A STUDY ON THE POTENTIAL OF RAMBUTAN AND WATERMELON IN CHEMOPREVENTION ACTIVITY ON OSTEOSARCOMA CELL LINES

WAN NUR HIDAYATI BINTI WAN SULAIMAN

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by

WAN NUR HIDAYATI BINTI WAN SULAIMAN

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DEDICATIONS

This thesis is especially dedicated to:

***My parents**, who are so precious to me,*

***My sisters and my brother**, who have filled my life with love, joy and happiness,*

***Mohd Farid Nasikin**, who supported me throughout these years*

&

***My friends**, who always there for me!*

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LIST OF ABBREVIATIONS

µg/ml	Microgram per milliliter
µl	Microliter
AIF	Apoptosis-inducing factor
AIP	Inhibitor of apoptosis
Apaf-1	Apoptotic protease activating factor 1
APS	Ammonium persulfate
ATP	Adenosine triphosphate
Bax	Bcl-2-associated X protein
Bcl2	B-cell lymphoma 2
BH	Bcl_2 homology
BH3	BH3-interacting-domain death
BID	BH3-interacting-domain death
CAK	CDK activating kinase
CCA	Cholangiocarcinoma
CDKI	Cyclin dependent kinases inhibitors
CDKs	Cyclin-dependent kinases
CO_2	Carbon dioxide
CRL 1543	Human osteosarcoma cells
ddH_2O	double-distilled water
DISC	Death inducing signalling complex
DMEM F/12	Dulbecco's Modified Eagle Medium F/12
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimetyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediamineteraacetic acid
FADD	Fas-associated death domain
FasL	Fas ligand
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate

g	Gram
G0	Resting phase
G0/G1	Sub G0 phase/Gap phase I
G1	Gap phase I
G2	Gap phase II
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCl	Hydrochloric acid
HepG2	liver hepatocellular carcinoma
HL-60	Human leukemia cells
HPLC	High performance liquid chromatography
HPV	Human papilloma virus
IC_{50}	Half minimal inhibitory (50%) concentration
JNK	c-Jun NH_2-terminal protein kinase
kDa	Kilodalton
m	Meter
M	Mitosis
MAPK	Mitogen-activated protein kinases
MAT1	Menage A Trosi 1
mg/ml	Milligram per milliliter
ml	Millilitre
mm	Millimetre
mTOR	Mammalian target of rapamycin
mtPTP	Mitochondrial permeability transition pore
NaCl	Natrium chloride
NHOst	Normal human osteoblast cells
PAGE	Polyacrylamide gel electrophoresis
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffer saline
PCD	Programmed cell death
PI	Propidium iodide

PS	Phosphatidylserine
RE	Rambutan extract
RIPA	Radioimmunoprecipitation assay
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RT	Room temperature
S	Synthesis
SD	Standard deviation
SDS	Sodium dodecyl sulphate
TBE	Trypan blue exclusion method
TBS	Tris-buffered saline
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor
TNFR1	Tumour Necrosis Factor Receptor 1
TRAIL	Tumour necrosis factor-related apoptosis-inducing ligand
UV	Ultra violet
WE	Watermelon extract

KAJIAN MENGENAI POTENSI RAMBUTAN DAN TEMBIKAI DALAM AKTIVITI KEMOPENCEGAHAN KE ATAS SEL OSTEOSARKOMA

ABSTRAK

Osteosarkoma adalah kanser malignan ‘kelas pertama’ yang biasanya menyerang kanak-kanak dan golongan muda. 85% daripada pesakit masih menghadapi masalah metastasis walaupun terdapat kemajuan dalam rawatan seperti kemoterapi dan pembedahan. Rawatan kanser menggunakan agen kemopencegahan yang bukan sahaja boleh merencat pertumbuhan sel kanser, tetapi juga dengan sasaran yang tepat pada peringkat molekular menjadi objektif yang paling penting dalam kajian ini. Tesis ini mengkaji potensi buah-buahan yang terdapat di Malaysia, *Nephelium lappaceum* (rambutan) dan *Citrullus lanatus* (tembikai) terhadap kesan kemopencegahan ke atas sel osteosarkoma (CRL 1543). RE dan WE menunjukkan proses perencatan terhadap pertumbuhan sel osteosarkoma dan nilai IC_{50} iaitu 0.005 mg/ml dan 0.5 mg/ml masing-masing. Proses perencatan sel dikaitkan dengan perencatan kitaran sel dan mencetuskan apoptosis. RE dan WE juga menunjukkan perubahan morfologi yang terbentuk melalui proses apoptosis. RE dan WE juga telah menyebabkan perencatan kitaran sel masing-masing pada fasa G2/M dan G0/G1. Pengenalpastian terhadap kebarangkalian terhadap laluan isyarat molekular sel penyebab kepada perencatan sel oleh apoptosis dan gangguan terhadap kitaran sel telah disiasat dan dikenal pasti laluan isyarat apoptosis oleh RE adalah melalui intrinsik melalui pengakrifan terhadap caspase-9 dan caspase-3. Manakala WE pula melalui laluan isyarat apoptosis extrinsik melalui pengaktifan

caspase-3 tetapi tidak pada caspase-9. Penurunan paras protein antiapoptosis, $Bcl_{2,}$ oleh RE dan WE melengkapi penemuan tersebut. Peningkatan paras protein $p27^{Kip1}$ dan penurunan paras protein cyclin D1 adalah konsisten dengan penemuan dimana WE telah menyebabkan gangguan kitaran sel pada fasa G0/G1. RE pula tidak menyebabkan sebarang perubahan terhadap paras protein cyclin D1 yang juga konsisten dengan gangguan terhadap fasa G2/M tetapi tidak pada G0/G1. Protein $p27^{Kip1}$ juga telah dilaporkan terlibat dalam proses apoptosis konsisten dengan peningkatan paras protein $p27^{Kip1}$ oleh RE. RE dan WE didapati tidak toksik terhadap sel normal lantas membuktikan bahawa kedua ekstrak ini mempunyai kesan yang baik sebagai kemopencegah terhadap CRL 1543. Secara keseluruhannya, data yang terkumpul dari tesis ini membuka ruang baru terhadap potensi bahan buangan daripada kulit rambutan dan tembikai sebagai agen kemopencegahan terhadap CRL 1543.

