CYTOTOXICITY AND CELL DEATH MECHANISMS AGAINST CANCER CELL LINES ELICITED BY THE EXTRACTS OF *PHYSALIS MINIMA* L.

by

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
ATP	adenosine triphosphate
Bad	Bcl-X_L /Bcl-2-associated death promoter homologue
Bak	Bcl-2 homologus antagonist killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-X_L	Bcl-2 homologue splice variants derived from same gene
BCP	1-bromo-3-chloropropane
bp	base pair
CAD	caspase-activated DNase
cDNA	complementary deoxyribonucleic acid
CD95	Fas/Apo-1/Apoptosis-antigen 1/ TNFRSP6/ tumor necrosis factor receptor super family member 6
ced	cell death abnormal
DFF40	DNA fragmentation factor 40 (endonuclease)
DFF45	DNA fragmentation factor 45
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxyribonucleoside triphosphate
EDTA	ethylenediaminetetracetic acid
FCS	fetal calf serum
GADD45	growth arrest and DNA damage inducible gene 45
HEPES	N-2 hydroxyethypiperazine-N'-2-ethanesulphonic acid
ICAD	inhibitor of CAD
IGF-BP3	insulin growth factor binding protein 3
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	infrared
kb	kilo bases
LB	Luria Bertani
LC-MS	liquid chromatography – mass spectrometry
Mdm-2	mouse double minute-2
M-MLV	Moloney Murine Leukemia Virus
MOPS	3-(N-morpholino)propanesulphonic acid

mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2 -(4sulfophenyl)-2H-teterzolium
NCI	National Cancer Institute
OD	optical density
P21/cip/wif	cyclin dependent kinase inhibitor
PARP	poly (ADP-ribose) polymerase
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidylinositol-3-kinase
PK	protein kinase
PMS	phenazine methosulphate
RNA	ribonucleic acid
RNase	ribonuclease
RPMI	Rosewell Park Memorial Institute
RT-PCR	reverse transcriptase PCR
SEM	standard error mean
TBE	tris-borate-EDTA
TLC	thin layer chromatography
T_m	melting temperature
UV	ultraviolet
v/v	volume to volume
w/v	weight to volume
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

KESITOTOKSIKAN DAN MEKANISME KEMATIAN SEL TERHADAP TURUNAN SEL KANSER YANG DIELISITKAN OLEH EKSTRAK *PHYSALIS MINIMA* L.

ABSTRAK

Terapi herba semakin penting dalam perubatan alternatif untuk merawat kanser. Maka, tujuan kajian ini ialah untuk menentukan sitotoksisiti beberapa tumbuhan ubatan dan untuk menyelidiki mekanisma kematian sel yang dielisitkan oleh ekstrak yang paling poten dan fraksinya. Sejumlah 20 ekstrak daripada empat tumbuhan antikanser (*Pereskia grandifolia*, *Vernonia cinerea*, *Elephantopus scaber* dan *Physalis minima*) telah disaring terhadap tiga jenis turunan sel. Ekstrak kloroform *Physalis minima* telah menunjukkan kesan sitotosik yang paling tinggi terhadap sel kanser NCI-H23 (sel kanser peparu adenokarsinoma manusia), T-47D (sel kanser payudara manusia) dan Caov-3 (sel kanser ovari manusia), dengan nilai EC_{50} yang rendah iaitu 2.80μg/ml, 3.80μg/ml dan 5.10μg/ml masing-masing. Mekanisme kematian sel yang berlainan ditunjukkan oleh ekstrak tersebut terhadap jenis sel kanser yang berbeza. Didapati bahawa sel NCI-H23 dan T-47D yang diujikan dengan ekstrak tersebut menunjukkan tahap DNA fragmentasi yang lebih tinggi berbanding dengan sel Caov-3. Pendedahan kepada ekstrak ini juga menghasilkan pengawalaturan pengekspresan mRNA c-myc, caspase-3 dan p53 yang signifikan terhadap semua jenis sel kanser. Penganalisaan ultrastruktur dan perwarnaan aneksin V juga mempamerkan kehadiran kematian sel secara apoptosis terancang di dalam semua sel kanser yang diperlakukan dengan ekstrak ini. Di samping itu, morfologi bukan apoptosis (vakuolar) juga diperhatikan dalam sebilangan sel Caov-3 dan sebahagian kecil sel NCI-H23 serta T-47D yang diperlakukan dengan ekstrak. Asai pengecualian tripan biru telah mengabaikan nekrosis sebagai punca utama kematian sel. Oleh itu, ekstrak kloroform *Physalis minima* mengelisitkan gabungan mekanisme kematian sel terancang pada sel Caov-3. dan mengaruhkan lebih banyak kematian sel secara apoptosis pada sel NCI-H23 dan T-47D.

Penyisihan ekstrak kloroform *Physalis minima* telah menghasilkan 16 fraksi yang berbeza. Hanya sebilangan fraksi daripada ekstrak kloroform ini menunjukkan aktiviti sitotosik

terhadap sel T-47D, dengan fraksi F11 merupakan fraksi yang paling poten, dengan nilai EC_{50} yang paling rendah (3.60μg/ml). Fraksi F10, F11 dan F13 mempamerkan DNA fragmentasi yang tipikal berkaitan dengan apoptosis dalam sel T-47D. Asai pengecualian tripan biru menunjukkan bahawa nekrosis tidak memainkan peranan utama dalam mengaruhkan kematian sel T-47D oleh fraksi-fraksi tersebut. Kajian lanjutan telah menunjukkan bahawa fraksi F11 dan ekstrak kloroform paling banyak mengaruh kematian sel secara apoptosis dalam sel T-47D, berdasarkan perwarnaan kebanyakan sel dengan aneksin V yang lemah dan pengawalaturan pengekspresan mRNA c-myc, caspase-3 dan p53 secara signifikan. Selain itu, sebilangan sel T-47D yang diperlakukan dengan fraksi F10 dan F13 pula diwarnakan oleh kedua-dua aneksin V serta propidium iodida, yang seterusnya mencadangkan kewujudan gabungan mekanisma kematian sel berprogram. Pisalin B, F dan K telah dikenalpastikan sebagai sebatian utama di dalam fraksi F11. Kombinasi sebatian pisalin ini dalam ekstrak dan fraksi mungkin bertanggungjawab terhadap aktiviti sitotosik dan kematian sel T-47D secara terancang. Penemuan ini patut diteruskan penyelidikannya agar *Physalis minima* dapat digunakan dalam terapi kanser kerana kesan antikansernya yang melibatkan apoptosis dan autofagi terhadap turunan sel kanser.

