halliwell,
2011). For example, superoxide radicals are scavenged by superoxide dismutase, resulted in formation of hydrogen peroxide, which is detoxified by catalase or glutathione peroxidases. However, rise in intracellular ROS level or decrease in antioxidant detoxification mechanism leads to the disruption of redox homeostasis which also known as oxidative stress. Mounting findings reported that excess oxidative stress eventually acts as an inducer to damage the cell structures such as DNA, proteins and lipids either via necrosis or apoptosis (Mieyal et al., 2008; Zamzami et al., 1996). For years, there remain paradoxical statements that oxidative stress may bring about the survival or the death of the cells. Interestingly, accumulating findings have reported that oxidative stress may cause initiation of cancer, proliferation, grow arrested or cell death trigger (Ekshyyan and Aw, 2005). In cancer, redox alterations are very intricate and noteworthy because of the plethora of factors that are associated with the stress response and redox regulation. Therefore, there is an emerging interest and attention raised on oxidative stress to become one of the targets as anticancer therapy.

2.7.2 Reactive Oxygen Species (ROS)

ROS is generally a term for oxygen containing free radicals. Some examples of ROS are superoxide anion, hydroxyl radicals, hydrogen peroxide and singlet oxygen which act as donors or receivers of free electrons. The organelles or components that are involved in continuous production of reactive oxygen and nitrogen species are the mitochondria (O$_2^-$, H$_2$O$_2$, and OH$^-$) of most cells, cytochrome P450 (O$_2^-$ and H$_2$O$_2$), macrophages (O$_2^-$, H$_2$O$_2$, and NO$^-$), and peroxisomes (H$_2$O$_2$) (Genestra, 2007; Klaunig and Kamendulis, 2004). ROS are central players in oxidative stress and they are primarily produced as byproducts through cellular metabolism, mitochondrial respiratory chain (electron transport chain system) which take place in the mitochondria. Other pathways
that contribute to the formation of ROS are inflammatory signaling and endoplasmic reticulum stress. During mitochondrial oxidative metabolism about 5% of oxygen is primarily converted into $\text{O}_2^-$, whereas 95% of it is reduced to water. Production of ROS can be via numerous routes depicted in Figure 2.5 (Valko et al., 2007).

Concurrently, generation of ROS is deleterious and cause adverse effects to mitochondria. Continuous supply of ATP from mitochondria is of great importance for cellular metabolism. Hence, any damage that results in impairment of the respiratory chain function might also have great impact on cell viability. In order to protect cells from oxidative damage, mitochondria contain a highly structured biochemical defense mechanism which is antioxidants principally participating to neutralize the ROS stimulated-damage effect and detoxify ROS. Nevertheless, ROS has been reported to play dual role in biological system which can be beneficial as well as deleterious to cell (Valko et al., 2004).
Apoptosis is executed by caspases in response to intracellular damage or physiological cues. Caspase cascades have been delineated that can be activated via death receptor-mediated pathway or triggered by multiple stimuli such as stress, DNA damage and microtubule disruption (Abraham and Shaham, 2004). The ROS stress stimuli affect the mitochondria which in turn lead to the execution of apoptosis. In response to stress...
stimuli, mitochondria-apoptotic signaling pathway is activated and translocates bcl-2 family proteins, manipulates the permeability of mitochondrial membrane and subsequently cytochrome c release or through activation of cell surface death receptors (Fan et al., 2005). As delineated above, ROS which appears to be important regulatory signals are involved in cell death mechanism.

According to the reviews, only low concentrations of ROS are produced in normal cells thus can be effectively neutralized by antioxidants. On the contrary, a modest amount of intracellular ROS initiates cell differentiation and promotes cell proliferation (Boonstra and Post, 2004; Schafer and Buettner, 2001) and mediates a diverse signal transduction mechanism such as regulating enzymes activity, mediating inflammation by triggering cytokine production and removing pathogens (Trachootham et al., 2009). In addition, elevation of intracellular ROS level which can cause oxidative damage to many vital components such as lipids, proteins and DNA, promotes the initiation, invasion, progression and metastasis of cancer by modulating the DNA sequence through point mutations, deletions, amplifying genomic instability and rearrangements and eventually leading to the activation of several oncogenes and tumor suppressor genes (Murakami et al., 2000; Ott et al., 2007; Raj et al., 2011). Multiple lesions such as modification on pyrimidines and purines bases and single and double-stranded breaks result in DNA damage initiated by ROS (Gromer et al., 2004; Mates et al., 2010). Other than DNA, lipid, polyunsaturated fatty acid, phospholipids are one of the ROS target. ROS will initiate lipid peroxidation during which peroxyl radicals are rearranged via cyclisation to produce aldehyde product such as malondialdehyde. Later, malondialdehyde can conjugate with DNA, lipid and protein to form malondialdehyde adducts as depicted in Figure 2.4 (Valko et al., 2006). In addition, ROS also elicits modification on the conformation and activity of proteins as well as result in protein-protein linkages and fragmentation. Particularly few side chains of amino acid residues of proteins are more
vulnerable to oxidation by ROS namely tryptophan, cysteine and methionine (Adler et al., 1999).

Excess of intracellular ROS beyond the antioxidant detoxification capacity can be lethal to cells. Hence, such circumstance will render the cells either more susceptible to be damaged or irreversibly damaged and eventually apoptosis occurs (Behrend et al., 2003; Pelicano et al., 2004; Wu, 2006). Collectively, these findings suggest that oxidative stress in cancer cells holds promising potential to be exploited as novel and selective chemotherapeutic agents. In the early 1960’s, Bereneis and colleagues reported that procarbazine is oxidized in solution to form ROS such as $\text{H}_2\text{O}_2$ with resultant damage to DNA (Berneis et al., 1963), and they showed that procarbazine was synergistic with radiation through production of free radicals (Berneis et al., 2004). In addition, several preclinical studies reported that chemotherapeutic agents including cisplatin and etoposide which are commonly used have been proven to trigger apoptosis by formation of ROS (Kurosu et al., 2003; Muller et al., 1998). Hence, one of the cancer treatment modes is to manipulate ROS levels without instigating significant toxicity to normal cells (Schumacker, 2006).
2.7.3 Antioxidants

Antioxidants are exploited to remove the free radicals and scavenge ROS which causes oxidative stress. Generally, antioxidants are categorized as exogenous (natural dietary or synthetic) and endogenous compounds which are produced in human body. Antioxidants can neutralize the free radicals by donating their own electrons. In human body, antioxidants may possibly perform in three distinct modes which are preventing as well as maintaining generation of reactive species to a minimum for example, desferrioxamine, scavenging reactive species either by using catalytic or non-catalytic molecules such as ascorbic acid, alpha-tocopherol and lastly repairing damaged target molecules including glutathione (Kohen and Nyska, 2002). Mitochondria are highly supplemented with antioxidants comprising of glutathione (GSH), superoxide dismutase and glutathione peroxidase, which occur on dual sides of their membranes in order to reduce oxidative stress in the organelle (Cadenas and Davies, 2000).

Paradoxically, bountiful studies have reported that high dosage of antioxidants does not drive the survival of the cell but possess detrimental effects on human health (Halliwell, 2011). Basically, antioxidant system in human body is sorted into enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants included catalase, thioredoxin reductase, glutathione peroxidase as well as superoxide dismutase whereas non-enzymatic antioxidants comprise of direct acting antioxidants, which are incredibly imperative in defense against oxidative stress. Most of non-enzymatic antioxidants comprise of Vitamins C and E, ascorbic and lipoic acid, polyphenols and carotenoids, derived from dietary sources. A marginal of these molecules are synthesized and found in the cell itself. Indirectly acting antioxidants mostly include chelating agents and bind to redox metals to prevent free radical generation (Gilgun-Sherki et al., 2001).
2.8.4 Glutathione (GSH)

Glutathione (GSH), L-γ-glutamyl-L-cysteinyl glycine, which is an important antioxidant to avert apoptosis and oxidative stress. Cellular GSH production is through ATP dependent γ-glutamyl cycle catalyzed by γ-glutamate-cysteine ligase (GCL) and glutathione synthetase (GS) with the presence of glycine, glutamate and cysteine amino acids (Meister and Tate, 1976). Several pathways including de novo synthesis from precursor amino acids, (cysteine, glutamate and glycine), regeneration from GSSG by glutathione reductase (GR), and utilization of exogenous GSH sources maintain the intracellular GSH homeostasis (Circu and Aw, 2012). GSH is oxidized into glutathione disulfide, GSSG, resulting in intracellular redox imbalance as reflected in a decreased GSH-to-GSSG ratio, a condition often associated with oxidative stress.

GSH is involved in cellular signaling, regulation and redox activation of transcription factors, sensitivity against xenobiotics and cell protection against free radicals (Jones et al., 2000). GSH has great reducing power, with the ability to donate electrons to free radicals thereby acting as antioxidant. Tumor mitochondria are known to possess excessive amount of GSH (Ortega et al., 2011). Modulation of the GSH system has been well studied in vitro. Depletion of GSH has been demonstrated to sensitize tumor cells to oxidative cytolysis (Arrick and Nathan, 1984; Arrick et al., 1982; Nathan et al., 1981). Moreover, collective findings demonstrated that depletion of GSH precedes the onset of apoptotic signaling by rendering the susceptibility of cell to xenobiotics (Jones, 2010).

Depletion of GSH is usually coupled with the increment of ROS level as well as corresponds to mitochondrial dysfunction which usually linked to mitochondrial membrane potential. To produce and maintain GSH level, ATP energy supply is needed as the synthesis is ATP-dependent pathway. Therefore, dysfunction of mitochondria
will perturb mitochondrial respiratory chain system including deficiency of ATP energy. Concomitantly, GSH turnover was affected due to impairment in producing ATP. Numerous findings reported that depletion of the GSH pool was associated with reduction of mitochondrial respiratory complexes activity, aggrandization of the intracellular ROS level, collapse of mitochondrial membrane potential and discharge of apoptogenic factors (Ghosh et al., 2005). Thus, tipping the balance of cellular redox via pharmacologic exploitation in favor of elevation in intracellular ROS and/or depletion of protective reducing metabolites (such as GSH) may result in oxidative stress and followed by apoptosis induction within cancer cells (Engel and Evens, 2006).

2.8 Caspases

Caspases are designated as interleukin-1β-converting enzyme family of proteases as well as aspartate-specific cysteine proteases which are highly homologous to Caenorhabditis elegans cell death gene CED-3. So far, fourteen caspases have been discovered, which all share common catalytic triad residues consisting of the active site Cys285, which resides in the conserved pentapeptide active site ‘QACXG’, His237 and the backbone carbonyl of residue 177 (X can be R, Q or D) (Fan et al., 2005). Another striking characteristic of caspase family is its specificity for substrate cleavage after an Asp residue. Caspases are synthesized as zymogens which are catalytically inactive. Zymogens (procaspases) consist of three domains which are an N-terminal pro-domain of variable lengths followed by a large (p20) and a small subunit (p10). In order to activate the procaspases, apoptotic stimulus is necessary to trigger proteolytic cleavage or autocatalysis. During activation, each polypeptide chain is cleaved and produces a large and a small subunit, which then form a dimer. The mature enzyme oligomerized to form a heterotetramer which consists of two p20/p10 heterodimers and two active sites. The N-terminal of the prodomain in procaspases contains a highly diverse structure
required for caspase activation; and they are all capable of autoactivating as well as activating other caspases, to produce a heterodimer with a big and a small subunit, and two heterodimers forming an enzymatic active heterotetramer (Launay et al., 2005). All the procaspases of the inflammatory mediators, apoptosis executioner caspases and apoptosis activator caspases possess long prodomains in procaspases. The long prodomain consists of the death effector domain (DED) in procaspases-8 and -10, or the caspase recruitment domain (CARD) in procaspases-1, -2, -4, -5, -9, -11, and -12 (Figure 2.5). There are three subfamilies of caspases with different roles namely, apoptosis activator (Caspase-2, -8, -9 and -10) apoptosis executioner (Caspase-3, -6 and -7) and inflammatory mediator (Caspase-1, -4, -5, -11, -12, -13 and -14) which is encapsulated in Table 2.1.