A STUDY ON THE POTENTIAL OF RAMBUTAN AND WATERMELON IN CHEMOPREVENTION ACTIVITY ON OSTEOSARCOMA CELL LINES

ABSTRACT

Osteosarcoma is a primary malignant bone tumour that commonly found in children and young adults. Despite of the significant advances of treatment such as chemotherapy and surgery were developed against osteosarcoma, 85% of the patients still suffer from metastases. The improvement in cancer treatment using cancer chemopreventive agent which is not only can inhibit the cancer cells proliferation but with define molecular target has become the main objective in this study. This study evaluated the potentiality of the Malaysian local fruits, *Nephelium lappaceum* (rambutan) and *Citrullus lanatus* (watermelon) in exhibiting chemopreventive effects on osteosarcoma cells (CRL 1543). RE and WE showed the growth inhibitory effect and with IC_{50} value at 0.005 mg/ml and 0.5 mg/ml respectively. The cell deaths are believed to be associated to the induction of apoptosis and cell cycle arrest. Morphological changes displaying apoptosis also can be observed upon treatment with RE and WE. Moreover, RE and WE also caused alteration to the cell cycle regulation on CRL 1543 at G2/M and G0/G1 phase respectively. Identification of the possible signalling pathways of cell death by apoptosis and cell cycle at molecular level were investigated and the apoptosis induction by RE followed an intrinsic via activating caspase-9 and caspase-3. Meanwhile, WE induced apoptosis by extrinsic pathway via activating caspase-3 but not on caspase-9. The downregulation of antiapoptotic Bcl_2 protein expression by RE

and WE also complemented the findings. The upregulation of $p27^{Kip1}$ and the downregulation of cyclin D1 protein expressions were consistent with the finding of WE caused cell cycle arrest at G0/G1 phase. RE otherwise showed no significant changes for cyclin D1 protein expression consistent with the G2/M blockage but not in G0/G1 phase. $p27^{Kip1}$ has also been reported to induce apoptosis consistent with the increased of protein expression of $p27^{Kip1}$ by RE. RE and WE showed no toxicity effect on normal cells thus proved the extracts features a potential chemopreventive agent on CRL 1543. Overall, the data collected provide new insight of using the waste products by rambutan and watermelon rinds which can be used as chemopreventive agents on CRL 1543.

CHAPTER 1

INTRODUCTION

1.1 Introduction

Cancer disease is the second of cause death after cardiovascular disease. It has been reported that the incidence is over 6 million of cases annually across the globe (Srivastava *et al.,* 2005). Although the survival of the patients had increased due to the rapid advancement of neoadjuvant chemotherapy and surgery, however, drug resistance acquired by cancer cells has led to treatment failure. Factors like rapid emergence of the new diseases and the development of the drug resistance have brought to the exploration of natural products as alternative strategies for cancer treatment (Cragg *et al.,* 1997).

Natural products exist in large diversity in nature and have become the source for ameliorate diseases for a long time ago (Graham *et al.,* 2000). Its accumulations also have been recorded in the pharmacopeia (Newman *et al.,* 2000). Almost 25 years ago, World Health Organization (WHO) estimated that 80% of the people in the developing countries, which means around 3.5 to 4 billion of people in the world, rely on plants as traditional medicines for their primary health care. To add more, around 85% of it involves in plant extracts (Farnsworth, 1988). Recently, the advancement in science and technology has brought to the discovery of the commercial drugs and until today, 50% of all drugs in clinical use are still represent by natural products (their derivatives and

analogs) while another 25% of all drugs comes from higher plant-derived natural products (Balandrin, 1993; Raskin & Ripoll, 2004).

Currently, chemoprevention has caught many attentions in the field of scientific investigations. Chemoprevention is defined as either suppressing, delaying or reversing the carcinogenesis process by using natural product thus served as a new strategy for the improvement to the survival rate of patients (Sporn & Suh, 2002). Plants give many advantages to human because it is not only included in the dietary consumptions but also used as the traditional medicines. Natural product contains several bioactive plant compounds that are also the product of metabolism, in which their function in life processes acts a similar way to the compounds that operate in human and animals (Ameenah, 2006). These bioactive plant compounds are including plant polyphenols which has been reported to display antiproliferative and cytotoxicity effect to several tumors but not on normal cells (Denise *et al.,* 1996; Georgine *et al.,* 1997). Besides that, an ideal chemopreventive agent should display a significant bioavailability, safety, inexpensive and can target on more than one mechanism of actions. Thus, dietary consumption of foods and herbal medicines is a convenient method of administering potentially chemopreventive agents in a cost-effective manner.

The mechanism of cancer cell death induced by bioactive compounds from natural product is one of the approaches for chemopreventive activity. The chemopreventive agents have the ability to block or delay the promotion or progression of pre-malignant or malignant cells by modulating cell proliferation or differentiation (Sun *et al.,* 2004).

Thus, the alteration on the cellular signalling pathways such as on apoptotic cell death and disturbation on cell cycle regulations by chemopreventive agents can provide information in natural drug discovery (Fulda & Debatin, 2006; Khan *et al.,* 2009; Vermeulen *et al.,* 2003).

Apoptosis, a form of programmed cell death, plays a critical role in both development and tissue homeostasis and it can be characterised by morphological and biochemical hallmarks, including cell shrinkage, nuclear DNA fragmentation and membrane blebbing (Hengartner, 2000). Activation of apoptosis signalling pathway is the key point by chemopreventive agents in cancer chemoprevention and defects in apoptosis signalling pathways will lead to the tumour resistance (Debatin, 2004). It is resulted by the activation caspases, a family of cysteine proteases that act as common death effector molecules in various forms of cell death (Degterev *et al.,* 2003; Fulda & Debatin, 2006). Thus, a better understanding of this mode of tumour cell death by chemopreventive agents will provide a molecular basis for new strategies targeting caspase-dependent and independent death pathways.