CYTOTOXICITY AND CELL DEATH MECHANISMS AGAINST CANCER CELL LINES ELICITED BY THE EXTRACTS OF *PHYSALIS MINIMA* L.

ABSTRACT

Herbal therapy is fast becoming an important alternative medicine for cancer treatment. Therefore, the aims of this study were to determine cytotoxicity of some medicinal herbs and to investigate cell death mechanism elicited by the most potent extract as well as its fractions. A total of 20 extracts form four anticancer plants (*Pereskia grandifolia*, *Vernonia cinerea*, *Elephantopus scaber* and *Physalis minima*) were screened against three different cancer cell lines. *Physalis minima* (Leletup-direct translation from Malay) chloroform extract was shown to exhibit remarkable cytotoxic effects on NCI-H23 (human lung adenocarcinoma), T-47D (human breast carcinoma) and Caov-3 (human ovarian carcinoma) cell lines, with EC_{50} derived at 2.80μg/ml, 3.80μg/ml and 5.10μg/ml, respectively. Different cell death mechanisms exerted by this extract on different cell lines. It was found that DNA fragmentation level of the extract-treated NCI-H23 and T-47D cells was higher than Caov-3 cells. Acute exposure to the extract produced a significant regulation of c-myc, caspase-3 and p53 mRNA expression in all cell lines. Ultrastructural analysis and annexin V staining also demonstrated the presence of apoptotic programmed cell death in the extract-treated cell lines. Furthermore, the appearance of non-apoptotic (vacuolar) morphology was observed in some treated Caov-3 cells and in minority of treated NCI-H23 and T-47D cells. Trypan blue exclusion assay ruled out necrosis as the main cause of death. Thus, cell death mechanism elicited by the *Physalis minima* chloroform extract appeared to be a mixture of programmed cell death in Caov-3 cells and a major apoptotic cell death in both NCI-H23 and T-47D cells.

Fractionation of *Physalis minima* chloroform extract revealed 16 different fractions. Only some of the fractions exhibited cytotoxic activities against T-47D cells, with F11 being the most potent, exhibiting the lowest EC_{50} (3.60μg/ml). Fractions F10, F11 and F13 exhibited typical DNA fragmentation associated with apoptosis in T-47D cells. Trypan blue exclusion assay

demonstrated that necrosis did not play a major role in eliciting T-47D cell death of these fractions. Further investigations revealed both fraction F11 and chloroform extract induced major apoptotic cell death in T-47D cells, which was based on majority of weakly diffused annexin V stained cells and significant regulation of c-myc, caspase-3 and p53 mRNA expression levels. Meanwhile, a considerable number of T-47D cells treated with fractions F10 and F13 were stained with annexin V and propidium iodide, which further suggested the presence of a mixture programmed cell death. Physalins B, F and K were identified as major constituents in fraction F11. Combination of these physalin compounds in the extract and fraction may have contributed to the cytotoxic activities and programmed cell death of T-47D cells. These findings warrant further research on *Physalis minima* plant for use in cancer therapy due to its apoptosis- and autophagy-dependent anticancer effect on cancer cell lines.

Chapter 1
Introduction

1.1 Complementary and alternative medicine (CAM)

Recently, a greater emphasis has been given towards the use of herbs as complementary and alternative medicine (CAM) that deals with cancer management (Powell *et al*., 2003). CAM is a generic term for a vast range of modalities and practices that are outside the mainstream of conventional medicine (Kelly, 2004; Verhoef *et al*., 2005). These therapies are spiritual healing, homoeopathy, herbal remedies, megavitamins, acupuncture, relaxation, meditation and psychologic methods (Fisher & Ward, 1994; Eisenberg *et al*., 1998; Pud *et al*., 2005). Herbal medicines are among the most widely used form of CAM (de Smet, 2002; Barnes, 2003; Algier *et al*., 2005). The increasing of positive view in herbal medicines compared with conventional therapies, largely because they are perceived as being "natural" and "safe" (Pirmohamed, 2003). A large number of breast and ovarian cancer patients have taken herbal therapies as complementary medicines concurrently with conventional treatments (Lee *et al*., 2000; Powell *et al*., 2002). The fact that they believe herbal treatments are able to boost their immune system, prolong life, relieve symptoms and ameliorate on the desirable side effects of Western therapies (Richardson *et al*., 2000).

Among alternative therapies, traditional Chinese medicine (TCM) is probably the best established and codified (Lim *et al*., 2005), dating back several thousand years (Fen *et al*., 2001). The origins and development of TCM are based on accumulation of daily experience and expert knowledge in herbal medicines (Chang, 1992). In TCM, the ying-yang is employed to explain the pathology changes in the human body and to guide the clinical diagnosis, and treatment (Cheng, 2000). Herbal medicines are used to restore or maintain balance between these elements and to grant vital energy (qi) in human body, which has both yin and yang aspects (Borrel, 2001; Fakim, 2006).