Figure 2.5: Schematic illustrated the caspases with the domains such as CARD, DED which involved in recruitment and activation, large (p20) and small (p10) catalytic subunits. Group I represented as inflammatory caspases; group II represented as apoptosis initiator caspases; group III represented as apoptosis effector caspases. Adapted from Rupinder et al., 2007.
Table 2.1: Table illustrated subfamily members of caspases. Adapted from Fan et al., 2005.

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Role</th>
<th>Members</th>
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</thead>
<tbody>
<tr>
<td>I</td>
<td>Apoptosis initiator</td>
<td>Caspase-2, Caspase-8, Caspase-9, Caspase-10</td>
</tr>
<tr>
<td>II</td>
<td>Apoptosis executioner</td>
<td>Caspase-3, Caspase-6, Caspase-7</td>
</tr>
<tr>
<td>III</td>
<td>Inflammatory mediator</td>
<td>Caspase-1, Caspase-4, Caspase-5, Caspase-11, Caspase-12, Caspase-13, Caspase-14</td>
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Caspase activation is important in the execution of apoptosis. Generally, there are two pathways which involve the caspase activation. One of the pathways is the death signal-induced, cell surface death receptor-mediated pathway while another is the stress-induced, mitochondria-mediated pathway (i.e. a caspase-9-dependent pathway) (Figure 2.6). Death receptor mediated pathway, activation of caspase-8 following its recruitment to the DISC is a critical event that transmits the death signal. These signals were initiated by the complex of the cell death signals and the corresponding death receptors such as FasL complexes with Fas or TNF complexes with TNFR-1. These will further recruit and activate the pro-caspase-8 or pro-caspase-10. Activated caspase-8/ -10 can either directly activate executioner caspase-3/ -6/ -7 and lead to apoptosis induction by the apoptotic substrates or indirectly activates caspase-3/ -6/ -7 through cleavage of Bid and inducing cytochrome c release from the mitochondria and activation of initiator caspase-9 (Fan et al., 2005).
On the contrary, mitochondrial-mediated pathway is mediated by cellular stress. This is associated with caspase-cascade activation induced by the formation of a multimeric Apaf-1cytochrome c complex that is completely functional in recruiting and activating procaspases-9. Proapoptotic proteins in the cytosol will be activated, which subsequently evoke the opening of mitochondrion permeability transition pores (MPTPs) in the presence of cellular stress (e.g. DNA damage). Consequently, cytochrome c localized in mitochondria will be released into the cytosol. When cytosolic dATP (deoxyadenosine triphosphate) or ATP occurs, apoptotic protease activation factor-1 (Apaf-1) and procaspases-9 oligomerizes. Activated caspase-9 will further cleave and activate downstream caspases such as caspse-3, -6 and -7 (Budihardjo et al., 1999; Fan et al., 2005).
Figure 2.6: Caspase-dependent death receptor-mediated and mitochondrial-mediated apoptosis mechanism (Ashkenazi, 2008).
2.9 Bcl-2 protein family

Mitochondria are vital organelles involved in the apoptosis signal transduction pathway. Thus, these organelles operate either to accelerate or directly induce apoptosis by releasing key effector proteins including cytochrome c and other proapoptotic molecules. It has been shown that the apoptogenic activities of mitochondria are regulated by members of the expanding Bcl-2 proteins family (Tsujimoto et al., 1985). One of the pivotal regulatory factors in apoptosis is the members of the Bcl-2 family. Based on the functional and structural factors, the members can be classified into two groups. Group I proteins are anti-apoptotic proteins, including Bcl-2, A1/Bfl1, Mcl-1, Boo/Diva, Bcl-xL, NR-13, Bcl-w and Nrf3 in mammals (Fu and Fan, 2002; Gross et al., 1999; Milosevic et al., 2003). All of the proteins possess four short Bcl-2 homology (BH) domains which comprise of BH1, BH2, BH3 and BH4 (Figure 2.7). The most evident machineries of their anti-apoptotic functions is inhibiting proapoptotic proteins of the Bcl-2 family by complexing with them. Group II proteins are proapoptotic proteins, consisting of Bad, Bax, Bak, Bcl-xS, Bid, Bim, Bcl-rambo, Bik, Nip3Bok/Mtd, BNIP3, Hrk and Blk, in mammals (Milosevic et al., 2003). When apoptotic mechanism initiates, Bax and Bak, which are originally localized in the cytoplasm will translocate to the mitochondrial outer membrane. Bax and Bak will experience conformational alteration, oligomerization and insertion into the mitochondrial outer membrane to promote the permeability of MPTPs following the translocation. Group I proteins are able to bind the active conformation of Bax to inhibit it from inserting into the mitochondrial outer membrane to maintain the normal permeability of MPTPs, and prevent the release of mitochondrial proapoptotic factors, such as cytochrome c, AIF and Smac/DIABLO (Lu et al., 2003; Milosevic et al., 2003; Read et al., 2002). Through cytochrome c, AIF, and others, Bcl-2 family proteins can lead to dissipation of Δψm and indirectly regulates the caspase activities in related apoptotic pathways (Read et al., 2002). Based on numerous
studies, findings have shown that alteration of Δψm is associated with a polyprotein channel, permeability transition pore which consists of the mitochondrial voltage-dependent anion channel (VDAC). It is also found that VDAC is utmost important for the translocation of Bax/Bak to induce the release of cytochrome c and loss of Δψm consequently execution of apoptosis (Shimizu et al., 1999). These findings showed that BH3-only proteins including Bid and Bad can induce apoptotic signaling to the mitochondria via posttranslational modifications (Korsmeyer, 1999). Bid which is localized in the cytoplasm is cleaved by caspase-8 and become truncated Bid (tBid), followed by the trigger of cytochrome c release. This cell death mechanism is stimulated through Fas/FasL or TNF receptor pathway. In contrast, phosphorylated Bad is localized in the cytoplasm and dephosphorylation of Bad results in translocation to the mitochondria. Dephosphorylated Bad binds to Bcl-xL and inhibits its antiapoptotic activity (Wang et al., 1999; J. Zha et al., 1996). Bcl-xL has recently been shown to prevent the oligomerization of Apaf-1 with pro-caspase-9 and thereby inhibits the activation of caspase-9 (Chinnaiyan et al., 1997).
Figure 2.7: Bcl-2 proteins family members classified into proapoptotic and anti-apoptotic. ‘TM’ refers to a hydrophobic region in the carboxyl terminus of several of these proteins that was originally assumed to be a transmembrane domain. Adapted from D’Amelio et al., 2010.
2.11 Nephelium ramboutan-ake

The annual harvest amount of rambutans, mangosteens, pulasan for fresh consumption, canned or processed produce vast amount of waste. Therefore, increasing attention has been paid to utilize the wastes in discovering potential substances with antioxidant, antimicrobial, antidiabetic and anticancer potential. Interesting recent research has revealed that fruit rinds such as grape, pomegranate, mangosteen peels and rambutan rinds possess antioxidant properties (Ling et al., 2010; Palanisamy et al., 2011; Seeram et al., 2005; Thitilertdecha et al., 2010), anti-hyperglycemic properties (Palanisamy et al., 2011) and anti-cancer properties (Larrosa et al., 2006; Moongkarndi et al., 2004; Seeram et al., 2005). These natural compounds could provide promising bioactive candidates. According to present knowledge, there are some evidences that phytochemicals from the tropical fruits’ rinds such as punicalagin from pomegranate, α-mangostin from mangosteen and ellagic acid from rambutan are effective in the treatment of colon adenocarcinoma cells, prostate carcinoma cells and human bladder cancer T24 cells with respectively (Adams et al., 2006; Hung et al., 2009; Li et al., 2005). Thus, pulasan rinds become target of interest in this current research. Fruit of Nephelium ramboutan-ake (Pulasan) is a tropical fruit, which its rind is normally discarded as waste after consumed (Figure 2.8). The pulasan is the common name for Nephelium ramboutan-ake syn. Nephelium mutabile in Peninsular Malaysia. N. ramboutan-ake is a plant belonging to the family of Sapindaceae which is closely allied to the rambutan, lychee and longan. The sapindaceae are mostly trees and shrubs, and tendril-bearing vines. This family consists of about 140 genera and 1500 species. The rambutan (N. lappaceum) and the pulasan (N. ramboutan-ake) are from the South East Asian archipelago. They prefer high humidity and rainfall and little variation from a 28°C daily mean temperature. These species can be found in India, Burma, Indonesia, Malaysia, Philippines and Thailand. Pulasan is a small ovate fruit and is a little larger
than the rambutan, with fewer fruit per panicle, but appearance and eating quality is very similar. Pulasan has dark red rind; while others appear in yellowish green and purplish red. The rambutan has soft spines, whereas pulasan is covered with short stumpy spines (Wong et al., 1996)

![Image of Nephelium rambutan-ake fruits.](image)

**Figure 2.8:** The outer and inner look of the *N. rambutan-ake* fruits.

**Scientific classification**

Domain: Eufaryota

Kingdom: Plantae

Phylum: Tracheophyta

Class: Magnoliopsida

Order: Sapindales

Family: Sapindaceae

Genus: Nephelium

Specific epithet: rambutan-ake

To date, no actual chemical and bioactivity studies have been carried out on *Nephelium rambutan-ake*. However, in this present study, the rind of *N. rambutan-ake* was found to possess anticancer properties. The cytotoxic activity of *N. rambutan-
ake aqueous fraction was evaluated and the potential underlying mechanisms were characterized in HT-29 human colon cancer cells.
CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Solvents

95% ethyl alcohol, ethyl acetate, hexane and absolute ethanol were purchased from Merck.

3.1.2 Cell lines

HT-29 (Human colorectal adenocarcinoma cell, HTB-38), HCT-116 (Human colorectal carcinoma cell, CCL-247), Ca Ski (Human Caucasian cervical carcinoma cells, CRL1150), MDA-MB-231 (Human breast adenocarcinoma cells, HTB-26) and Chang liver cells (Human liver cell, CCL-13) were purchased from American Type Culture Collection, Manassas, VA, USA.

3.1.3 Growth medium

Growth medium such as DMEM (Dulbecco’s Modified Eagle’s Medium) and RPMI (Roswell Park Memorial Institute) 1640 medium were purchased from Sigma, St. Louis, MO, USA.

3.1.4 Antibodies, chemicals, drugs and reagents

The following purchased items are listed according to the manufacturer:

Sigma: dimethyl sulfoxide (DMSO), HEPES, Sodium bicarbonate (Na₂CO₃), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Phosphate buffer (PBS), iron (III) chloride (FeCl₃), Potassium hydroxide, Hoechst 33342, Propidium Iodide, Curcumin, reduced glutathione (GSH), glutathione reductase enzyme (6 unit/mL), 5,5’-dithiobis-2-nitrobenzoic acid (DTNB 1.5 mg/mL), 2′-7′-dichlorofluorescein diacetate (DCFH-DA), paraformaldehyde, tert-butyl hydroperoxide (TBHP), Ethylene diamine
tetrachloroacetic acid (EDTA.4Na,4H2O), potassium phosphate buffer, trypan blue and RNase A.