On the other hand, cell cycle regulation in normal cells will control and maintain homeostasis within cells by determining whether a cell will continue to proliferate, enter into a quiescent state or undergo apoptosis (Foster, 2008). The cell cycle regulation in normal cells is tightly controlled by checkpoints and any malfunction in this regulation is believed to be one of the hallmarks for cancer. It is regulated by the action of cyclin-dependent kinases (CDKs) that will form a complex with a regulatory

cyclin protein and will be activated at specific points in the cell cycle (Devault *et al.*, 1992; Schafer, 1998). The cell cycle consists of four phases (G1, S, G2 and M) and the alterations on these phases in cell cycle regulations can become activated due to many factors such as DNA damage, defects during replication of DNA or due to the exogenous stress signals (Gabrielli *et al.,* 2012; Vermeulen *et al.,* 2003). This will bring to the development of tumour cells. Thus, another approach of chemoprevention is by targeting the signal signalling pathways related to the cell cycle events by chemopreventive agents in tumour cells either at G1, S or G2/M phase that will eventually induce apoptotic cell death to the tumour cells (Sarkar & Li, 2004).

Rambutan (*Nephelium lappaceum*) and watermelon (*Citrullus lanatus*) rinds are basically inedible due to its lack of taste and were treated as agriculture by-product. However, studies have shown that both rinds hold potential bioactive compounds. According to Palanisamy *et al.,* 2008, rambutan contains polyphenolic compound that possess the antioxidant capacity. Watermelon also has been reported to possess a small amount of polyphenolic compound and a low amount of vitamin C when compared to other fruits (Gil *et al.,* 2006). Epidemiology studies have also reported that watermelon contains lycopene which is an antioxidant compound from member of carotenoid family can decrease risk of various cancers such as lung, prostate and colon cancer (Cai *et al.,* 2004; Giovannucci, 1999; Rao & Agarwal, 1998). The antioxidant compound has the ability to scavenge of free radicals and thus inhibit oxidation reactions (Jacob *et al.,* 2008). The overproduction of free radicals in human's body might lead to the oxidative damage of biomolecules such as proteins, DNA or lipid, that eventually leads to chronic

diseases such as atherosclerosis, aging, diabetes and including cancer (Barry, 1994; Steinmetz & Potter, 1996). Therefore, the mechanism of tumour cell death by apoptosis and deregulation of cell cycle related to these bioactive compounds from rambutan and watermelon in human system are worth to be studied for the development of new chemopreventive agents and to our best knowledge, no studies have been done on rambutan and watermelon rinds on osteosarcoma or known as bone cancer either *in vitro* or *in vivo*.

1.2 Problem statement

Cancer is a major health problem in Malaysia. According to Penang Cancer Registry in 1994, it is reported that the age standardised incidence rate for all cancers in Malaysia was 115.9 per 100 000 for males and 119.7 per 100 000 for females and it is expected to rise with an increase in aging population. In 1998, Malaysia's population was 21.4 million, of whom 4% were aged 65 years and above. The incidence of cancer is expected to rise with an increase in aging population. In 1957, the proportion aged of more than 60 years was 4.6%, had increased to 5.7% in 1990 and it is projected to be 9.8% in 2020 (Karim, 1997). Currently, the treatment for cancers are varies. It includes surgery, radiotherapy, chemotherapy and hormonal therapy. However, the conventional treatment does not show an improvement and the prognosis of cancer is still poor. Thus the new therapeutic approach is seriously needed to improve the quality of health of the patients.

Therefore, chemoprevention can be the most reliable treatment nowadays because it uses natural product to either suppress, delaying or reversing the process of carcinogenesis. By understanding the mechanism of cell death and targeting the cell signalling pathways involving apoptosis and cell cycle in tumour cells will give important information in cancer chemoprevention. This has given the idea to consult a research involving the comparative chemopreventive effect between rambutan (*Nephelium lappaceum*) and watermelon (*Citrullus lanatus)* rind extracts in order to see the potentialities of rambutan and watermenon as chemopreventive agents on human osteosarcoma cancer cell lines.

1.3 Research objectives

The main idea of this research is to get a better understanding on chemopreventive effect of rambutan and watermelon on human osteosarcoma cancer cell lines at molecular level. In order to achieve the main idea of the research, there are few specific objectives need to be outlined.

i. To determine the inhibitory concentration 50% (IC_{50}) and its antiproliferative effect of rambutan and watermelon rind extracts on human osteosarcoma cancer cell lines.
ii. To observe any morphological changes upon treatment with rambutan and watermelon rind extracts on human osteosarcoma cancer cell lines.
iii. To investigate the effect of rambutan and watermelon rind extracts on apoptosis on human osteosarcoma cancer cell lines.
iv. To investigate the effect of rambutan and watermelon rind extracts on cell cycle regulation on human osteosarcoma cancer cell lines.
v. To investigate the effect of rambutan and watermelon rind extracts on apoptosis markers such as caspases, MAPK (mitogen-activated protein kinase) and Bcl2 proteins and cell cycle proteins such as cyclin D1 and p27Kip1.
vi. To determine the cytotoxicity effect of rambutan and watermelon extracts on normal osteoblast (NHOst) cell lines.

The flowchart of the experimental design carried out in this study is shown in APPENDIX A

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer

Cancer disease is a second of natural cause of death after cardiovascular disease where the incidence are over 6 million of cases has been reported annually across the globe (Srivastava *et al.,* 2005). Cancer is always defined simply as the uncontrolled proliferation of abnormal cells. Although it is correct, but this is actually a very general definition that does not explain its extremely complex process where it also involves pathological state of cancer. The multifunctional and dynamic molecular event of cancer processes requires the changing effect of neoplastic cells and its interaction with their surrounding stroma and also with the immune system (Carbone & Pass, 2004). Cancer in general is characterised by two main characteristics. Firstly, it has an uncontrolled cells growth that eventually form a new growth, known as tumour or neoplasm. Secondly, the cells can migrate from original site to distant site and develops into another type of tumour tissues by spreading to other organs, resulting in metastasis. However, Hanahan and Weinberg, (2000) had characterised cancer by the following six hallmarks as in Figure 2.1.