Guided by therapeutic experience of TCM, a number of antineoplastic drugs have been found (Han, 1988; Han, 1994; Cai *et al*., 2004). Indirubin from *Indigofera tinctoria* (Hoessel *et al*., 1999; Bradbury, 2005) and homoharringtonine from *Cephalofaxus hainanesis* (Luo *et al*., 2004; Mai & Lin, 2005) have exhibited significant antileukemia activities. Experimental therapeutic studies indicated that irisquinone from *Iris pailasti* (Li *et*

al., 1981) and 10-hydroxy comptothecin from *Camptothera accuminata (*Li *et al*., 2004) produced definite activities on rodent tumors. Daidzen (Jing & Han, 1993) and ginsenoside (Ota *et al*., 1997; Helms, 2004) have shown to be effective in inducing cell differentiation in leukemia cells (HL-60) and melanoma cells (B-16), respectively.

Of note, TCM has been integrated with Ayurvedic herbs (traditional Indian medicines) in CAM for suppressing various tumors (Tillotson *et al*., 2001; Patwardhan *et al*., 2005). Indian traditional medicine is mainly based on various system including Ayurveda, Siddha, Unani, Naturaphaty and homocophaty (Satyavati, 1990). These traditional systems have provided a great deal of information on the folklore practices and traditional aspect of natural products (Mukherjee *et al*., 1998). The Ayurvedic therapies have a unique principal, which includes pathogenesis of tumors, therapeutic methodologies and combination of herbal ingredients (Premalatha & Rajgopal, 2005). Plant alkaloids are the primary active ingredients of Ayurvedic medicines (Borchardt, 2003). Other pharmacologically active compounds are being found in Ayurvedic plants, such as polyphenols, tannins and triterpenoids, which produce potential therapeutic effect in cancer remedies (Kaur *et al*., 2005).

Besides Ayurveda, the Malay folk medicinal plants are also being used as an alternative approach for the cancer treatment (Chen, 1981). The local traditional Malay medicine is actually found on the basic principle of Indonesian traditional medicine and has been modified to suit the current needs (Zakaria & Mohamad, 1994). Herbal decoctions consisting of multiple herbs each possessing tremendous potential for a cancer cure are commonly being used in Malay traditional medicine (Ong, 2004). Scientific studies have shown that several of Malay traditional vegetables (ulam) used as herbal medicines are reported to be cytotoxic against various types of cancer cells (Mohamed *et al*., 2005). A finding has demonstrated that several of Malay traditional plants contain high bioactive compounds, which are potentially be used for cancer therapy and other diseases (Rao, 2001).

As for all local traditional medical systems, herbal medicines have extensively been used as an alternative approach for preventing cancer. Therefore, the experience of these

traditional medicines could provide a potential resource to explore chemopreventive herbs and may also lead to the isolation of novel anticancer compounds.

1.2 Anticancer Agents

Written records of the use of pure natural drugs in cancer chemotherapy date back about 30 years. A number of anticancer agents have been discovered by screening natural products from plants, microorganisms and marine organisms (Cragg & Newman, 1999). Anticancer drug development has been oriented towards seeking target-specific or target-selective compounds using a screen composed of human tumor cell lines organized by tissue types (Cragg & Suffness, 1988). The new targets should be preferentially evaluated as sites for anticancer drug (Verweij, 1996). The result from the interaction of drugs with cellular targets, mechanism of damage repair and gene expressed within tumor and non-tumor cells are considered the cellular responses to anticancer agents (Danesi *et al.*, 2001). With the technological advancement in molecular biology and genomics, majority of drug discovery research is currently based on the molecular approach (Harvey, 1999).

1.2.1 Plant derived anticancer agents

Despite many research advancements in cancer chemotherapy, plant natural products still make an enormous contribution to drug discovery (Hamburger & Hostettmann, 1991; Lee, 1999). There are approximately 60 available cancer chemotherapeutic drugs that were derived from plants (Kinghorn *et al.*, 1999). Several plant derived agents are currently having great significance in cancer treatment. One of the best known classes of these agents is the dimeric *Vinca* alkaloid, which was isolated from the periwinkle *Catharanthus roseus* (Noble, 1990). The *Vinca* alkaloids (vinblastine and vincristine) are useful primarily in the treatment of Hodgkin's disease and childhood leukemia, respectively (Sneden, 1984; Hamburger and Hostettmann, 1991). Another two new clinically approved semisynthetic *Vinca* alkaloid derivatives, vindesine and vinorelbine are now widely used and licensed for the treatment of non-small cell lung cancer, metastatic breast cancer and ovarian cancer

(Ashizawa *et al*., 1993; Leveque *et al*., 1993; Romero *et al*., 1994; Kruczynski & Hill, 2001). These compounds work by interfering microtubule polymerization and subsequently arrest mitosis in the metaphase (Himes, 1991; Jordan *et al*., 1991; Panda *et al*., 1996; Wilson *et al*., 1999). Epipodophyllotoxins, etoposide and teniposide are anticancer drug derived from the mandrake plant *Podophyllum peltatum* (Imbert, 1998; Hande, 1992; Zhang *et al.*, 2005). These agents are topoisomerase II inhibitors, which prevent the cleavage and resealing of DNA strands (Ross *et al.*, 1984; Giaccone, 1995; Gordaliza *et al.*, 2001). Etoposide has produced high cure rates in testicular cancer and lung cancer, while teniposide is mainly used to treat leukemia, lymphoma and Kaposi's sarcoma (Selvin *et al.*, 1989; Johnson *et al*., 1997a; Gordaliza *et al.*, 2000).

Other prominent antimicrotubule agents, such as paclitaxel and docetaxel, arise from the taxol extracts of the pacific yew *Taxus brevifolia* (Panvichian *et al*., 1998; Jordan, 2002; Marchetti *et al*., 2002; Montera *et al*., 2005). They are effectively used to treat lung, breast and ovarian carcinomas (Kingston, 1994; Cortes & Pazdur, 1995; Crown & O'Leary, 2000; Perez *et al.*, 2001). In addition, the camptothecin derivatives, irinotecan and topotecan, have exhibited impressive antitumor activity against colorectal and ovarian cancers, respectively (Giovanella *et al.*, 1989; Jonsson *et al.*, 2000; Oguma, 2001). These compounds were obtained from the stembark of Nyssacca (*Camptotheca accuminata*) and act by inhibiting topoisomerase I (Slichenmyer *et al.*, 1993; Johnson *et al.*, 1997b; Liu *et al.*, 2000; Wu, 2003).