PAA Lab: fetal bovine serum, 100 µg/mL penicillin and 50 µg/mL amphotericin B.

Innovative Cell Technologies: Accutase

BD-Bioscience: Annexin V-FITC, annexin V binding buffer, Cytofix/Cytoperm, FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody, rabbit anti-human Bax polyclonal antibody and IgG1 isotype control.

Calbiochem: NADPH solution

Abcam: FITC-conjugated goat anti-rabbit F(ab')2 polyclonal secondary antibody

Abserotec: caspase-3/7 (FAM-DEVD-FMK), caspase-9 (FAM-LEHD-FMK) and caspase-8 (FAM-LETD-FMK)

3.1.5 Assay kits

The following kits purchased are listed according to manufacturer:

Stratagene: Mitochondrial membrane potential detection kit

Sigma: ApoBrdU apoptosis kit

3.1.6 Miscellaneous

The following items according to manufacturer:

Orange Scientific: cell culture flasks (25 and 75 cm²), cell culture dishes (60mm²) and cell culture multiwall plate (96 well, flat bottom).

DB Biosciences: conical centrifuge tubes (15 and 50ml)

Nalge Nune International: Cryovials.

Sartorius: 0.2 µm filter membrane and sterile syringe.

Fisher Scientific: glass slides and cover glass slips, haemocytometer and cover slips
3.1.7 Instruments/Equipments

Centrifuge (Eppendorf), CO₂ incubator (RS biotech), Microplate reader Asys UVM 340 (Biochrom Ltd), Flow cytometry (Accuri C6, Becton Dickinson), Rotary evaporator with vacuum (Buchi), Fluorescent microscope DM16000B (Leica).

3.2 Methods

3.2.1 Plant material

The *N. ramboutan-ake* (Syn. *Nephelium mutabile*) used in this study was identified by Dr Yong Kien Tai, curator of herbarium of the University of Malaya. The voucher specimen of *N. ramboutan-ake* was deposited and numbered No.KLU 47703 in the herbarium of the University of Malaya.

3.2.2 Preparation of crude *N. ramboutan-ake* extract and fractions

Powdered *N. ramboutan-ake* rind (1.2 kg) was soaked with 95% ethyl alcohol (2.0 L, three times) at room temperature for 3 days. The extract obtained was filtered from the residue through Whatman No.1 filter paper. The filtrate was concentrated using a rotary evaporator (Büchi) under reduced pressure at 40 °C. The dark red crude extract (35.81 g) which was in powder form was obtained and stored in specimen vials. The ethanol extract was partitioned with hexane (250 mL, room temperature). The hexane insoluble fraction was further partitioned with ethyl acetate and water in 1:1 ratio (500 mL: 500 mL, room temperature) to yield an ethyl acetate-soluble fraction. The ethyl acetate-soluble fraction was evaporated under reduced pressure at 45 °C, while the aqueous filtrate was lyophilized to yield the aqueous fraction. The *N. ramboutan-ake* rind ethanol extract (NREE), *N. ramboutan-ake* rind ethyl acetate fraction (NREAF) and *N. ramboutan-ake* rind aqueous fraction (NRAF) were dissolved in DMSO prior to each
assay. The final concentration of DMSO in all the experiments did not exceed 0.5% v/v. All samples were filter sterilized with 0.22 μm filters before use.

3.2.3 Cell Culture

3.2.3.1 Maintenance of cells

In the present study, the HT-29, HCT-116, Ca Ski MDA-MB-231 cells and Chang liver cells were maintained in RPMI 1640 medium whereas HepG2 cells were maintained in DMEM medium. All media were supplemented with 10% v/v heat-inactivated fetal bovine serum, 100 μg/mL penicillin and 50 μg/mL amphotericin B. The media was filter sterilized using a 0.22 μm filter membrane. The cells were cultured as monolayer and maintained in 5% CO₂ incubator at 37 °C in a humidified atmosphere.

3.2.3.2 Subculture of cells

The cells were subcultured every 2 or 3 days when the cell growth reached confluency to protect the cells from dying by providing more space and sufficient nutrients for continuous growth. The cells were routinely checked under an inverted microscope for any contamination. To subculture the cells, the medium was discarded and the cells were rinsed twice with phosphate buffer saline (PBS). Subsequently, 2ml of Accutase solution was added to cover the adherent cells and left in incubator for 3-5 min. The flask was observed under inverted microscope to ensure the cells were fully detached. The bottom of the flask was gently tapped to dislodge any remaining attached cells. Medium was added when the cells were detached and transferred into a 15ml conical centrifuge tube. Cell suspensions were centrifuged at 1000rpm for 5 min. Medium was added into cell pellet after discarding the supernatant. Cells were diluted with medium and added into new flasks for further culture.
3.2.3.3 Cryopreservation of cells

The confluent cells were harvested and centrifuged at 1000rpm for 5 min. The supernatant was discarded and pellet was resuspended in freezing medium which consist of 70% of respective culture medium, 20% of FBS and 10% of DMSO. The aliquots of the cell suspension was then transferred to sterile cryovial and fully labeled indicating the cell line, date, passage number and user identity. The vials were first frozen at -20°C and then transferred to -80°C prior to the placement into liquid nitrogen storage vessel for long-term storage.

3.2.3.4 Cell revival

For reviving cells, cryovials were took out from the liquid nitrogen storage and quickly defrosted by immersed in a 37°C water bath and gently shook until thawed. Once fully thawed, cells were pipetted into 15ml conical centrifuge tubes containing medium prior to centrifugation. After centrifugation, the supernatant was removed and resuspended in fresh medium. The cell suspension was then transferred into 25cm² cell culture flask and incubated in CO₂ incubator to allow cells to adhere. The medium was removed after 24 hours to eliminate traces of DMSO and replaced with fresh medium. The cells were cultured for at least 3 passages prior to any experimental usage.

3.2.3.5 Cell counting

A trypan blue exclusion assay was performed to count the viable cells using haemocytometer. Trypan blue is a dye that stains viable cells except dead cells with damaged plasma membrane. To determine the cell number, adherent cells were harvested and resuspended in a desired volume of the medium. A 100ul of 4% tryphan blue solution was then added (dilution factor=2). The cells were mixed and left for 2 min at room temperature followed by the addition of 20ul of cell suspensions into the two haemocytometer chambers covered with cover slip. The haemocytometer was then observed under an inverted phase-contrast microscope using 10x or 20x magnifications.
Only the number of viable cells was scored excluding the non-viable cells which stained blue. The number of viable cells per ml was calculated as the formula stated below: 
Average cell count per square x 10\(^{4}\) x dilution factor, where dilution factor =2 and 10\(^{4}\) = correction factor. To obtain the total viable cell number in the original suspension the viable cell number/ml was multiplied by the total volume in the original suspension.

3.2.3.6 Treatment of cells

Stock solutions (20mg/ml) of plant extract and fractions were prepared by dissolving in DMSO. These solutions were filter sterilized and stored in glass container at -20°C. For the treatment, the stock solutions were serially diluted by using medium and added to the cells. Exponentially growing cells were then treated with the vehicle DMSO. The final concentration of DMSO was 0.5% (v/v). Cells were harvested at different time points and further subjected to various assays.

3.2.4 Evaluation of the cytotoxic activity of extract and fractions of *N. ramboutan-ake*

The cytotoxic activity of crude ethanolic extract and fractions of *N. ramboutan-ake* were assessed by using MTT cell viability assay.

3.2.4.1 *In Vitro* MTT cell viability assay

The cell viability was investigated using the MTT assay. Viable cells were seeded into 96-well plates and were allowed to adhere overnight prior to treatment with varying concentrations (3.125–200 μg/mL) of *N. ramboutan-ake* rind extract and fractions. For the untreated cells (control), vehicle dimethyl sulfoxide (DMSO) was added instead of the sample. After 72 h incubation, MTT (20 μL, 5 mg/mL) was added to each well and the plates were incubated for another 4 h at 37 °C. Following incubation, the culture medium was removed by gentle aspiration and replaced with DMSO (150 μL), to dissolve the formazan crystals. The amount of formazan product was measured at 570 nm and 650 nm as a background using a microplate reader (Asys UVM340). The
percentage of cell viability = (absorbance of treated cells/absorbance of untreated cells) × 100%. MTT assay was used to determine the cell viability based on reduction of yellow tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to a purple formazon by mitochondrial dehydrogenase enzyme especially by NADH reductase which can be measured spectrophotometrically as shown in Figure 3.1 (Liu and Schubert, 1997). The purple formazon was dissolved by using dimethyl sulfoxide (DMSO) to give purple coloured solution. Hence, the amount of formazon produced reflected the number of metabolically active viable cells (van de Loosdrecht et al., 1994).

**Figure 3.1:** Reduction of tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) adapted from Ebada et al., 2008.

### 3.2.4.2 Total cell count

A total of 1 x 10^6 cells were seeded into 60mm^2 culture dish and left for overnight. After 24 h, the medium was replaced with fresh medium containing varying concentrations of active fraction ranging from 50- 200µg/mL. The cells were further incubated for 12, 18, 24, 48 or 72 h. After each incubation period, the cells were harvested by using Accutase solution and centrifuged at 1000rpm for 5 min. The cell pellets were diluted with a dilution factor of 50 in 0.4% of trypan blue solution. The viable cell count was
determined by using haemocytometer. Figure 3.2 shown distribution quadrants of haemocytometer and formula used for cell count.

<table>
<thead>
<tr>
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<th>C</th>
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<td>D</td>
<td>E</td>
<td>F</td>
</tr>
<tr>
<td>G</td>
<td>H</td>
<td>I</td>
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**Figure 3.2:** Quadrants showed under the view of inverted light microscope.

Formula: \((A+C+E+G+I)/5 \times 10^4 \times \text{Dilution factor}\)

### 3.2.5 Assessment of apoptotic effects of the bioactive fraction of *N. ramboutan-ake*

According to the MTT assay result the most active fraction of *N. ramboutan-ake* was subjected to several assays to evaluate the apoptotic-inducing effects and elucidate the apoptosis underlying mechanisms.

#### 3.2.5.1 Nuclear morphology detection using Hoechst 33342/PI

HT-29 cells \((1 \times 10^6 \text{ cells/mL})\) were seeded in 60 mm² culture dish for 24 h and treated with NRAF or vehicle DMSO (control). After indicated periods, the cells were harvested and washed with PBS. The cell suspension was stained with Hoechst 33342
and PI solution at 37 °C in dark for 30 min followed by the observation under inverted fluorescence microscope and photographs were captured (Leica DM16000B, Wetzlar, Germany).

3.2.5.2 Terminal Deoxynucleotidyl Transferased UTP Nick End Labeling (TUNEL) Assay

For detection of DNA breakage, a TUNEL assay was performed following the protocol provided by the manufacturer (Sigma). In brief, HT-29 cells (1 × 10^6 cells/mL) were seeded in 60 mm² culture dish and treated with NRAF or vehicle DMSO (control). NRAF-treated cells were harvested, washed with PBS and fixed with 1% (w/v) paraformaldehyde in PBS on ice for 15 min. After fixation, the cells were washed and then incubated in DNA labeling solution [containing terminal deoxynucleotidyl transferase enzyme, bromodeoxyuridine (BrdU), and TdT reaction buffer] for 60 min at 37 °C. The cells were then rinsed and incubated with FITC-conjugated anti-BrdU antibody for 30 min at room temperature in the dark. Subsequently, the propidium iodide/RNase A solution was added to the cells and further incubated for another 30 min in the dark. The cells were then analysed by using Accuri C6 flow cytometry and the fluorescence intensity in X-axis and Y-axis were detected in FL1-A and FL2-A channel respectively.