Cancer is caused by many factors contributing to the disease by which environmental factors and endogenous factors are the examples that may contribute to the cancer initiation and development. In general, tumour occurs when the cell divides excessively in the body. Typically, neoplasm occurs when the cell division is uncontrollable and

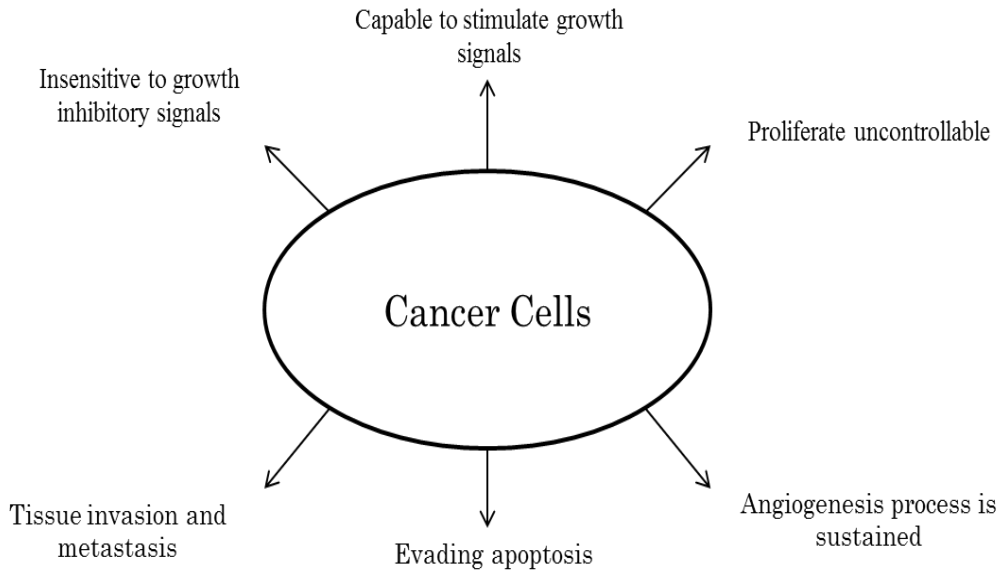

Figure 2.1: Hallmarks of carcinogenesis. The complex process by which normal cells will turn into malignant tumour including i) it has enough capability to stimulate growth signals, ii) it is insensitive to growth inhibitory signals, iii) potentially to proliferates uncontrollable, iv) angiogenesis process is sustained, v) as the ability to evade the apoptosis and vi) tendency to invade surrounding tissue and travel beyond the site of origin (Hanahan & Weinberg, 2000).

unbalanced (Bhadauria *et al.,* 2012). Since cell division requires in the biological activity where it replace the older cells that is damaged or dysfunctional to new functional cells in balance, if the cell division and death is disturbed, a tumour may form. Tumour varies to the normal cells because they differ in many aspects such as in the growth control, morphology, cell-to-cell interactions, membrane properties, cytoskeletal structure, protein secretion and gene expression (Joyce & Pollard, 2009). However, tumour can be cancerous (malignant) or non-cancerous (benign). The processes of which the normal cells turn into cancer cells are called carcinogenesis.

2.1.1 Pathogenesis of cancer

Cancer can be resulted from the complex process of carcinogenesis where it involves a series of individual steps upon activation of protooncogenes and tumour suppressor genes. They can be divided into three distinct molecular and cellular alteration stages, which are initiation (normal cell becomes transformed or initiated cell), followed by promotion (initiated cell become preneoplastic cell) and later the progression (preneoplastic cell become neoplastic cell) stage. The process of carcinogenesis is summarised in Figure 2.2.

The process of carcinogenesis begins with initiation process which resulted from rapid and irreparable process which includes normal cells that exposed to any carcinogenic agents. The metabolic activation has distributed it to organs and tissues thus subsequently interact covalently with target cell DNA, leading to the genotixic damage that remains unrepaired or misrepaired. These transformed cells will undergo many

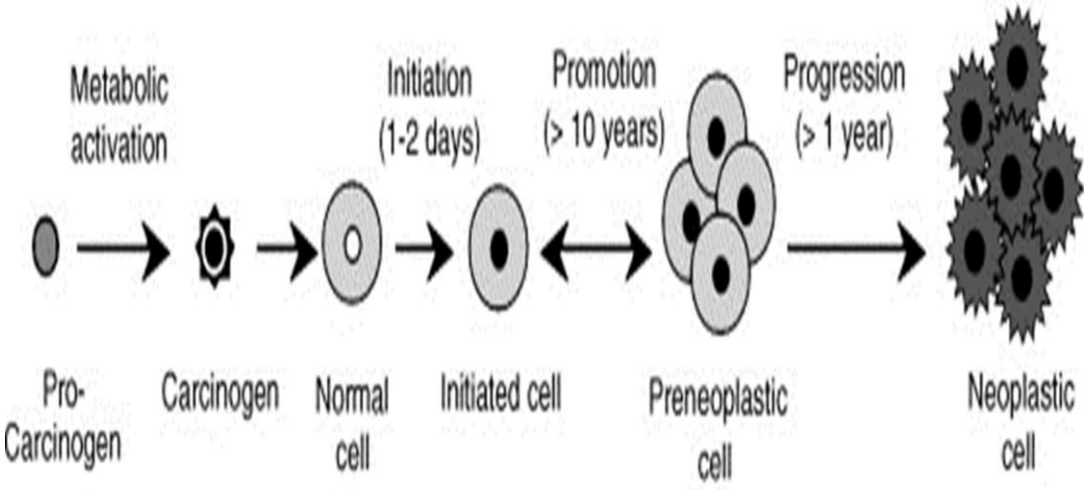

Figure 2.2: Schematic representation of multi-stage carcinogenesis. These process involved steps from initiation, promotion and later by progression process (adapted and modified from Annelyse *et al.*, 2005).

changes to form preneoplastic cells (Young-Joon, 1999). The initiation process however, is not rapid as compared to initiation process. It is the expansion of damage cells to actively proliferate and in this stage, oxidative stress and chronic inflammatory become the key point in promoting tumour proliferation and angiogenesis that are necessary for tumour growth (Coussens & Werb, 2002).