1.2.2 Microbe derived anticancer agents

Antitumor antibiotics are among the best and important cancer chemotherapeutic agents with the widest spectrum of activity in human neoplasm (Cragg *et al*., 1997). The majority of these agents are originally isolated from fermentation products of *Streptomyces peucetus* (Spiegel, 1984). These microbially derived agents are topoisomerase I and II inhibitors that intercalate between paired bases of the DNA and thereby have inhibitory effects on DNA or RNA synthesis (Bachur *et al*., 1992; Sinha, 1995; Sutter *et al.*, 1997;

Binaschi *et al.*, 2000). Mitomycin (Peterson *et al*., 1995; Paz *et al*., 1999) and bleomycin (Hay *et al*, 1991; Scarpato *et al*., 1998) are known to cause DNA damage via the formation of DNA cross-links and the production of oxygen free radicals, respectively. Both drugs have been used in the treatment of head and neck squamous cell carcinoma (Haffy *et al*., 1997), Hodgkin lymphomas, testicular carcinoma (Mir *et al*., 1996; Azambuja *et al*., 2005) and colon cancer (Pan & Gonzalez, 1997; Spanswick *et al*., 1998).

The anthracycline antibiotics, doxorubin, daunomycin and adriamycin are primarily employed in clinical antineoplastic drugs (Blum & Carter, 1974; Arcamone, 1980; Westwell, 2002). Although these agents are active against a variety of solid tumor and haematologic malignancies, their clinical use is limited by tumor resistance and toxicity to healthy tissue (Hortobagyi, 1997; Husseini *et al*., 2002; Lin *et al*., 2005). Subsequently, new anthracycline anticancer agents such as ansamycin (Schulte & Neckers, 1998), amrubicin (Ogawa, 1999), esorubicin, epirubicin and idarubicin (Weiss, 1992; Arcamone *et al*., 1997; Kim *et al*., 1999) have been synthesized to increase the antitumor activity and to decrease the undesirable side effects. In fact, almost all clinically active anthracyclines are anthraquinones (Hande, 1998). Among a series of anthracenediones, mitoxantrone is the most active and has demonstrated a spectrum of antitumor activity similar to the anthracyclines, but with less cardiotoxicity (Posner *et al.*, 1985; Faulds *et al*., 1991).

1.2.3 Marine derived anticancer agents

In recent years, promising compounds are being tapped from the world's oceans. Several new compounds derived from marine organisms have entered preclinical and clinical evaluation as anticancer candidates (Cooper, 2004). For example, didemnin B, aplidine and ecteinascidine 743 are derived from tunicates (Schwartsmann *et al.*, 2001). Didemnin B is a cyclic desipeptide isolated from the tunicate *Tridemnum solidum*. It has exhibited potent preclinical antitumor activity. However, its clinical use was hindered by cardiotoxicity and neuromuscular toxicity (Shin *et al*., 1991; Rinehart, 2000). Aplidine, a related desipeptide was subsequently isolated from the Mediterranean tunicate *Aplidium albicans*. Several

studies indicated that aplidine appears to be more potent antitumor activity than didemnin B and lacks severe secondary effects in preclinical models (Urdiales *et al.*, 1996; Rinehart, 2000).

Numerous ecteinascidins have been derived from the marine tunicate *Ecteinascidia turbinata*. Preclinical studies have demonstrated that ecteinascidin 743 is active against a variety of solid tumor cell lines and has promising activity in phase I and phase II clinical trials (Minuzzo *et al*., 2000; Damia *et al*., 2001; Erba *et al.*, 2001). Other agents originating from marine sources are dolastatin and bryostatin, which have currently entered phase I and II clinical trials (Pitot *et al*., 1999; Poncet, 1999; Pagliaro *et al.*, 2000). These compounds have shown activity against malignant melanoma and colorectal cancer, respectively (Pathak *et al*., 1998; Propper *et al.*, 1998; Zonder *et al*., 2001).

1.2.4 Metal complexes and hormonal agents

The platinum compounds, cisplatin and carboplatin, are two of the most commonly used anticancer drugs in treatment of solid tumors including lung, ovarian, cervix, head and neck cancers (Petering *et al.*, 1984). Of note, the platinum-based chemotherapy has administered in combination with other anticancer agents (such as paclitaxel, etoposide, vincristine, vinblastine and bleomycin) for significant regimens in testicular, ovarian and lung carcinomas (Williams *et al*., 1987; Eisenhauer & Vermorken, 1998; Rogers *et al*., 2002; Reck *et al*., 2003). These platinum compounds act by forming cross linking of DNA strands and inhibiting of DNA replication (Spiegel, 1984; Lan & Ng, 2002). Nedaplatin and platinum (II) complexes are new platinum analogs which recently have pronounced preclinical antitumor activities against solid tumors, virtual low rate of nephrotoxicity and relatively less neurotoxicity (Alberts *et al*., 1997; Young *et al*., 2002). Another new generation of platinum agent, oxaliplatin, was reported to have poor activity as a single agent against breast cancer, but interesting results in combination regimens (Awada *et al.*, 2003).

Hormonal therapy is a systemic therapeutic approach in the management of postmenopausal women with metastatic breast cancer (Dellapasqua & Gertsch, 2005).