3.2.5.3 Annexin V/PI staining for the assessment of phosphatidylserine externalization

The early and late apoptosis induced by NRAF was further investigated using Annexin V/PI staining. Thus, HT-29 cells (1 × 10^6 cells/mL) were seeded in 60 mm² culture dish and treated with NRAF or vehicle DMSO (control). After HT-29 cells (1 × 10^6 cells/mL) were treated with NRAF, both adherent and suspension cells were harvested, washed twice with PBS, re-suspended in annexin V binding buffer (BD) and stained at room temperature in the dark for 30 min with annexin V-FITC (BD) and PI (Sigma) as
described in the manufacturer’s protocol. After treatment with various concentration of NRAF ranging 25 μg/mL to 200 μg/mL, the HT-29 cells were then analyzed by flow cytometry using quadrant statistics for apoptotic and necrotic cell populations. The fluorescence intensity in X-axis and Y-axis were detected in FL1-A and FL2-A channel respectively. Annexin V was used to detect both the early and late stages of apoptosis while PI was used to detect late apoptosis and necrosis. The discrimination between viable (both annexin V and PI negative), late apoptosis (both annexin V and PI positive), and necrotic (annexin V negative and PI positive) cells was achieved by quantitatively estimating the relative amounts of the annexin V/PI-stained cells in the cell population.

3.2.5.4 Measurement of mitochondrial membrane potential ($\Delta\psi_m$)

The change in mitochondrial membrane potential ($\Delta\psi_m$) was assessed by using the cell-permeable, mitochondrial-specific fluorescent probe 5, 5',6,6'-tetrachloro-1,1',3,3'-tetaethylbenzimidazolylcarbocyanine iodide (JC-1) dye (Figure 3.3). As usual, HT-29 cells ($1 \times 10^6$ cells/mL) were seeded in 60 mm$^2$ culture dish and treated with NRAF. For the untreated cells (control), vehicle dimethyl sulfoxide (DMSO) was added instead of the sample. After treatment with NRAF, the cells were harvested, washed and stained with medium containing JC-1. The cells were incubated at 37 °C in the incubator for 15 min. Subsequently, the cells were washed again and resuspended in the medium. Finally the cells were subjected to flow cytometry analysis by detecting the green and red fluorescence signals. JC-1 aggregates in the mitochondria of healthy cells produced red fluorescence as observed in FL2-A channel while in the cells with altered mitochondrial membrane potentials, the JC-1 dye maintained as monomeric form in the cytoplasm where it fluoresced green and detected in FL1-A channel.
**Figure 3.3:** The chemical structure of $5,5',6,6'$-tetrachloro-$1,1',3,3'$-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) dye.

### 3.2.5.5 Determination of intracellular total glutathione (GSH) content

Intracellular GSH level was determined after treatment with various concentrations ranging from 50 μg/mL to 200 μg/mL of NRAF and vehicle DMSO (control). The treated cells were harvested, centrifuged, washed with ice-cold PBS and re-suspended in 500 μL of 5% 5-sulfosalicylic acid before incubating on ice for 15 min with intermittent vortexing. The cell suspension was centrifuged at 10,000 × g to collect the supernatant. The supernatant (10 μL) was then subjected to glutathione assay in 96-well plate format in a 150 μL of working solution. The final concentrations of the reaction mixture were 95 mM potassium phosphate buffer (pH 7.0), 0.95 mM EDTA, 228 μL of glutathione reductase enzyme (6 unit/mL) and 228 μL of $5,5'$-dithiobis-2-nitrobenzoic acid (DTNB 1.5 mg/mL) were then added and left for 5 min prior to addition of 50 μL of NADPH solution (0.16 mg/mL). Absorbance was measured at 1 min intervals for 10 min at 412 nm with an Oasys UVM340 microplate reader and compared with a glutathione standard curve. The results were expressed as pmoles of glutathione per milligram of protein (pmole GSH/mg protein).
**3.2.5.6 Measurement of intracellular reactive oxygen species (ROS)**

The fluorescent probe 2’-7’-dichlorofluorescein diacetate (DCFH-DA) used to monitor intracellular accumulation of ROS. HT-29 cells (1 × 10^6 cells/mL) were seeded in 60 mm² culture dish. Cells were either pre-treated with or without 1mM N-acetylcysteine (NAC) prior to the treatment of NRAF or vehicle DMSO (control) while tert-butyl hydroperoxide (TBHP) was served as positive control. After 4 h treatment with various concentrations (50 μg/mL to 200 μg/mL) of NRAF, the cells were washed and incubated with medium containing 50 μM DCFH-DA for 1 h. Cells were then harvested and washed again with PBS. The cell suspension was re-suspended in PBS and the fluorescence intensity was measured by flow cytometry and detected in FL1-A channel. This fluorogenic probe is a lipophilic dye that is permeable to the cell membrane and then undergoes deacetylation by intracellular esterases to produce 2’, 7’-dichlorodihydrofluorescein (DCFH) (Halliwell and Whiteman, 2004; H. Wang and Joseph, 1999). DCFH is believed to be oxidized by intracellular oxidizing ROS or hydrogen peroxide and results in the formation of a fluorescent compound, DCF in the presence of cellular peroxidases (Crow, 1997; Myhre et al., 2003), which the whole mechanism was illustrated in the Figure 3.4 (Gomes et al., 2005).
3.2.5.7 Determination of Bax and Bcl-2 protein expression level

The protein expression level of Bax and Bcl-2 was assessed by immunofluorescence staining using flow cytometry. This method was based on Roussi et al. (Roussi et al., 2007) with some modifications. After HT-29 cells (1 × 10^6 cells/mL) were treated with NRAF or DMSO (control), both adherent and suspension cells were harvested, washed twice with PBS and then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA, USA). NRAF-treated cells (1 × 10^6 cells) were resuspended in fixation/permeabilization solution (500 μL) and incubated for 20 min at 4 °C. The cells were washed twice with Perm/Wash buffer and incubated for 15 min in this buffer.
(1 mL). To detect Bax or Bcl-2, the fixed and permeabilized cells were incubated with Perm/Wash buffer (100 μL) containing the antibodies. For Bcl-2 protein, the cells were stained directly for 30 min with FITC-conjugated mouse anti-human Bcl-2 monoclonal antibody or IgG1 isotype control (10 μL, BD Biosciences) at 4 °C. For indirect Bax staining, the cells were incubated for 30 min with either rabbit anti-human Bax polyclonal antibody or IgG1 isotype control (BD Biosciences) at 4 °C. After washing, the cells were further incubated for 30 min with FITC-conjugated goat anti-rabbit F(ab’)2 polyclonal secondary antibody (Abcam) at 4 °C. The cells were then washed with Perm/Wash buffer and analyzed using C6 Accuri flow cytometer and detected in FL1-H channel.

3.2.5.8 Measurement of caspase-3/7 and caspase-9 activities

HT-29 cells (1 × 10^6 cells/mL) were seeded in 60 mm² culture dish and treated with NRAF or vehicle DMSO (control). After treatment, cells were harvested and cell suspensions were stained with 30× FLICA solution (caspase 3/7-FAM-DEVD-FMK; caspase 9-FAM-LEHD-FMK; caspase 8-FAM-LETD-FMK) for 1 h at 37 °C under 5% CO₂ in darkness. Cells were then washed twice with wash buffer followed by resuspending of cell pellet in wash buffer. FAM-DEVD-FMK FLICA, FAM-LEHD-FMK and FAM-LETD-FMK will bind to caspase-3/7, caspase-9 and caspase-8, respectively that are present in the cells and appear as green fluorescence. Increase in fluorescence intensity indicating caspase-3/7, caspase-9 and caspase-8 activities were detected by flow cytometry and detected in FL1-A channel.
3.2.6 Phytochemical content analysis

The aqueous fraction of N. ramboutan-ake was subjected to preliminary qualitative phytochemical screening according to the Sofowora, Trease and Bibi methods (Bibi, 2010; Sofowora, 1993; Trease, 1989). This analysis was to determine the presence of phytoconstituents such as flavonoids, tannins, saponins, alkaloids and sterols.

3.2.6.1 Test for alkaloids

The extract (0.5 g) was added to 1% aqueous hydrochloric acid (5 mL) on a steam bath and filtered. A few drops of Dragendorff’s reagent were added into 1 mL of the filtrate. Turbidity or precipitation with this reagent was taken as evidence for the presence of alkaloids.

3.2.6.2 Sakowski test for sterols

The plant extract (0.5 g) was dissolved in chloroform (1 mL); concentrated sulphuric acid (1 mL) was added carefully along the sides of the test tube. Production of a red colour indicates the presence of steroids.

3.2.6.3 Frothing test for saponins

Plant extract (0.5 g) was dissolved in boiling water (1 mL) followed by cooling (room temperature) and shaking. Appearance of froth indicates the presence of saponins.

3.2.6.4 Test for tannins

Plant extract (0.5 g) was boiled in distilled water (20 mL) in a test tube and then filtered. Two to three drops of FeCl₃ (0.1%) was added to filtrate. Appearance of brownish green or blue black coloration showed the presence of tannins.

3.2.6.5 Test for flavonoids

Prepared extract (0.5 g) was shaken with petroleum ether (5 mL) to remove the fatty materials. The defatted residue was dissolved in 80% of ethanol (20 mL) and filtered.
The filtrate (3 mL) was mixed with 1% KOH (4 mL). A dark yellow color was observed indicating the presence of flavonoids.

3.7 Statistical analysis

In all the experiments, data were expressed as means ± standard error. A significant difference from the respective control for each experiment was assessed using Student’s t-test, with p values <0.05 being regarded as statistically significant.
CHAPTER 4
RESULTS

4.1 Reduction of HT-29 cell viability by NRAF

The in vitro cytotoxicity activity of the *N. ramboutan-ake* rind on colorectal cancer cells (HT-29 and HCT116), cervical cancer cells (Ca Ski), breast cancer cells (MDA-MB231) and normal cells (Chang) was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The crude ethanol extract and fractions of *N. ramboutan-ake* rind exhibited a dose-dependent cytotoxic effect in all the cancer cell lines except Chang cells (Figure 4.1). Among the fractions, NRAF appeared to exhibit the lowest inhibitory concentration (IC$_{50}$) value against all cell lines, followed by the NREE and NREAF (Figure 4.2). HT-29 treated with NRAF displayed the lowest IC$_{50}$ value of 16.66 ± 0.55 µg/mL whereas exposure to NREE and NREAF exhibited higher IC$_{50}$ values of 20.70 ± 0.49 µg/mL and 32.24 ±1.81 µg/mL, respectively (Table 4.1). NRAF yielded a lower IC$_{50}$ value than curcumin, a positive control in this study (Table 4.1). The IC$_{50}$ values of the extract and fractions against five different cancer cell lines are encapsulated in Table 4.1. NRAF also revealed significant dose-dependent reduction of cell viability in Ca Ski cells (31.14 ± 0.41 µg/mL), HCT-116 (33.90 ± 1.06 µg/mL) and MDA-MB231 (41.53 ± 0.32 µg/mL). However, cell viability of normal Chang’s cells only started to reduce at a concentration of 400 µg/mL.
Table 4.1: IC$_{50}$ values of extract and fractions of *Nephelium ramboutan-ake* rind against different cancer cell lines and normal cell line.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Ethanol (µg/mL)</th>
<th>Ethyl acetate (µg/mL)</th>
<th>Aqueous (µg/mL)</th>
<th>Curcumin* (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HT-29</td>
<td>20.70 ± 0.49</td>
<td>32.24 ± 1.81</td>
<td>16.67 ± 0.55</td>
<td>21.32 ± 0.17</td>
</tr>
<tr>
<td>HCT-116</td>
<td>35.73 ± 0.56</td>
<td>47.23 ± 2.84</td>
<td>33.90 ± 1.06</td>
<td>NA</td>
</tr>
<tr>
<td>Ca Ski</td>
<td>34.38 ± 0.66</td>
<td>44.90 ± 0.58</td>
<td>31.14 ± 0.41</td>
<td>NA</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td>51.09 ± 1.32</td>
<td>61.65 ± 0.42</td>
<td>41.53 ± 0.32</td>
<td>NA</td>
</tr>
<tr>
<td>Chang</td>
<td>NA</td>
<td>NA</td>
<td>&gt;400</td>
<td>NA</td>
</tr>
</tbody>
</table>

The data represent mean ± S.E. of three independent experiments (n=9).