While the last stage of carcinogenesis which is the progression, involves gradual production of the new clones of tumour cells and conversion of tumour cells into invasive cells, thus leading to increase of its metastasis potential (Pan *et al.,* 2011). This progression process (angiogenesis/invasion/metastasis) involves rate-limiting steps that are influenced by non-malignant cells of the tumour microenvironment (Figure 2.3).

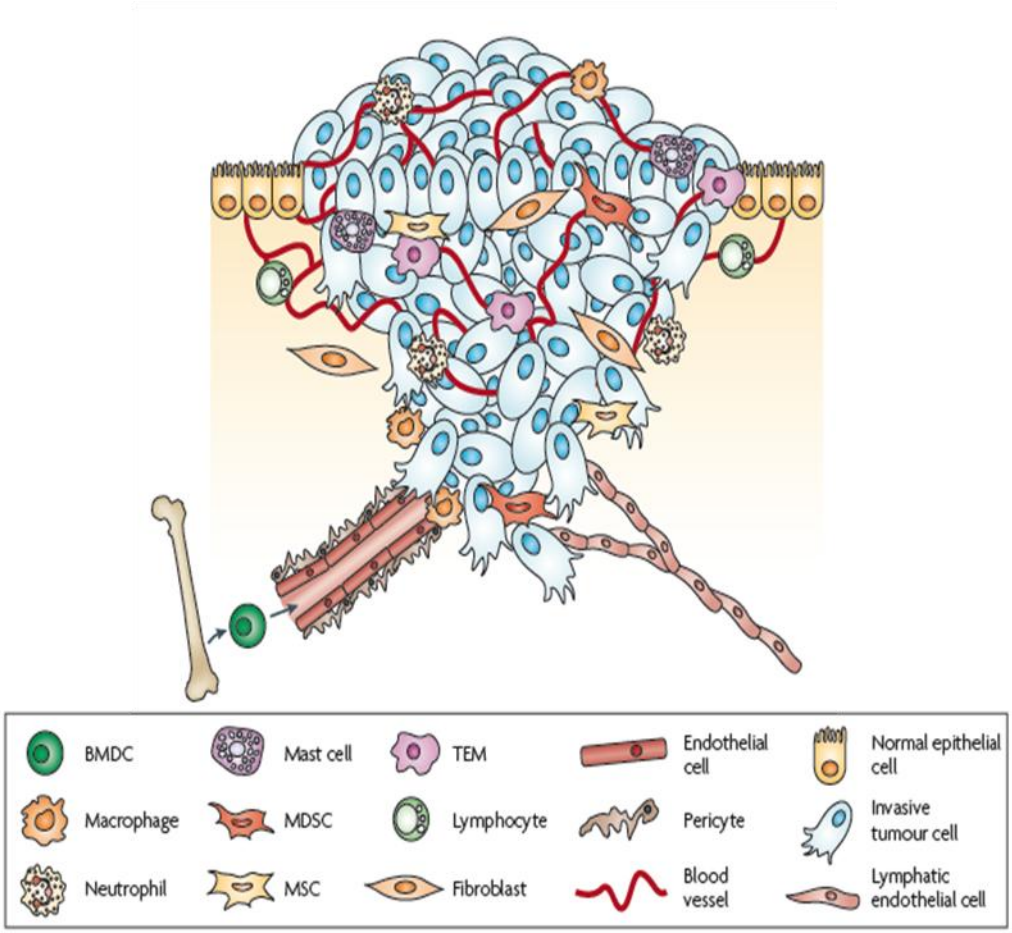

Figure 2.3: The primary tumour microenvironment. The tumour microenvironment comprises of a numerous cells such as the endothelial cells of blood and lymphatic circulation, stromal fibroblasts and a variety of bone marrow-derived cells including macrophages, myeloid-derived suppressor cells, TIE2-expressing monocytes and mesenchymal stem cells (adapted from Joyce & Pollard, 2009).

2.2 Osteosarcoma (human bone cancer)

Osteosarcoma is a common primary malignant bone tumour that is commonly known as childhood diseases. However, osteosarcoma can continue throughout adulthood (Figure 2.4). It arises from primitive bone-forming mesenchymal cell that histopathologically shows osteoid formation (Schajowicz *et al.,* 1995). Only 5% of osteosarcoma occurs in jaws (Rosen *et al.,* 1982) and craniofacial osteosarcoma that often located in the mandible or maxilla (Vege *et al.,* 1991; Wanebo *et al.,* 1992) accounts only 6-13% of all osteosarcomas (Caron *et al.,* 1971; Clark *et al.,* 1983; Garrington *et al.,* 1967) and often located in the mandible or maxilla. Osteosarcoma in jaws is rare, but more aggressive malignancy compared to osteosarcoma of the long bones. Both types of osteosarcoma differ in its biological behaviours, but share the same histologic features. Classical osteosarcoma of long bones most often affects adolescents and young adult meanwhile craniofacial osteosarcoma typically occurs in the third or fourth decade of life (Bertoni *et al.,* 1991; Caron *et al.,* 1971; Clark *et al.,* 1983; Delgado *et al.,* 1994; Mark *et al.,* 1991).