Tamoxifene, the first selective estrogen receptor modulator (SERM), was primarily synthesized as a drug against hormone responsive breast cancer (Mocanu & Harrison, 2004). This nonsteroidal antiestrogen has been used as adjuvant in the treatment of early stage breast cancer for over 20 years (Awada *et al*., 2003). One known mechanism is that the tamoxifene molecule competes with estrogen for binding to estrogen receptors, thus the effect of estrogen to promote the growth of breast cancer cells is diminished (Rong *et al.*, 2005). Tamoxifene is also found to be effective in the treatment of hormone non-responsive breast cancers that do not express estrogen receptors (Salami & Tehrani, 2003). In addition to breast cancer, tamoxifene has been used to treat other cancers such as hepatocellular carcinoma (Simonetti *et al*., 1997), ovarian cancer (Trope & Kaern, 2000) and prostate cancer (Bergan *et al*., 1999). However, tamoxifene's uterine adverse effects pushed the ongoing research to develop new agents with higher affinity for the estrogen receptor (Neven & Vergota, 2001).

Following tamoxifene, a number of new antiestrogens have been developed in attempt to increase its efficacy and reduce the partial agonist properties. The first generation SERMs, idoxifene (Nuttall *et al.*, 2000), toremifene (Holli, 2002; Chen *et al.*, 2002) and droloxifene (Hasmann *et al.*, 1994), have shown minimal activities in tamoxifene-resistant diseases (Robertson *et al.*, 2005). Both have stimulatory effects on the uterus (Gonzalez *et al.*, 1998; Morello *et al.*, 2002; Harvey *et al.*, 2005). Efficacy results for second generation SERMs such as raloxifene (Gasco *et al.*, 2005) and arzoxifene (Freddie *et al.*, 2004) are not high, although raloxifene exhibits promise in the chemoprevention of breast cancer (Neven *et al.*, 2005). Consequently, there is a need for new endocrine therapeutic approaches for breast cancer, especially for use in disease that is resistant to tamoxifene. Fulvestrant is a new type of steroidal estrogen receptor antagonist with no agonist effects (Robertson, 2004). Fulvestrant has a unique mechanism of action, which binds, blocks and leads to estrogen receptor degradation (Steger *et al.*, 2005). This agent exerts high efficacy result compared with the SERMs palliative approach in the treatment of advanced breast cancer (Howell & Abram, 2005). Furthermore, the newer aromatase inhibitors, exemestane, anastrozole and

letrozole have demonstrated greater efficacy than tamoxifene as first line treatments for metastatic breast cancer with significantly better toxicity profiles (Coombes *et al.*, 2003; Dowsett & Haynes, 2003).

1.3 Programmed cell death

Cell death is a fundamental process in normal development, tissue homeostasis and integrity of multicellular organisms (Hakem & Harrington, 2005). The tight regulation of both cell proliferation and cell death is required to generate the proper numbers and types of cells during differentiation and to maintain this balance in the mature animal (Ellis *et al*., 1991). Unwanted cells are eliminated during metamorphosis, embryogenesis, pathogenesis and tissue turnover (Hakem & Harrington, 2005). In vertebrates, naturally-occurring cell deaths have been extensively observed in almost all tissue (Cole *et al*., 1993), in the nervous system (Becker & Bonni, 2004) and in the immune system (Krammer, 2000). These cell deaths, which involve a genetically programmed process of the cell to promote a cascade of cell suicide mechanism in response to specific signals, are known as programmed cell death (Gorski & Marra, 2002).

There are numerous cell death mechanisms that are tissue-specific and cell type-specific (le Blanc, 2003). Typically, programmed cell death is regulated by a variety of extracellular and intracellular signals which is governed by the environment of the cell (le Blanc, 2003). Under critical physiologic conditions, programmed cell death is initiated in specific cell types by endogenous tissue-specific agents and exogenous cell-damaging agents (Neuman *et al*., 2002). Various exogenous activations of programmed cell death, physical agents (such as, radiation, physical trauma, cold shock and chemotherapeutic drugs) and infectious agents (such as viruses and bacterial toxin) act on most types of cells (Duckett *et al.*, 1998). Internal imbalances can also trigger apoptosis, including growth factors withdrawal, treatment with glucocorticoids, ablation of trophic hormone and loss of matrix attachment (Caron-Leslie *et al*., 1991; Neuman *et al*., 2002).

Programmed cell death plays an essential role in normal development, especially in the epigenetic self-organization process, in sexual dimorphism (the counterpart of certain sex type) and in morphogenesis (the sculpting of the form of embryos) (Saran, 2000). Programmed cell death is crucial in the adult, by allowing tissues homeostasis primary defense against viral infections (Fan *et al*., 1998) and the regulation of aging process (Monti *et al*., 1992). Programmed cell death also functions to eliminate cells that are produced in excess, abnormal, misplaced, non-functional or potentially dangerous to the organism (Jacobson *et al*., 1997). Conversely, programmed cell death deregulation has been proposed to participate in the pathogenesis of several diseases, including tumorigenesis, autoimmunity, neurodegenerative disorders and infectious diseases (Reed, 1999).

The term apoptosis usually refers to a morphological type often observed in programmed cell death. It presents the defining characteristics of a cell death program, including cell shrinkage, membrane blebbing, nuclear fragmentation and segmentation of the cell into apoptotic bodies (Ameisen, 2002). Indeed, many research groups began to consider programmed cell death and apoptosis as a single entity (Melino, 2002). However, recent studies have proven that the concept of programmed cell death as a sequence of events are based on cellular metabolism, but not necessarily those that led to the morphology of apoptosis (Sloviter, 2002). In other words, some programmed cell death may not involve the mechanism of apoptosis. For this reason, the term ‘apoptosis’ should never be considered synonymous with programmed cell death (Guimaraes & Linden, 2004).