NA: Not available.

* Curcumin served as positive control
Figure 4.1: The cytotoxicity effect of NRAF extract and fractions against selected cancer cell lines at 72 h. Bar chart represented the effect of NRAF on the viability of HT-29, HCT-116, Ca Ski, MDA-MB-231 and Chang liver cells. The data expressed as mean ± S.E. of three independent experiments (n=9). Asterisks indicate significantly different value from control (*p<0.05).
Figure 4.2: Graph represented the cytotoxicity effect of *N. ramboutan-ake* rind ethanol extract (NREE), ethyl acetate fraction (NREAF) and aqueous fraction (NRAF) against HT-29 cell line. The data expressed as mean ± S.E. of three independent experiments (n=9). Asterisks indicate significantly different value from control (*p<0.05).*
4.2 The effect of NRAF on nuclear morphological changes of HT-29 cells by Hoescht 33342 and PI staining

Physiological cell death is characterized by apoptotic morphology, including chromatin condensation, nuclear shrinkage, membrane blebbing, DNA fragmentation and apoptotic body formation. To determine and distinguish whether the cell death was associated with apoptosis, HT-29 cells were double stained with Hoescht 33342 and Propidium Iodide (PI) after exposure to NRAF followed by observation under fluorescence microscopy. The cell morphology was categorized into live, early apoptotic, late apoptotic and necrotic cells. For the control untreated group, the nuclei appeared round in shape with intact chromatin which was homogeneously stained with faint blue fluorescence by Hoescht 33342 dye only (Figure 4.3(a)). However, the presence of 50 µg/mL NRAF caused nuclear shrinkage and typical chromatin condensation which displayed early apoptosis, exhibited intense bright blue fluorescence nuclei at 24 h (Figure 4.3(b)). For late apoptotic cells, they were dual-stained and displayed purple and blue fluorescence (Figure 4.3(b), Arrow 2). At a higher concentration (100 µg/mL) of NRAF for 24 h incubation led to nuclear shrinkage with more apparent nuclear changes, exhibiting more obvious cell shrinkage and loss of nuclear architecture (Figure 4.3(c), Arrow 3) such as DNA fragmentation, nuclear condensation and late apoptosis (Figure 4.3). This progressed into fragmented nuclei in the process of forming apoptotic bodies (Figure 4.3(c), Arrow 4).
Figure 4.3, continued
Figure 4.3: Nuclear morphological changes of HT-29 cells by NRAF. (a) Untreated control cells. (b) After exposure to 50 μg/mL of NRAF and (c) 100 μg/mL of NRAF for 24 h, when stained with Hoechst 33342 and PI. Arrows indicated early (e.g., chromatin condensation, cell shrinkage and nuclear fragmentation) and late apoptotic morphological changes. Magnification: 630×. Arrow 1 indicates chromatin condensation, 2 late apoptosis, 3 cell shrinkage and 4 DNA fragmentation.
4.3 Induction of DNA fragmentation detected by TUNEL assay

Another important biochemical hallmark of apoptosis is DNA fragmentation. Thus, TdT-mediated dUTP Nick End Labelling (TUNEL) assay was utilized to determine whether induction of DNA fragmentation occurred after exposure of NRAF on HT-29 cells. Results indicated that NRAF-treated cells induced DNA fragmentation (Figure 4.4). The control untreated cells showed negative TUNEL staining (Figure 4.4). The elevation in the percentage of TUNEL-positive cells was apparent as the concentrations of NRAF (50 – 200 μg/mL) gradually increased at 24h treatment (Figure 4.4). The percentage of apoptotic cells (apoptotic index) was distinctly increased up to 31.07 ± 0.79% compared to the control group (1.1 ± 0.5%). Taken together, these results indicated that NRAF induced typical apoptosis in HT-29 cells.
Figure 4.4: Effect of NRAF on DNA fragmentation of HT-29 cells. HT-29 cells were treated with different concentrations of NRAF (50 µg/mL, 100 µg/mL and 200 µg/mL) at 24 h incubation period. Positive TUNEL staining was shown by the M2 region in which cells were stained with FITC-conjugated anti-BrdU antibody. Histograms are representatives of three separate experiments (n=3).
4.4 Externalization of phosphatidylserine by using Annexin V/PI staining

The loss of plasma membrane asymmetry is due to the externalization of phosphatidylserine (PS) during early apoptosis (Koopman et al., 1994), onto the cell surface, which is considered one of the prime markers of apoptosis. This led us to investigate the effect of NRAF on early events of apoptosis. Assessment of early and late apoptosis induced by NRAF was further labeled by using Annexin V/Propidium Iodide staining to detect the occurrence of translocation phosphatidylserine to outer plasma membrane. As shown in dual parametric dot plots (figure), the four quadrants correspond to viable at lower left (Annexin−/PI−), early apoptotic at lower right (Annexin+/PI−), late apoptotic at upper right (Annexin+/PI+) and necrotic cells at upper left (Annexin−/PI+), respectively. After exposure to varying concentrations of NRAF (25 μg/mL to 200 μg/mL), the HT-29 cells population significantly showed a shift from viable stage to early and late stage of apoptosis in a dose-dependent manner. The percentage of early apoptotic cells increased from 0.97 ± 0.02 % (control) to 5.84 ± 0.16 %, 6.9 ± 0.69 %, 8.07 ± 0.32 % and 7.78 ± 0.13 % after treatment with 25, 50, 100 and 200 μg/mL of NRAF, respectively for 24 hours incubation period (Figure 4.5 (a)). On the other hand, the percentage of late apoptotic cells was significantly increased in a dose-dependent manner from 1.3 ± 0.04 % (control) to 20.88 ± 0.87, 30.63 ±0.72, 38.86 ± 0.93 and 48.04 ± 0.38 %, at 25, 50, 100 and 200 μg/mL respectively at 24 h treatment. Compared to the untreated cells, NRAF-treated cells showed significant increase in the percentage of total annexin V positive cells which consists of early and late apoptotic cells in a dose-dependent manner (Figure 4.5 (c)). With increasing concentrations of NRAF (25, 50, 100 and 200 μg/mL), the percentage of total annexin V positive cells (Annexin+/PI− and Annexin+/PI+), increased from 2.27 ± 0.02 % (control untreated cells) to 26.71 ± 1.02 , 37.56 ± 1.25, 46.93 ± 0.61, 55.82 ± 0.27 %, respectively.
Figure 4.5, continued
Figure 4.5, continued
Figure 4.5: Dose-dependent induction of early and late apoptosis by NRAF (25-200 µg/mL) at 24 h. (a) showed the flow cytometric fluorescence patterns of Annexin V-FITC/PI staining. (b) indicated the percentage of viable, early apoptotic, late apoptotic and necrotic cells. (c) depicted the percentage of annexin V positive cells. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p<0.001).
4.5 Alteration of mitochondrial membrane potential (ΔΨm)

Bountiful evidence proposes that the relationship between the alterations of mitochondrial function and apoptosis as well as a decreasing mitochondrial transmembrane potential, Δψm, is correlated with mitochondrial dysfunction. Numerous studies also revealed that apoptosis proceeds concomitantly with an irreversible dissipation of mitochondrial membrane potential (ΔΨm). In addition, loss of mitochondrial membrane potential is a crucial event during the mitochondrial-mediated apoptosis. To evaluate the possibility that the NRAF-treated HT-29 cells could induce loss of ΔΨm, JC-1 probe, a cationic dye, was used which specifically accumulates within mitochondrial compartment in a ΔΨm-dependent manner (Chen et al., 2007). When the mitochondrial depolarization precedes the onset of apoptosis, JC-1 converts from red to green fluorescence. As shown in Figure 4.6 (a), the upper quadrant represents red JC-1 aggregates whereas the lower quadrant represents green JC-1 monomers. Results (Figure 4.6) reflected that the population of the cells shifted progressively from the upper quadrant to the lower quadrant after exposure to NRAF whereas in control untreated cells, most of the red JC-1 aggregate fluorescence emerged in the upper right quadrant. The percentage of red JC-1 aggregate fluorescence was drastically reduced and appeared as green JC-1 monomer fluorescence after exposure to 25 µg/mL NRAF. It was noted that the percentage of red JC-1 aggregate fluorescence gradually reduced inversely proportional with increasing concentrations and reached almost complete loss of JC-1 aggregate fluorescence at the highest concentration of 200 µg/mL. Figure 4.5(a) depicted the percentage of control for 25, 50, 100 and 200 µg/mL of NRAF with the values of 72.60 ± 0.58, 45.21 ± 0.42, 39.11 ± 0.72, 21.23 ± 0.26 %, respectively. As indicated by JC-1 red/green ratio (Figure 4.6 (b)), NRAF resulted in a substantial dose-dependent loss of ΔΨm.
Figure 4.6, continued
Figure 4.6: Dose-dependent attenuation of mitochondrial membrane potential in HT-29 cells elicited by NRAF. (a) Showed the flow cytometric fluorescence patterns analysis of JC-1 (5, 5’, 6, 6’-tetrachloro-1, 1’, 3, 3’ tetraethylbenzimidazolylcarbocyanine iodide) staining. (b) Showed that NRAF has resulted in a substantial dose-dependent reduction of red/green fluorescence corresponding to loss of Δψm. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p<0.001).
4.6 Effect of NRAF induced formation of ROS