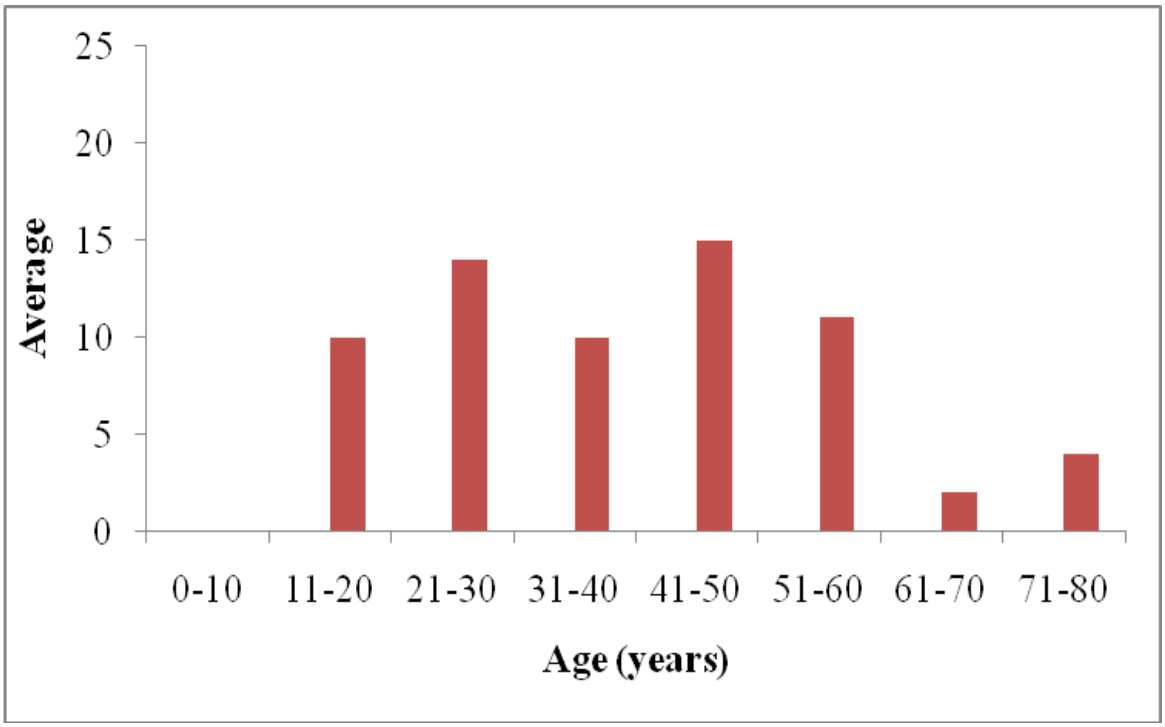

Figure 2.4: Age-related incidence of osteosarcoma (adapted and modified from Clark *et al.*, 1983).

2.2.1 Symptoms and treatment for osteosarcoma

Patients with craniofacial osteosarcoma may have the symptoms such as changes in the fit of dental prosthesis, the teeth become loose or changes in tooth position. The signs and symptoms of craniofacial osteosarcoma are also including regional swelling, pain and paraesthesia (Clark *et al.,* 1983). However, these signs and symptoms are non specific, thus there is often a delay before correct diagnosis can be made (August *et al.,* 1997; Patel *et al.,* 2002). Due to poor prognosis in patients with osteosarcoma, it is very important to find other alternative ways in order to treat the patients with osteosarcoma. Metastasis also common and occurs within 1 to 2 years and it remains the primary cause of poor survival for patients with osteosarcoma. Chemotherapy followed by surgery becomes the only method of treatment for patients with this disease (Link *et al.,* 1986; Trieb *et al.,* 2003; Valabrega *et al.,* 2005). However, surgery is also associated with lower survival rate meanwhile chemotherapy does not improve the cancer prognosis. Otherwise, early diagnosis and radical surgery are the better ways to improve survival rate. Although the prognosis of craniofacial osteosarcoma is poorer than osteosarcoma in long bones, research on the combination of adjuvant therapy and radical surgery need to be improved (Mardinger *et al.,* 2001). This is due to the combination of surgery and aggressive adjuvant chemotherapy did improved the overall survival for patients with osteosarcoma, however, more than 90% of the patients with osteosarcoma and 30-4-% patients with non metastatic disease still experience relapse after the treatment (Goorin *et al.,* 1991; Link *et al.,* 1986).

2.3 Natural products for cancer treatment

Plant kingdom provides a great source of medicine since the beginning of human civilisation and during that time, plants have become dominant in treating ailment and diseases. The earliest written document of medicinal plants for various illnesses has been recorded in at least 4000 years ago to the Sumerians (Jin-Ming *et al.*, 2003). The ancient civilisations of the Chinese, Indians and North Africans also provide written evidences for the use of natural sources for curing various diseases (Phillipson, 2001). For instance, raw garlic was prescribed for circulatory disorders, mandrake was prescribed for pain relief and tumeric possesses blood clotting properties and these traditional remedies are still being used until now (Phillipson, 2001). However, in the nineteenth century, the isolating and purifying compounds from natural sources alone rather than accepting the medicinal power of the crude extracts has started to become famous (McCurdy & Scully, 2005). Taxol (Paclitaxel), camptothecin, morphine and quinine are the examples of drugs derived from active components isolated from natural sources (Phillipson, 2001). Morphine has been isolated from *Papaver somniferum* while atropine is obtained from *Atropa belladonna* meanwhile Taxol is obtained from the stem bark of *Taxus brevifolia* (Western yew). Taxol has brought interest to the scientists since it showed potent anti-leukemia and anti-tumor properties in cell assays (Wani *et al.*, 1971). Between the years 1981-2006, about a hundred anticancer agents have been developed, of which, twenty five are natural product derivatives, eighteen are natural product mimics, eleven candidates are derived from a natural product pharmacophore, and nine are pure natural products (Newman & Cragg, 2007). Thus natural sources make a very significant contribution to the health care system.

2.3.1 Secondary metabolites

Secondary metabolites are actually a generic name for over 30,000 different substances that can be produced by plants. Secondary metabolites have a lot of purposes and functions to the plant such as in giving colour to the plants, protecting the plant from the pest, as plant's own hormones as well as can act as attractants. Since these active secondary metabolites have given a great impact to human health, it has been noted to be prescribed in drugs and it is known that 25% of all drugs come from natural products (Raskin & Ripoll, 2004). Secondary metabolites do not have nutrient characteristic to human they exist in a small amount in the human body. However, their effect in human health is actually enormous and many of these effects are not well known and required a lot of investigations. The wide variety of bioactive compounds and nutrients in fruit and vegetables has been hypothesised to have the ability in inhibiting three stages of carcinogenesis; initiation, promotion, and progression. These bioactive compounds have been prove in preventing cancer development by affecting cell cycle regulation and progression, inhibit the activation of pro-carcinogens, quench exogenous and endogenous radicals, stimulating the immune system, and also modulating hormone metabolism (Smith-Warner *et al.,* 2006). Figure 2.5 summarises the structural classes of pharmacologically active secondary metabolites from plants such as phenolics compound, terpenoids and steroids (Schmidt *et al.,* 2007).