1.3.1 Programmed cell death in inverterbrate

The molecules that participate in the basic steps of programmed cell death are best defined through genetic studies in the nematode *Caenorhabditis elegans* (Horvitz, 2003). Among the 1090 somatic cells generated during *Caenorhabditis elegans* hermaphrodite development, 131 of its cells undergo programmed cell death (Conradt & Horvitz, 1998). Four essential genes have been identified ced-3, ced-4, ced-9 and egl-1 that regulate the commitment step that decides the ultimate life or death fate of cell (Shaham *et al.*, 1999).

Most significantly, ced-3 and ced-4 are necessary for the activation of cell death, while ced-9 inhibits it (Han *et al.*, 1998). Ced-9 is a repressor of cell death, which binds to Ced-4, prevents it from activating Ced-3 (Hugunin *et al.*, 1996). Recent studies reported that *Caenorhabditis elegans* contains an antagonist of Ced-9 called Egl-1, that by binding to it and prevents it from suppressing cell death, thus functioning as a transdominent inhibitor of Ced-9 (Conradt & Horvitz, 1998). Indeed, the completion of the 'death program' will depend on the interactions between each of these four proteins and their respective expression level is regulated by cell signaling during development (Seydoux & Priess, 2005). Subsequently, the dead cells are immediately engulfed and degraded by neighboring cells (Horvitz, 2003). Seven genes (ced-1, -2, -5, -6, -7, -8 and -10) are involved in initiation of phagocytosis while gene nuc-1 is involved in degradation of pycknotic DNA of dead cells (Wu *et al.,* 2000; Seydoux & Priess, 2005).

In *Drosophila melanogaster*, large numbers of cells die during metamorphosis as well as embryonic development exhibited the morphological characteristics of apoptosis (White *et al.*, 1994). Three genes, rpr (reaper), hid (head involution defective) and grim, have been shown to activate caspase activity in *Drosophila* (Grether *et al.*, 1995; Vucic *et al.*, 1998). By contrast, identification of Diap1 (inhibitor of apoptosis protein - IAP), has shown to inhibit caspase activity and to suppress apoptosis when overexpressed (Hay *et al.*, 1995).

The observation on the cell death in *Caenorhabditis elegans* and *Drosophila melanogaster* contributed more to the understanding of the molecular mechanism of programmed cell death (Kanuka *et al.*, 1999). In fact, the genetic basis of these systems has an added advantage over the other systems and encouraged researchers to search for the functional homologues of these genes in mammals (Hale *et al.*, 1996).

1.3.2 Mammalian homologues of programmed cell death genes from *Caenorhabditis elegans*

Many homologues are new emerging between genes during invertebrates development and genes activation in mammalian tissues and tumors (Yuan & Horvitz, 2004).

Thus, the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila melanogaster* appear to share with mammals at least part of a common pathway to programmed cell death (Steller, 1995; Haining *et al.*, 1999). Molecular analysis revealed that the cell death protein encoded by ced-3 showed significant similarity to mammalian protein, namely interleukin-1β-converting enzyme (ICE) (Yuan & Horvitz, 2004). In fact, ICE protease has played a crucial role in apoptotic regulation of mammalian cells (Duan *et al.*, 1996). A single mammalian homolog of Ced-4 has been identified thus far, termed apoptotic protease activating factor (Apaf) (Zou *et al.*, 1997). However, the human Apaf-1 is structurally more complex than the nematode Ced-4 (Hu *et al.*, 1998). Similarly, the Ced-4 promotes apoptosis by binding to the Ced-3, while the Apaf-1 induces mammalian apoptosis by activating the proteases such as Caspase-3 (Izban *et al.*, 1999).

The gene product of ced-9 is involved in the tight regulation of ced-3 and ced-4 genes (Yan *et al.*, 2005). ced-9 itself encodes for a 280 amino acid protein showing 23% homolog with mammalian bcl-2 proto-oncogene product (Desoize, 1994). The bcl-2 family consists of around 20 gene products, including antiapoptotic proteins (Bcl-2, Bcl-X_L, Bcl-W, Mcl-1, Bcl-G and A1) and proapoptotic proteins (Bax, Bak, Bcl-X_S, Bid, Bad, Bik, Blk, and Bim) (Maser *et al.*, 2000). The family members have four conserved Bcl-2 homology (BH) domains: BH1, BH2, BH3 and BH4 (Wang *et al.*, 1999a). The BH3 domain is an important component in inducing apoptosis, while the BH4 domain is involved in the anti apoptotic functions (Zhang *et al.*, 2000).

Both BH1 and BH2 domains show ion channel activity for regulating the release of cytochrome C from mitochondria (Yang *et al.*, 1997). The release of cytochrome C is central in turning apoptosis on or off and is determined by the ratio of proapoptotic to antiapoptotic proteins (Kuwana *et al.*, 2002). Some of the bcl-2 family members can share the capacity to homodimerize and to neutralize each others through heterodimerization (Cohen-Saidon *et al.*, 2003). Thus, the susceptibility of cells to undergo apoptosis is determined by the way dimerize protein combine and mix and concentration of individual proteins (Oltvai *et al.*, 1993). Bcl-2 appears to be localized to the outer mitochondria membranes, nuclear, and

endoplasmic reticulum (Heiden & Thompson, 1999). Recent studies have associated commitment to apoptotic cell death with loss of mitochondrial membrane potential and have indicated that the membrane potential gradient can be maintained by Bcl-2 overexpression (Martinou & Green, 2001). Overexpression of Bcl-2 is associated with drug resistance in the chemotherapy (Wilson *et al.,* 1997). Conversely, the down-regulation of Bcl-2 by antisense oligonucleotides enhances drug sensitivity in the promotion of apoptosis (Sawada *et al.*, 2000). Thus, the effectiveness of the level of chemotherapy might depend on the level of Bcl-2 expression and the interaction with proapoptotic genes in overcoming cellular drug resistance in cancer cells (Cory & Adams, 2002; Davis *et al.*, 2003).