One of the oxidative stress factors that lead to cell death is the production of ROS. The level of intracellular ROS within the cells treated with various concentrations of NRAF was measured using a ROS-sensitive fluorometric probe, 2’-7’-dichloro-fluorescein diacetate (DCFH-DA) indicated by dichlorofluorescein (DCF) fluorescence which was proportional to the amount of intracellular ROS formed. Notably, at 4 h treatment, a significant generation of ROS appeared as early as 50 µg/mL of NRAF-treated cells (Figure 4.7). This was evident by the shift of the histogram from left to right, indicating an increase in the fluorescence intensity (Figure 4.7 (a)). A progressive shift of the histogram to the right with increasing concentrations of NRAF (50 µg/mL, 100 µg/mL and 200 µg/mL) was also observed, compared with control untreated cells (Figure 4.7 (b)). Results revealed that the level of ROS significantly increased to 1.96 ± 0.04, 2.48 ± 0.08 and 3.38 ± 0.03 folds from the control after treatment of HT-29 cells with 50, 100 and 200 µg/mL of NRAF, respectively. In order to elicit in more detail and unravel the involvement of intracellular ROS in NRAF-induced apoptosis, an antioxidant N-acetylcysteine (NAC) was used due to its role in attenuating apoptosis induction. Interestingly, 1mM NAC pretreatment abrogated the ability of NRAF to generate intracellular ROS (Figure 4.7 (c) and (d)). Additionally, the presence of NAC prevented the reduction of cell viability in NRAF-treated HT-29 cells (Figure 4.7 (e)).
Figure 4.7, continued
Figure 4.7, continued
Figure 4.7, continued
Figure 4.7, continued
**Figure 4.7**: Effect of NRAF on intracellular ROS level. (a) Showed induction of ROS production after treatment with different concentrations of NRAF (50 µg/mL, 100 µg/mL and 200 µg/mL). (b) Data analysis indicating shifts in the intracellular ROS level at different concentrations when compared to the negative control (untreated cells) and positive control (treated with TBHP) (c) Showed the intracellular ROS level in the presence and absence of 1mM NAC for 1 h on untreated and NRAF treated HT-29 cells. (d) Bar chart represented the fold change of intracellular ROS compared to control. (e) Bar chart showed the cell viability of untreated and treated cells (25-200 µg/mL of NRAF) with and without NAC for 24 h by using MTT assay. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p<0.001). Double asterisks indicate significantly different value from the treated-cells without the presence of 1mM NAC.
4.7 Depletion of glutathione content by NRAF

Reduced glutathione (GSH) is a major cellular antioxidant which protect cells against apoptosis and/or from oxidative stress by scavenging peroxides in the cytosol and mitochondria (Voehringer, 1999). Above and beyond, studies also verified that collapse of GSH content can render the cells more sensitive to the apoptosis inducing agents and hence induce the onset of apoptosis. Therefore, alteration of intracellular glutathione content which correlates with oxidative stress in cell death was assessed in the present work. NRAF enhanced oxidative stress by altering intracellular glutathione levels. The results revealed that cells stressed with varying concentrations of NRAF at 24 h treatment, displayed a concurrent decline in intracellular GSH content compared to the control untreated cells (Figure 4.8). The results revealed that the intracellular GSH level was high 952.50 ± 21.65 pmoles in the control untreated cells and has remarkably depleted to 505.00 ± 23.63, 404.17 ± 46.93 and 394.17 ± 24.85 pmoles with increasing concentrations of NRAF of 50, 100 and 200 µg/mL, respectively.
Figure 4.8: Effect of NRAF on intracellular total glutathione content of HT-29 cells at 24 h. There was a significant reduction in intracellular GSH content (> 50%) after treatment with varying concentrations of NRAF (50 – 200 µg/mL). The data expressed as mean ± SE of three independent experiments (n=9). Asterisks indicate significantly different value from control (*p<0.001).
4.8 Modulation of apoptotic proteins by NRAF

Since decades ago, collective studies showed the importance of Bcl-2 family member proteins in apoptosis. Present findings which demonstrated NRAF-stimulated collapse of the mitochondrial transmembrane potential in colon cancer cells HT-29 (Figure 4.6 (a) & (b)) and the appearance of this disruption via the formation of pores in the mitochondria by dimerized Bax or activated Bid, Bik or Bad proteins. Activation of these proapoptotic proteins is accompanied by the release of cytochrome c into the cytoplasm which induces caspase cascade activation that eventually induces apoptosis. In view of that, NRAF induced mitochondrial-mediated apoptotic pathway through the regulation of Bax and Bcl-2 proteins was examined. Subsequently, flow cytometric immunofluorescence staining was exploited to validate the Bax and Bcl-2 protein expression in individual cells. Results attained showed that treatment of NRAF exhibited a marked increase in fluorescence intensity (Figure 4.9 (a)) when stained with Bax antibody compared to the control as evident in the shift of histogram from left to right. This data implied that exposure 50 µg/mL of NRAF has upregulated the expression level of proapoptotic Bax protein by 2.22 fold at 24 h treatment as shown in Figure 4.9 (b). Conversely, Bcl-2 level remained low throughout the time-course treatment (Figure 4.9 (d)) and was evident as the shift of histogram from right to left (Figure 4.9 (c)) with respect to the control. Notably, the increase in Bax protein expression (Figure 4.9 (a)) has elevated the Bax/Bcl-2 ratio by 2.5 fold, at 24 h (Figure 4.9 (e)).
Figure 4.9, continued
Figure 4.9, continued
Figure 4.9, continued
Figure 4.9, continued
Figure 4.9: Effect of NRAF on protein expression level of Bax and Bcl-2 in HT-29 cells. Cells were treated with 50 µg/mL of NRAF for different exposure periods. (a) Representative histogram showed the expression of Bax in the treated-HT-29 cells at different exposure periods. (b) Representative histogram showed the expression of Bcl-2 in the treated-HT-29 cells at different exposure periods. (c) Bar chart showed the relative Bax protein expression level. (d) Bar chart showed the relative Bcl-2 protein expression level. (e) Bar chart showed the ratio of Bax/Bcl-2. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p<0.001).
4.9 NRAF induced caspase -3/7 and caspase -9 activation

Caspases are considered elemental in mediating various apoptotic responses. Activation of caspase-9 and caspase-8 insinuates the trigger of the respective intrinsic and extrinsic apoptotic pathways. Furthermore, many studies showed the correlation between the ratio of proapoptotic Bax to antiapoptotic Bcl-2 and the initiation of a caspase cascade including caspase-3/7. The present findings of the NRAF-induced upregulation of Bax protein and the resultant increase in the Bax/Bcl-2 ratio led us to explore the roles of caspase-9, -8 and -3/7 in the NRAF-induced apoptotic pathways. To examine the apoptotic pathway in NRAF-treated HT-29 cells, caspase -9, -8 and -3/7 activities were assessed by using fluorochrome caspase inhibitors, FAM-LEDH-FMK, FAM-LETD-FMK and FAM-DEVD-FMK, respectively. The fluorescence intensity is proportional to the caspase activity. Firstly, HT-29 cells were exposed to NRAF in time-course study to determine whether it will affect the activation of caspase-8 in HT-29 cells. Activity of caspase-8 observed was maintained relatively low in these cell lysates after NRAF treatment throughout all incubation periods (data not shown). On the contrary, astonishing results illustrated that both caspase-9 (Figure 4.10 (a)) and caspase-3/7 (Figure 4.10 (b)) activities remarkably increased when HT-29 cells were exposed to 50 µg/mL of NRAF in time-course study (12, 18 and 24 h incubation). The intensity of fluorescence increased directly proportional with the increasing incubation periods as evident in the shift of histogram from left to right indicating an increase in the fluorescence intensity. In other words, caspase-9 and caspase-3/7 were positively activated in a time-dependent manner. These circumstances apparently revealed a significant time-dependency in the increase of caspase-9 and caspase-3/7 activities. The NRAF induced 2.67 fold activation of caspase -3/7 as early as 12 h post-treatment and notably increased to 3.83 and 4.75 folds at 18 and 24 h, respectively (Figure 4.10 (c)). Similarly, NRAF also induced 2.67, 3.15 and 4.85 fold activation of caspase-9 at 12, 18
and 24 h, respectively (Figure 4.10 (d)). In this study, we observed activation of caspase-9 and caspase-3/7, but not caspase-8, after treatment with NRAF. These results suggested that NRAF induces apoptosis in HT-29 cells, associated with both caspase-9 and caspase-3/7 activations.
Figure 4.10, continued
Figure 4.10: Activation of caspase-3/7 and -9 after exposure to 50 µg/mL NRAF at different time intervals. (a) Represented the flow cytometric fluorescence patterns of caspase-3/7 (b) Represented the flow cytometric fluorescence patterns of caspase-9. (c) Effect of NRAF on the activation of caspase-3/7 in HT-29 cells. (d) Effect of NRAF on the activation of caspase-9 in HT-29 cells. Both caspase activities were assessed in a time-dependent manner (12, 18 and 24 h) at 50 µg/mL of NRAF. The data expressed as mean ± S.E. from three individual experiments. Asterisks indicate significantly different value from control (*p<0.05).
4.10 Phytochemical content analysis

In the present study, a qualitative phytochemical analysis of NRAF revealed the presence of flavonoids, tannins and saponins, while showing no presence of alkaloids and sterols (Table 4.2). This was observed through the colour intensity, turbidity and precipitation seen in the different reactions.

Table 4.2: Phytochemical content analysis of *N. ramboutan-ake* aqueous fraction (NRAF)

<table>
<thead>
<tr>
<th>Phytochemical test</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>++</td>
</tr>
<tr>
<td>Tannins</td>
<td>+++</td>
</tr>
<tr>
<td>Saponins</td>
<td>+++</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>_</td>
</tr>
<tr>
<td>Sterols</td>
<td>_</td>
</tr>
</tbody>
</table>

* – absent; + low, ++ moderate and +++ abundant
CHAPTER 5

DISCUSSION

Many fruit peels are recently shown to possess anti-cancer properties and cytotoxic effects against various cancer cell lines and widely used for therapeutic purposes. *Nephelium ramboutan-ake* rind is not reported previously for any biological activities. To date, this is the first study in deciphering the mechanisms involved in NRAF induced early events leading to the activation of signaling cascades culminating in apoptotic cancer cell death. In this study, the anticancer potential and the cell death mechanism conferred by NRAF were investigated.

The *N. ramboutan-ake* rinds were dried and soaked with 95% ethyl alcohol at room temperature for 3 days. Ethanol was selected as a solvent for the crude extraction due to the versatility in extracting the polar, semi-polar and non-polar compounds. Subsequently, hexane, a non-polar solvent, was used to fractionate and remove the non-polar in hexane soluble fraction from the polar compounds in hexane insoluble fraction. The fractionation was then proceeded by immersing the hexane insoluble fraction in ethyl acetate and water in the ratio of 1:1 to yield semi-polar compounds in the ethyl acetate-soluble fraction (NREAF) and more highly polar compounds in the aqueous fraction (NRAF). In the present study, the potential of NREE, NREAF and NRAF to exert cytotoxic activity and induce apoptotic effects against multiple human cancer cell lines was investigated.

The crude ethanol extract and fractions of *N. ramboutan-ake* rind exhibited varying dose-dependent cytotoxic activity against HT-29, HCT 116, MDA-MB-231 and CaSki cancer cell lines except the normal Chang cells (Figure 4.1(a)). Among the fractions, the aqueous fraction, NRAF exhibited the lowest inhibitory concentration (IC\(_{50}\)) against all cell lines, followed by the NREE and NREAF (Figure 4.1(b)).
Among the cancer cell lines, HT-29 cells showed the highest susceptibility with the lowest IC50 value after exposure to NREE, NREAF and NRAF. Interestingly, NRAF yielded a lower IC50 value than curcumin, a positive control in this study (Table 4.1). The growth suppressive effect was only observed in the normal Chang liver cells at 400 µg/mL and above. NRAF was found to significantly abrogate the growth of HT-29 cells in a dose-dependent manner and hence it was selected for further investigations to determine the apoptosis inducing effects.