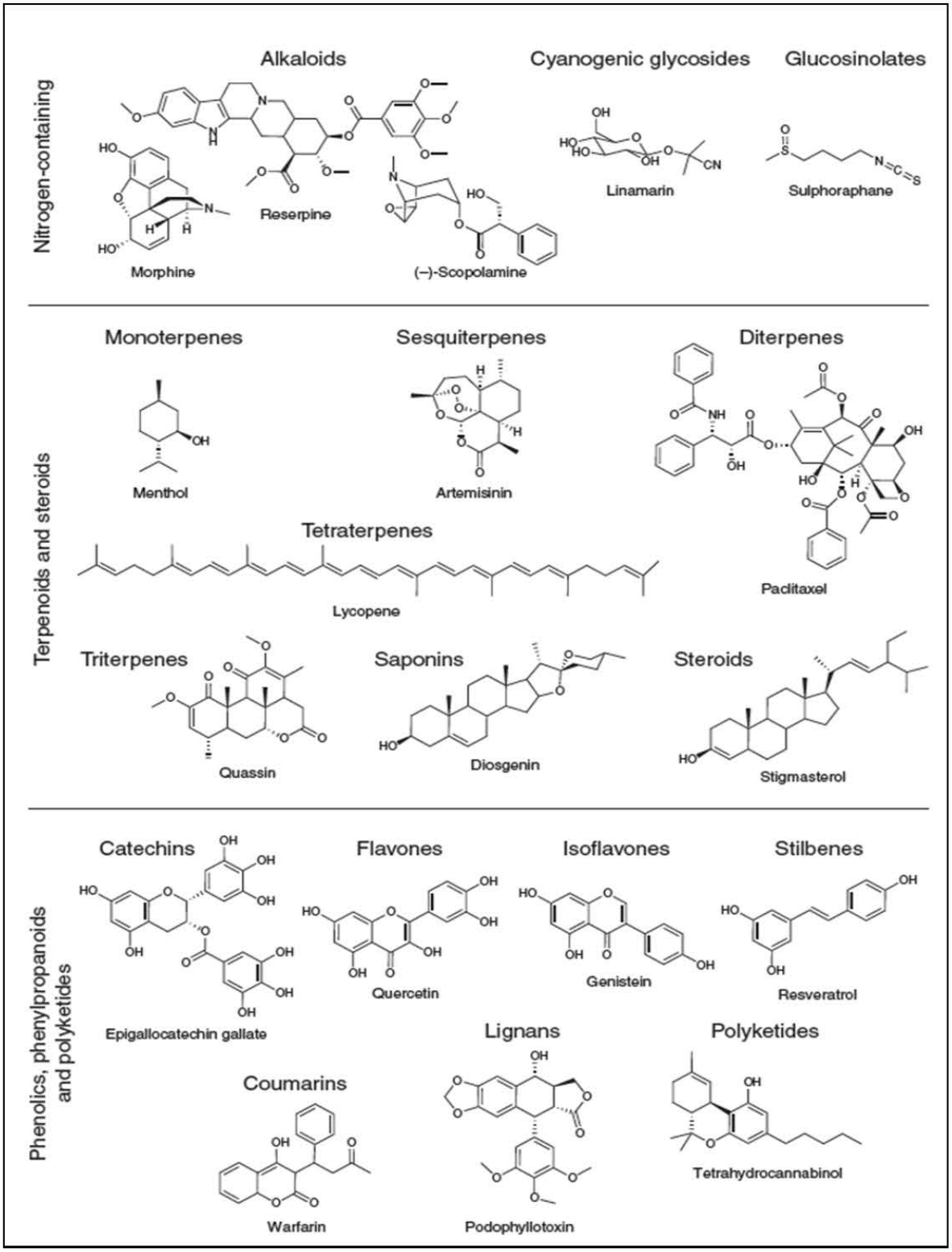

Figure 2.5: Classification of pharmacologically active secondary metabolites (adapted from Schmidt *et al.*, 2007).

2.4 Cancer chemoprevention

Currently, the conventional treatments have failed to exert control of the metastasis. Chemoprevention is a very attractive strategy which uses natural products in either suppress, delay or reverses the process of carcinogenesis (Sporn, 1976). The close relationship between dietary intake with their benefits in 'life-style-related-diseases' such as cancer and heart disease has been proven for decades. According to Williams, 1995, fruit juices possess health benefits that are associated with inhibiting certain cancers and heart disease. Moreover, according to epidemiological studies, the regular consumption of fruits and vegetables might provide health benefits due to their natural compounds such as carotenoids, flavonoids, phenolic compounds and vitamins (Gardner *et al.,* 2000).

Compounds with diverse structural and chemical components are always associated with reducing the risk of cancer. For instance, Vitamin C, E and β-carotenes are well known as powerful antioxidant properties (Krinsky & Johnson, 2005). However, instead of these antioxidant properties, phenolics and polyphenolics, which also known as secondary metabolites acts as a better scavenger of free radicals compared to vitamin C and E (Shi *et al.,* 2003). Thus, the mechanism of chemopreventive effect by these phytochemicals at molecular level is one of the promising approaches of cancer chemoprevention. The identification of new chemopreventive agents is still lacking and further investigation is needed since Malaysia is a great source of plant kingdom (Murakami *et al.,* 2000).

2.4.1 Mechanisms of chemoprevention

2.4.1.1 Apoptosis

Around 10 billion of cells die in each day in order to give ways to new cells arising through mitosis. This process is needed to maintain the homeostatic balance of the body. Apoptosis that also known as programmed cell death (PCD) was firstly reported in 1964 by Gewies and Grimm, (2003). It is a vital process in this life-death cycle. Apoptosis is necessary for the elimination of pathogen infected cells from the body, as well as activated- or auto aggressive immune cells (Heemels, 2000). It is a physiological process of removing the dead or damaged cells effectively during development which cannot be repaired during the cell cycle (Gewies & Grimm, 2003; Kerr *et al.,* 1972). Failure of these cells to be eliminated through apoptosis may lead to their immortalisation and possible malignancy (Evan & Littlewood, 1998). The word 'apoptosis' is from Greek word which means 'falling off or dropping off' in analogy of petals dropping off from a flower or leaves falling off from trees. This term 'apoptosis' emphasising that the death of living matter is a necessary part of the life cycle in organisms. Since apoptosis is a pathway that all cells possess to death, thus, defects in apoptosis pathway and the ability for the cells to evade death is one of the hallmarks of cancer (Okada & Mak, 2004).