1.4 Functions and types of cell death

Physiological cell death is a widespread phenomenon in the development of multicellular organisms (Clarke, 1990). Cell death is typically discussed dichotomously as either necrosis (accidental cell death) or apoptosis (programmed cell death) (Farber, 1994; Raza *et al*., 2002). Although programmed cell death has often been equated with apoptosis, it is well known that nonapoptotic form of programmed cell death also exists (Bursch *et al*., 2000; Kroemer *et al*., 2009). These nonapoptotic cells use different pathways for active self-destruction as reflected by different morphological and biochemical events (Bursch *et al*., 2000). Both apoptotic and non-apoptotic cell death have been defined in normal physiology and during tumorigenesis and these could potentially be contributed to the deletion of cancerous cells (Castro-Obregon *et al*., 2004).

1.4.1 Apoptosis

The term apoptosis was proposed by Kerr and his colleagues in 1972. It has been used to describe a specific morphological pattern of cell death observed as cells that were eliminated during embryonic development, neurode generation and normal physiological processes and pathological conditions (Kerr *et al*., 1972). Notably, apoptosis in the classical Greek word meaning ‘a falling off’, as leaves from a tree (Bowen *et al*., 1998; Elmore,

2007). The word connotes a controlled physiologic process of removing individual component of an organism without destruction to the organism (Bowen *et al*., 1998; Elmore, 2007). Apoptosis is characterized by a series of typical morphological changes, including cell shrinkage, nuclear condensation, chromatin margination, membrane blebbing and convolution of the nuclear and cytoplasmic membranes followed by the formation of apoptosis bodies (Kroemer *et al*., 2009). The cytoplasmic organelles remain well preserved (Leist & Nicotera, 1998). These apoptotic bodies were then rapidly engulfed by neighbouring cells with little or no inflammatory response occurring (Wiegand *et al*., 2001).The biochemical event of apoptosis is the cleavage of chromatin into nucleosomal fragments that are multiples of units comprising 180-200 base pairs (Arends *et al*., 1990). As a genetically programmed form of cell death, apoptosis is mediated by a family of cysteine proteases known as the caspase (Thornberry & Lazebnik, 1998). The activation of caspase is essential for the execution of apoptotic chromatin degradation (Cohen, 1997). Another important characteristic of apoptosis is externalization of cell surface phosphatidylserine, which can be recognized by phagocytes as a signal for engulfment (Koopman *et al*., 1994). If the body is not phagocytosed, it may undergo degradation which resembles necrosis in a process called secondary necrosis (Bowen *et al*., 1998).

1.4.2 Autophagy

Interest in non-apoptotic forms of programmed cell death has grown recently, especially autophagic cell death or termed as type II cell death. It plays a major role in the degradation of cellular components within the dying cell in autophagic vacuoles (Baehrecke, 2003; Degenhardt *et al.*, 2006). Autophagy has been extensively described to occur in *Drosophila* salivary gland (Myohara, 2004) and vertebrates including humans (Schweichel & Merker, 1973). It is usually associated with the elimination of large secretory cells during adjustment of sexual organs and ancillary tissues to seasonal reproduction. In yeast cells, autophagy cell death is induced under various nutrient starvation condition (Abeliovich & Klionsky, 2001). As to pathophysiology, autophagy has been associated with models of

tumorigenesis (Hermann *et al*., 1995) and human neurological disease (Liberski *et al*., 2004). Autophagy can be derived into macroautophagy and microautophagy, which differ in their mechanism of vacuole formation and delivery of material (van Doorn & Woltering, 2005). Briefly, the macroautophagy pathway in mammalian cells begins with the sequestration of larger portions in the cytoplasm to form an early autophagosomes (Devrim & Adi, 2004). The early autophagosome is generally considered to derive from the endoplasmic reticulum and also has been suggested to originate from the post-Golgi membranes (Denis & Codogno, 2003). On the contrary, microautophagy operates by invaginating a portion of pre-existing vacuolar membrane to engulf cytosol or organelles (van Doorn & Woltering, 2005). The sequestration process is under the control of GTPase, phosphatidylinositol kinase and involves ubiqitin conjugation system (Denis & Codogno, 2003). All autophagosomes eventually fuse with lysosomes, thereby, the final degradation of the sequestered cytoplasmic material is triggered (Devrim & Adi, 2004). Genetic screens in yeast and Drosophila have led to the discovery of APG (autophagy) gene and AUT (autophagocytosis) gene that encoded protein need for the induction and regulation of autophagy process (Abelivich & Klionsky, 2001; Baehrecke, 2003). In addition, beclin 1 is the mammalian functional homolog of yeast apg-6 gene that is involved in the early steps of autophagic vesicle formation (Huettenbrenner *et al*., 2003). This is the first reported autophagic gene in mammalian and has the properties of a tumor suppressor gene (Liang *et al*., 1999; Yang & Peng, 2008).

1.4.3 Non-lysosomal and cytoplasmic cell death

The third morphological type of programmed cell death is often seen in mature neurons as a form of atrophy and may be induced by withdrawing nerve growth factor (NGF) (Amin *et al*., 2000). The type III cell death is distinguished from the type II cell by its lack of lysosomal involvement (Liberski *et al*., 2004; van Doorn & Woltering, 2005). Instead, this cell death is characterized by swelling of intracellular organelles, following by the formation of empty spaces in the cytoplasm, which eventually merge with each other

(Lee & Baehrecke, 2001). As noted by Clarke (1990), the type III cell death has been further subdivided into types 3A (non-lysosomal cell death) and 3B (cytoplasmic cell death). Although both types of cell death (3A and 3B) exhibit dilation of intracellular organelles and formation of non-lysosomal vacuoles, they differ in the apparent manner of cell degradation (Clarke, 1990). For non-lysosomal cell death, the cytoplasmic membrane of a cell is degraded with nuclear vacuolation in the late stage (Hirai & Harada, 2004). Meanwhile, the nuclear of cytoplasmic degradation exhibits granular chromatin or karylosis and its cytoplasmic membrane rounds up in the late stage (Hirai & Harada, 2004).