The occurrence of apoptosis was initially detected based on the morphological alteration by Hoechst 33324 and Propidium Iodide (PI) staining to detect the nuclear morphological changes. Bisbenzimidazole dye Hoechst 33342 is known to penetrate the plasma membrane and is a specific DNA stain that binds preferentially to AT-rich regions of double-stranded DNA without permeabilization. H/PI dual staining allows apoptotic cells to be distinguished from necrotic cells. Therefore, this method allows the detection of archetypal apoptotic characteristics including DNA fragmentation, chromatin condensation, apoptotic bodies, cell shrinkage, membrane blebbing and others. H+/PI- dual stained early apoptotic cells with intense bright blue nuclei depicted archetypal apoptotic characteristics including nuclear shrinkage and chromatin condensation compared to the control untreated cells which appeared to be faint round blue nuclei with intact chromatin. Uptake of PI when necrosis (H+/PI+) or late apoptosis (H+/PI+) occur as a result of the terminal damage of the cell membrane while early apoptotic and viable cells are unable to uptake PI due to the consequences of cell membrane integrity. Nuclear condensation and/or fragmentation along with PI uptake can be observed in late apoptotic cells. Results have shown that with increasing concentrations of NRAF, the nuclear changes were more apparent, exhibiting more obvious loss of nuclear architecture. However, these aberrant nuclear changes were not
observed in the control untreated cells. These morphological findings strongly suggest the induction of apoptosis on HT-29 cells by NRAF.

An array of naturally derived anticancer agents possess the properties to mediate diverse anticancer machineries including DNA damage, immune response, induction of apoptosis and cell cycle progression arrest (Chiang et al., 2011; Michaud et al., 2011; Su et al., 2011). The occurrence of DNA fragmentation was detected by TUNEL assay. This assay was developed as a method to identify individual cells that were experiencing apoptosis, by using the polymerase terminal deoxynucleotidyl transferase (TdT) to label the ends of degraded DNA (Gavrieli et al., 1992), which catalyzes the template-independent addition of deoxynucleotide triphosphates to the 3’-OH ends of DNA. Endonucleases result in DNA degradation during late stage of apoptosis, consequently produce fragments of DNA strand breaks (DSBs) with the exposure of 3’-OH ends (Compton, 1992). NRAF-induced cell death in HT-29 cells through apoptosis mechanism was verified by the evidence of DNA fragmentation. DNA fragmentation exhibited in TUNEL assay-labeled nuclei cells often correlates with the morphological observation which is regularly exploited as an apoptosis biochemical index. The results indicated augmenting percentage of TUNEL-positive cells with increasing concentrations of NRAF. The increase in TUNEL positivity occurred in conjunction with the emergence of apoptotic nuclear morphological alterations as illustrated in Hoechst 33342/PI staining. These results indicated that DNA fragmentation, particularly the breakage of DNA strands, might be a factor to the aberrant nuclear changes.

A perturbation in the equilibrium between the status of cell proliferation and apoptosis may initiate the development of tumor cells. The main purpose of cancer chemopreventive is to restore such equilibrium. Results thus far have demonstrated the typical morphological and biochemical changes that occur during late apoptosis. Subsequently, this led to the investigation of the early apoptosis events after exposure of
NRAF. Copious studies have reported that advanced DNA fragmentation is preceded by early events such as externalization of phosphatidylserine (PS) following the mitochondrial membrane potential collapse. During early apoptosis, phosphatidylserine will translocate from inner to outer surface of plasma membrane occurs as a consequences of cell surface phospholipid asymmetry disruption (Koopman et al., 1994), which is considered one of the distinctive markers of apoptosis. Hence, the apoptotic programmed cell death elicited by NRAF was verified by dual staining with Annexin V-FITC/PI and analyzed by flow cytometry. Annexin V staining patterns indicated different stages of apoptosis consists of early and late apoptosis in the NRAF-treated cells whereas PI which is a DNA-binding dye identified late apoptosis and necrosis. This assay is based on the scientific fact that apoptotic cells have exposed phosphatidylserine molecules (Fadok et al., 1992) and has higher affinity to bind with annexin V while necrotic cells possess compromised membranes and thus take up PI (Vermes et al., 1995). Annexin V is a Ca\(^{2+}\) dependent phospholipid-binding protein that detects the PS externalization of plasma membrane (Callahan et al., 2000). During early stage of apoptosis, the faintly annexin V-stained cells probably are as a result of limited phosphatidylserine exposure (Chiu et al., 2003). The heavily stained annexin V and PI cells indicated the late stage of the apoptosis, whereby the cells which losing their plasma membrane integrity and more binding sites of PS were detected (Koopman et al., 1994). After treatment with different concentration of NRAF, the percentage et al of annexin V positive cells (early and late apoptotic cells) significantly increased in a dose-dependent manner indicating the initiation of apoptosis. Collectively, the above mentioned findings further suggest the apoptotic induction by NRAF.

The disruption of mitochondrial integrity is another distinctive marker of apoptosis as well as one of the early events leading to apoptosis. Mitochondrial dysfunction usually triggers specific cellular signaling to induce apoptosis. Generally, there are two main
focal apoptotic signaling machineries comprising of cell surface death receptor and mitochondrial-mediated pathway. Dissipation of mitochondrial membrane potential is a crucial event during the mitochondrial-mediated apoptosis (Fu et al., 2006; Han et al., 2006; Kroemer et al., 2007). In the present study, substantial dose-dependent loss of mitochondrial membrane potential was observed in NRAF-treated HT-29 cells at 24 h treatment as evident in the flow cytometric fluorescence patterns (Figure 4.6(a)) and a remarkable reduction in the ratio of JC-1 red/green fluorescence (Figure 4.6(b)). The loss of mitochondrial transmembrane potential ($\Delta\psi_m$) is a crucial dysfunction in apoptotic processes, which can result in the mitochondrial release of apoptogenic factors from mitochondria and reduction of ATP production, finally leading to cell apoptosis. Therefore, current findings suggested that NRAF-induced apoptosis in HT-29 cells involved mitochondrial dysfunction associated with dissipation of the $\Delta\psi_m$.

Reactive oxygen species (ROS) are a family of reactive oxygen molecule containing free radicals and are associated with the regulation of biological cell functions. Excessive ROS formation and/or limited antioxidant, perturbation of intracellular redox homeostasis, and irreversible oxidative modifications of lipids, proteins, or DNA subsequently results in growth arrest, senescence or apoptosis (Circu and Aw, 2010; Finkel and Holbrook, 2000; Martindale and Holbrook, 2002). Redox homeostasis in a cell is maintained through the equilibrium between the intracellular ROS and ROS scavenging antioxidants and enzyme systems. Intracellular ROS which is generated from mitochondria has been revealed to be an early signal in apoptosis induction (Simon et al., 2000), in concurrence with $\Delta\psi_m$ loss as an early event in apoptosis induction (Koopman et al., 1994). Besides, it is also regarded as the byproduct of a normal cellular oxidative process. It has been indicated that oxidative stress contributes to the initiation of apoptotic signaling. At this early apoptotic event, the damaged mitochondria which causes the loss of mitochondrial membrane potential, activates the
inhibition of the oxidation of reducing equivalents in the respiratory chain electron transfer system and leads to the formation of superoxide anion due to the transfer of one electron to $O_2$ (Turrens, 1997). From the results obtained, NRAF induced the dissipation of mitochondrial membrane potential (Figure 4.6(a)) accompanied with high intracellular ROS level in HT-29 cells. The intracellular ROS level was determined using a ROS-sensitive fluorometric probe, 2’, 7’-dichlorodihydrofluorescein diacetate (DCFH-DA), indicated by dichlorofluorescein (DCF) fluorescence which was proportional to the amount of intracellular ROS formed. Collectively, the results implied that accumulation of intracellular ROS by NRAF disrupts the integrity of the mitochondrial membrane, subsequently perturbing the electron transport system resulting in the production of ROS by one-electron transfer. In addition, results (Figure 4.7(e)) indicated that in the presence of NAC, ROS generation is a critical target in NRAF-induce apoptosis in HT-29 cells. This event is in parallel with numerous findings, for instance, resveratrol (Juan et al., 2008) and flavokawain B (Kuo et al., 2010) possess anticancer effects and are able to induce ROS-dependent mitochondrial apoptosis pathway in HT-29 and HCT116 cells in the presence of pretreatment with NAC. Hence, the present collective findings provide evidence that NRAF potentiates ROS-dependent apoptosis in HT-29 cells.

Among the diverse mechanisms proposed for apoptosis, great attention has been raised to intracellular redox status. Recently, it has been demonstrated that intracellular GSH is an intracellular antioxidant and is known to participate in maintaining cellular redox balance (Pramanik et al., 2011). Growing evidences have revealed the importance of intracellular GSH associated with apoptosis, in which intracellular GSH depletion precedes the onset of apoptosis triggered by numerous agents (Beaver and Waring, 1995; Duranteau et al., 1998; Macho et al., 1997; Wang et al., 2007). Additionally, depleting intracellular GSH renders cells more sensitive to apoptotic agents (Dai et al., 1999; van
den Dobbelsteen et al., 1996; Wang et al., 2007). Disorder of GSH redox state evokes the apoptosis and unfolding protein response (Frand and Kaiser, 2000). The current results presented here revealed that NRAF treatment caused a dose-dependent depletion in intracellular GSH level.

Mitochondria function normally in physiological conditions which contains adequate antioxidant level to prevent the initiation of oxidative damage and to remove excess ROS. However, under some circumstances, excessive generation mitochondrial ROS or a depletion of antioxidant such as reduced GSH occur resulting in the dysfunction of mitochondria (Pramanik et al., 2011). Evidences to date delineated that oxidation of GSH was mandatory for the occurrence of mitochondrial ROS generation, dissipation of mitochondrial membrane potential, and caspase-9 and -3 activation in cardiomyocyte apoptosis (Ghosh et al., 2005). Similarly, exposure of menadione in intestinal cells, early ROS production and mtGSSG formation eventually lead to mitochondrial dysfunction and apoptosis (Circu et al., 2008). Hereby, the results indicated a shift of redox equilibrium towards pro-oxidant state (Figures 4.7 and 4.8). The present findings provided interesting insight into the systematic effect of NRAF on intracellular redox state and apoptosis. This study revealed that the apoptotic mechanism of NRAF in HT-29 cells was partially related to ROS production, GSH depletion and $\Delta\psi_m$ loss. These phenomena are similar to the effects observed for a variety of clinical and pre-clinical anticancer drugs. For example, cisplatin can alter the intracellular ROS and $\Delta\psi_m$ in human HepG2 hepatoma cells (Chen et al., 2005). Curcumin, a pre-clinical anticancer drug, and $\alpha$-Hederin, experimental antitumor agent, (Swamy and Huat, 2003) can induce GSH depletion which caused dissipation of the $\Delta\psi_m$ with concurrent increased intracellular ROS prior to induction of apoptosis, release of cytochrome c and activation of caspases in cancer cells (Sandur et al., 2007). Collectively, these results strongly suggested that NRAF-induced depletion of intracellular thiols (Figure 4.8) distorted the
redox state, and then allowed reactive oxygen species to mediate a mitochondrial permeability transition. Withal, the current study has implied that oxidative stress due to early increased intracellular ROS production concomitant with GSH depletion subsequently result in the trigger of cellular damage and apoptosis.