The understanding of apoptosis plays an important role in the study of physiological process in cancer research, for instance, the mutation of p53 gene has been significant with the production of defective proteins that can be found in cancer cells that resulted in resistance to the apoptosis (Debatin, 2004). The resistance of cancer cells towards

therapeutic agents therefore, has brought to the new research focusing on devising ways to overcome this resistance and thus subsequently triggers apoptosis (Okada & Mak, 2004). The ability of p53 to induce apoptosis is a crucial defence against cancer where apoptosis is also a key to immune defence and elimination of cancerous cells (Yu, 2006). The activation of apoptosis pathway leads to the suicidal cell by characteristic processes that the dead cells will ultimately fragmented and engulfed by cells such as macrophages (Figure 2.6).

2.4.1.1.1 Morphology features of apoptosis and necrosis

Unlike necrosis, apoptosis produce some fragments called apoptotic bodies that phagocytic cells are able to engulf the damage cells. Necrosis otherwise is a traumatic but passive form of cell death resulting to the released of cellular contents into the surrounding environment, resulting to the damage to the surrounding cells and causes a strong inflammatory response in the corresponding tissues (Gewies, 2003). Apoptosis is characterised by the biochemical hallmarks and distinct morphology such as cell shrinkage, membrane blebbing, or deformation of the cells and loosen contact with the neighbouring cells. The apoptotic bodies otherwise contain cytosol, organelles and condensed chromatin (Okada & Mak, 2004). The DNA ladder with multiple fragments caused by internucleosomal DNA cleavage will be ending up by being engulfed by macrophages or other neighbouring cells to avoid the inflammatory response to surrounding tissues (Savill & Fadok, 2000).

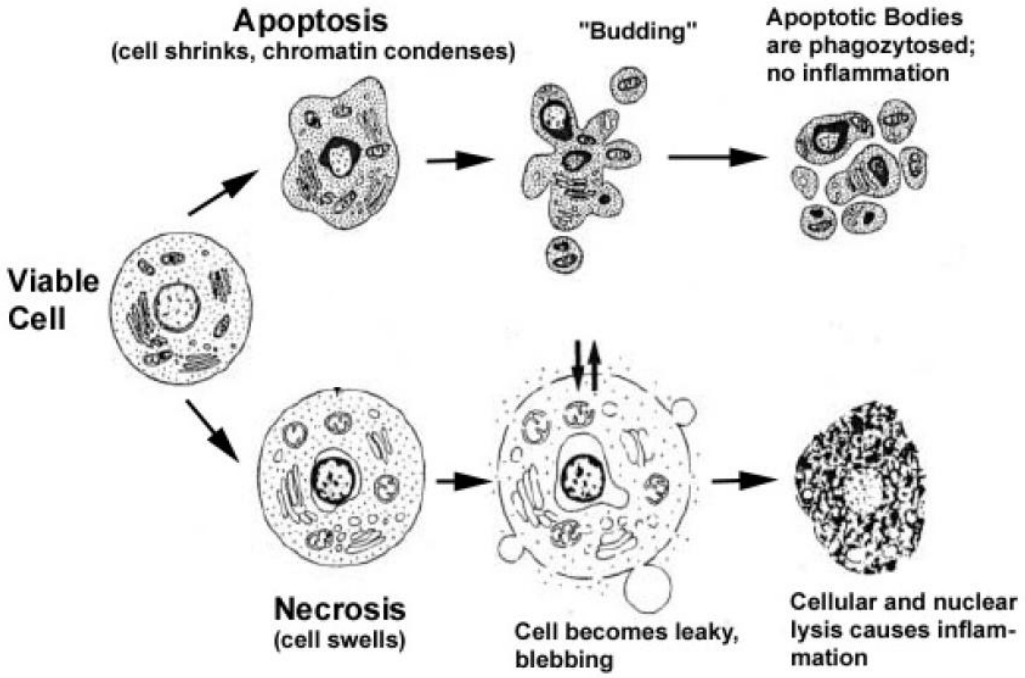

Figure 2.6: Hallmarks of the apoptotic and necrotic cell death process. Necrosis occurs when the cells are exposed to the physical or chemical disturbance resulting in the loss of membrane integrity, thus causing the cells to swell and rupture. Otherwise apoptosis is characterised by the biochemical hallmarks and distinct morphology such as cell shrinkage, membrane blebbing, or deformation of the cells and loosen contact with the neighbouring cells (adapted from Gewies, 2003).

2.4.1.2 Mechanism of apoptosis pathway

Apoptosis signal transduction can be divided into two pathways by which the intrinsic or mitochondria-mediated apoptosis and also the extrinsic or receptor linked apoptosis that occurs when the death receptor on the surface is triggered. Although these pathways initiated through different mechanisms but they seem to converge at the activation of pro-caspase 3, that will subsequently activates other pro-caspases along the caspase pathway that ultimately leads to apoptosis of the cell. Targeting apoptosis pathway is one of the important mechanisms for treatment regimes in order to kill tumour cells. Anticancer therapies are always associated with the activation of caspases, a family of cysteine-aspartic acid proteases. The biochemistry of apoptosis is summarised in three stages, the activation of initiator caspases, mitochondrial release of 'apoptogens' and finally the activation of effector caspases, which cleave recognised substrates to dismantle the dying cell (Yoshida *et al.*, 2003).

The activation of mitochondrial-dependent (intrinsic) pathway of apoptosis can be via loss of growth factor due to the response or signals such as chemotherapeutics drugs, DNA damage, oxidative stress or hypoxia. During apoptosis, various stimuli disturbed the membrane permeability of mitochondria and subsequently proteins in the mitochondrial intermembrane space are released into the cytoplasm. The protein is known as apotegenic factor which including cytochrome c, apoptosis-inducing factor (AIF), Smac/Diablo, Omi/HtrA2, endonuclease G, caspase-2 or caspase-9 (Debatin, 2004; Yoshida *et al.*, 2003).