ROS which performs as a secondary messenger elicit diverse redox-sensitive signaling cascades including mitochondrial intrinsic apoptotic cascade through interaction with Bcl-2 family proteins (Ji et al., 2011; Tsujimoto and Shimizu, 2007). Mitochondrial dysfunction is involved in the mitochondrial intrinsic pathway and often correlates to the attenuation of ΔΨm. This ascertains the mitochondrial permeability transition which is a vital step in the initiation of cellular apoptosis. There are multiple signaling mechanisms involved in the induction of apoptosis, modulation of Bcl-2 family members is one of the hallmarks of programmed cell death. Apoptosis is commonly dependent on the ratio of pro-apoptotic to anti-apoptotic protein members (McDonnell et al., 1996). Generally, the Bcl-2 family is categorized into proapoptotic (Bid, Bax and Bak) and anti-apoptotic (Bcl-2 and Bcl-xL) proteins which are imperative mediators of the mitochondrial-mediated pathway to modulate the permeabilization of mitochondrial membrane (Huang et al., 2003). These present findings indicated that NRAF up-regulated the Bax protein expression while down-regulating Bcl-2 protein expression, eventually leading to an increased in the Bax/Bcl-2 ratio. The level of Bax expression is essential determining index of apoptotic cell death, which also validates NRAF-induced apoptosis in HT-29 cells in mitochondrial signaling pathway. Once the mitochondrial-mediated pathway is activated, the insertion of Bax proapoptotic protein into mitochondrial membrane will lead to the formation of a protein complex with the mitochondrial voltage dependent anion channel (VDAC) (Shimizu et al., 1999). The Bax-VDAC complex will instigate the dysfunction of mitochondria through the dissipation of mitochondrial membrane potential (ΔΨm) (Green and Chipuk, 2008;
Green et al., 1998), by inducing the formation of the mitochondrial permeability transition pore (Bernardi et al., 2006) and subsequently the release of apoptosis promoting factors into the cytoplasm (Zha and Reed, 1997).

In a number of human cancers including prostate, lung and breast, the over expression of antiapoptotic Bcl-2 protein, or decrease of the proapoptotic protein expression, like Bax, has been observed (Ben-Ezra et al., 1994; Gee et al., 1994; Krajewska et al., 1996; Reed and Tomaselli, 2000). This results in resistance to a plethora of cell death stimuli including chemotherapeutic drugs (Cory and Adams, 2002). Bcl-2 maintains mitochondrial integrity whereas Bax destroys the mitochondrial integrity and causes loss of Δψm (Sharpe et al., 2004). Bax exerts proapoptotic activity by translocating from the cytosol to the mitochondria, where it evokes the opening of the mitochondrial voltage-dependent anion channel (VDAC) and the release of cytochrome c release from mitochondria to cytoplasm (Shimizu et al., 1999), whereas Bcl-2 exerts its apoptosis suppressor property, at least in part, by inhibiting the translocation of Bax to the mitochondria (Murphy et al., 2000; Nomura et al., 1999). Other studies have implicated that the elevation of Bax protein is closely related with the mitochondrial (intrinsic) pathway in apoptosis which involves the dissipation of membrane potential, cytochrome c release and subsequently leading to caspase activation (Jin et al., 2010; Ju et al., 2012).

The expression of the pro-apoptotic protein Bax is an early event that sensitizes the cells to undergo apoptosis. A number of models propose that cells can be committed to apoptosis by the upregulation of Bax alone (Finucane et al., 1999; Jurgensmeier et al., 1998; Xiang et al., 1996). In addition, several studies have proven that ROS directly down-regulates Bcl-2 and Bax translocation (Li et al., 2009; Zhang et al., 2006). The present findings have indicated an upregulation of Bax protein expression with a relatively low Bcl-2 protein expression and an increase in Bax/Bcl-2 ratio. We postulated that upregulation of Bax protein alone and/or increased Bax/Bcl-2 ratio
might be switching on the mitochondria-mediated apoptotic cascade. By up-regulating the Bax level in HT-29 cells, NRAF may promote the translocation of Bax from the cytosol to the mitochondrial membrane, leading to the release of cytochrome c. This triggers activation of downstream events which cytochrome c oligomerizes with apoptosis protease activating factor-1 (Apaf-1), deoxyadenosine triphosphate (dATP) and procaspases-9 to form apoptosome and further lead to caspase cascade activation that culminates in cellular apoptosis. The cascade is further amplified by the substantial disruption of Δψm elicited by NRAF. Collectively, findings suggested that the apoptosis induced by NRAF in HT-29 cells was mediated via mitochondrial-intrinsic pathway.

Copious studies have identified caspases as central players in the execution of apoptosis induced by a plethora of apoptotic stimuli. Caspases are aspartate-directed cysteine proteases that is important in the initiate and execute the process of apoptosis, necrosis and inflammation, failure of which may cause tumor development and several autoimmune diseases (Danial and Korsmeyer, 2004; Ghavami et al., 2009). Once activated, caspases activate other downstream caspases, leading to the execution stage of apoptosis. Apoptosis can be mediated through two pathways for caspase cascade, one involving caspase-8 and another involving caspase-9, eventually lead to the activation of caspase-3 (Fan et al., 2005; Zou et al., 1997). Besides, several studies have been reported that the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2 was correlated to the initiation of a cascade which results in the activation of caspases, such as caspase-3/7 (Yang et al., 1997). Thus, caspase activation is the key event in apoptotic cell death. The current findings on the NRAF-induced upregulation of Bax protein and the resultant increase in the Bax/Bcl-2 ratio led us to investigate the roles of caspase-3/7 and caspase-9 in the NRAF-induced apoptotic pathways. These experimental evidences suggest that NRAF induces apoptosis in HT-29 cells, accompanied by the activation of caspase-9 and caspase-3/7. This trigger was initiated by the caspase-9 and followed by
the effector caspase, caspase-3/7 which is one of the key mediators of apoptosis. Caspase-3 activates other caspases, cleaves cytoskeletal proteins, or activates the caspase activated deoxyribonuclease. Caspase-3 particularly cleaves an inhibitor of caspase-activated deoxyribonuclease (ICAD) which complexes with caspase-activated deoxyribonuclease (CAD). This releases the CAD from the complex and translocates into nucleus and brings about DNA fragmentation ultimately culminates in orderly cell demise (Fan et al., 2005; Gossau and Chen, 2004). Thus, these results can be substantiated by the morphological alteration observed in Hoescht 33342/PI staining as well as DNA fragmentation detected by TUNEL assay.

The qualitative phytochemical analysis for NRAF indicated the presence of flavonoids, tannins and saponins. These phytochemical compounds are known to play important roles in the bioactivity of medicinal plants. Flavonoids are reported to possess therapeutic properties and reduce disease risk. Flavonoids which are present in *N. ramboutan-ake* also exhibit anti tumor and anti cancer properties (Lopez-Lazaro, 2002). Besides, tannins is another class of phytochemical constituent was intensely present in NRAF. Tannins have been reported to have remarkable suppressive effort on tumors and cancers (Li *et al.*, 2003). Saponins were also present in *N. ramboutan-ake* extract and several pre-clinical studies have supported the usefulness of saponin in anticancer and antiproliferative effects (Liu *et al.*, 2000; Raju and Bird, 2007). The cytotoxic and anticancer property of NRAF against HT-29 shown in the present study is in accordance with the phytochemical constituent which may be associated with the intense presence of tannins and saponins in this fraction. In this context, further investigation is needed to identify the active compounds that are responsible for this bioactivity. Comprehensive phytochemical analysis for the isolation and identification of active compounds in NRAF is currently undertaken to provide a rational conclusion on its usage as a potential anticancer therapeutic agent.
CHAPTER 6

CONCLUSION

The results attained in the present study have demonstrated that the NRAF effectively inhibits the cell growth of HT-29 cancer cells through apoptosis in a dose-dependent manner. Taken together, NRAF mediated apoptosis through the imbalance of intracellular redox state (ROS generation and depletion of intracellular GSH), perturbation the mitochondrial function via loss of mitochondria membrane potential, followed by the alteration of the ratio of the Bax/Bcl-2, activation of caspase-9 and -3/7, resulting in DNA damage and eventually leads to apoptosis in HT-29 cells. These results indicated that NRAF warrants further comprehensive study to identify and isolate the bioactive compounds as well as in vivo study investigation by using tumor mouse models. In summary, for the first time the present study suggests that NRAF induced mitochondrial-mediated ROS-dependent apoptosis in HT-29 cells (Figure 6.1). Hence, cumulative results suggested that NRAF may be useful for the development of novel integrative and complementary medicine by promoting apoptotic cell death in HT-29 human colorectal cancer cells.
Figure 6.1: Schematic illustration of NRAF-induced mitochondrial-mediated ROS-dependent apoptosis in human colorectal adenocarcinoma HT-29 cells.
REFERENCES


APPENDIX

Poster

Antiproliferative and apoptosis effects of *Nephelium mutabile* Blume in HT-29 Human Colorectal adenocarcinoma cells.

Chim Kei Chan and Habsah Abdul Kadir
Biomolecular Research Group, Biochemistry Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, Malaysia.

Email: joanne_cck@hotmail.com

The aim of this study was to investigate the cytotoxic, antiproliferative and apoptosis effects of *Nephelium mutabile* (pulasan) rind in selected human cancer cell lines. The ethanol extract of the rind of *N. mutabile* was found to exert its effect on the growth of selected malignant cell lines including HT-29, HCT-116 and Caski cell lines by using MTT assay. The ethanol extract has effectively reduced the viability of the selected human cancer cell lines (HT-29 < HCT-116 < Caski) in a dose dependent manner. For a possible apoptosis mechanism, further analysis was conducted using flow cytometry and fluorescence microscopy. The cell growth was arrested in the sub-G1 phase when HT-29 cells were treated with the ethanol extract of *N. mutabile*. Early apoptosis was evident by the externalization of phosphatidylserine and disruption of mitochondrial membrane potential suggesting that apoptosis was induced by the ethanol extract of *N. mutabile* at a concentration as low as 50µg/ml. Apoptotic morphological changes were observed by Hoechst33342/PI staining showing chromatin condensation when the cells were treated with the ethanol extract of *N. mutabile*. Besides, the disruption of mitochondrial membrane potential resulted in the depletion of GSH intracellular content. The present findings suggest that *N. mutabile* rind merits further investigation as a potential therapeutic agent for the treatment of cancer.

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Aqueous Fraction of *Nephelium ramboutan-ake* Rind Induces Mitochondrial-Mediated Apoptosis in HT-29 Human Colorectal Adenocarcinoma Cells

Chim Kei Chan, Bey Hing Goh, Muhamad Noor Alfarizal Kamarudin and Habsah Abdul Kadir *

Biomolecular Research Group, Biochemistry Program, Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

* Author to whom correspondence should be addressed; E-Mail: aakhak@yahoo.com; Tel.: +603-7967-4363; Fax: +603-7967-4178.

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**Abstract:** The aim of this study was to investigate the cytotoxic and apoptotic effects of *Nephelium ramboutan-ake* (pulasan) rind in selected human cancer cell lines. The crude ethanol extract and fractions (ethyl acetate and aqueous) of *N. ramboutan-ake* inhibited the growth of HT-29, HCT-116, MDA-MB-231, Ca Ski cells according to MTT assays. The *N. ramboutan-ake* aqueous fraction (NRAF) was found to exert the greatest cytotoxic effect against HT-29 in a dose-dependent manner. Evidence of apoptotic cell death was revealed by features such as chromatin condensation, nuclear fragmentation and apoptotic body formation. The result from a TUNEL assay strongly suggested that NRAF brings about DNA fragmentation in HT-29 cells. Phosphatidylserine (PS) externalization on the outer leaflet of plasma membranes was detected with annexin V-FITC/PI binding, confirming the early stage of apoptosis. The mitochondrial permeability transition is an important step in the induction of cellular apoptosis, and the results clearly suggested that NRAF led to collapse of mitochondrial transmembrane potential in HT-29 cells. This attenuation of mitochondrial membrane potential ($\Delta \psi_m$) was accompanied by increased production of ROS and depletion of GSH, an increase of Bax protein expression, and induced-activation of caspase-3/7 and caspase-9. These combined results suggest that NRAF induces mitochondrial-mediated apoptosis.

**Keywords:** *Nephelium ramboutan-ake*; pulasan; cytotoxicity; apoptosis