1.4.4 Necrosis

In contrast to apoptosis, necrosis has been traditionally thought to be a positive form of cell death (accidental cell death) and initiated by lethal stimuli such as toxic trauma or extreme physical damage (Sanz & Tavernarakis, 2005). It is characterized morphologically by cytoplasm swelling, destruction of organelles (mitochondrial, endoplasmic reticulum and lysosome), disruption of the plasma membrane, leading to the release of intracellular components and induces inflammatory responses that affect neighboring cells (Proskuryakov *et al*., 2002; Guimaraes & Linden, 2004). Hence, necrosis is an inadequate means to maintain homeostasis, because it can lead to auto-immune reactions (Huettenbrenner *et al*., 2003). Necrosis is the term currently used by pathologist to designate the presence of death tissues or cells, regardless of the prelethal process (Trump & Berezesky, 1996). Therefore, necrosis does not describe a form of cell death, but rather it refers to secondary morphological changes to cell death induced by the main mechanisms, including apoptosis (Trump & Berezesky, 1996). In the absence of phagocytosis, apoptotic bodies may eventually lose their membrane integrity and proceed to secondary necrosis (Bowen *et al*., 1998). Nevertheless, there is growing evidence that necrosis by cell death could be programmed in nature. The programmed necrosis has been observed only under condition in which apoptosis is inhibited either chemically or genetically (Edinger & Thompson, 2004). A recent paper by Okada and colleagues has demonstrated that the Abl kinase inhibitor

(Gleevec) kills cells not only by activating the apoptotic pathway but also by initiating programmed necrosis (Okada *et al*., 2004). In fact, other studies have highlighted the important implications of programmed necrosis in patients treated with alkylating agents (Zong *et al*., 2004) and demonstrated a crucial physiologic role for programmed necrosis in response to viral infection (Chan *et al*., 2003a).

1.4.5 Oncosis

The term oncosis (from "onkos", meaning swelling) was first used in 1910 to describe ischemic cell death in osteocytes, and recently reintroduced to define the cell death distinct from apoptosis (Moore, 2000). Oncosis is characterized as a prelethal phase leading to cell death accompanied by cellular swelling, organelles swelling blebbing and an increase in the membrane permeability (Boulanger *et al*., 2004). The process of oncosis ultimately interferes the ionic pumps in plasma membrane and depletes the cellular energy stones (ATP) (Majno & Joris, 1995). Currently, the translocation of cytosolic phospholipase A_2S to cellular membrane has been documented to be involved in the hydrolysis of membrane phospholipids and decrease the membrane integrity (Ma *et al*., 2001). Furthermore, cell death by oncosis may result in necrosis with karyolysis whereas apoptotic cell death to necrosis with karyorrhexis (fragmentation of the cell nucleus) and cell shrinkage (Zhang *et al*., 1998).

1.4.6 Paraptosis

Paraptosis is another non-apoptotic form of programmed cell death which does not involve DNA fragmentation, caspase activation and apoptotic body formation (Sperandio *et al*., 2000). This type of cell death involves extensive cytoplasmic vacuolization, which begins with progressive swelling of the mitochondria and endoplasmic reticulum (Wang *et al*., 2004). Paraptotic cell death is distinguished from the apoptosis by its lack of response to various caspase inhibitors and Bcl-X_L treatment (Chen *et al*., 2002). However, this alternative form of cell death is inhibited by a catalytic mutant of caspase-9 zymogen

(Sperandio *et al*., 2000). There is increasing evidence that paratotic cell death exists in parallel with apoptosis, but they may be subjected to different controls (Wyllie & Golstein, 2001). Similarly, recent study has revealed that both polymorphonuclear leukocytes and macrophages are involved in the killing of T9-C2 tumor cells with vacuolization of the mitochondria and endoplasmic reticulum (ER) resembling paraptosis (Chen *et al*., 2002). For instance, the overexpression of PDCD5 (an apoptosis-promoting protein) has enhanced caspase-independent, paraptosis-like cell death induced by TAJ/TROY (tumor necrosis factor receptor protein) in HEK293, Hela and 293 T cells (Wang *et al*., 2004).

1.4.7 Pyroptosis

Pyroptosis is a novel form of eukaryotic cell death that is induced by infection with *Salmonella* and *Shigella* species that inherent proinflammatory (Boise & Collins, 2001; Sansonetti *et al*., 2000). The term pyroptosis from Greek roots “pyro”, relating to fire of fever and “ptosis” (to-sis) to denote a falling, to describe pro-inflammatory programmed cell death (Cookson & Brennan, 2001). This pathway of cell death is mediated by the activation of caspase-1, which clearly distinguishs it from necrosis (Hilbi *et al*., 1997; Hersh *et al*., 1999). Caspase-1 is unique among caspase because it activates the proinflammatory cytokines (Fantuzzi & Dinarello, 1999; Monack *et al*., 2000), but has no autonomous function in apoptosis (Li *et al*., 1995). Subsequently, the proinflammatory pathway is leading to cell lysis and release of inflammatory cellular content (Brennan & Cookson, 2000). This is an alternative pathway to remove potentially dangerous cells in a development organism, like the process that takes the life of *Salmonella*- infected macrophages (Cookson & Brennan, 2001). In addition, the observation caspase-1-dependent during cell death in central nervous (Friedlander *et al*., 1997), immune (Shi *et al*., 1996) and cardiovascular systems (Kolodgie *et al*., 2000) indicated that pyrotopsis might play a crucial role in variety of biological system.

1.4.8 Mitotic catastrophe

The term mitotic catastrophe (mitotic cell death) was first coined by Paul Russell and Paul Nurse to describe the lethal fate of *Schizosacharomyces pombe* cells (Russell & Nurse, 1986). More currently, mitotic catastrophe has been used to define the type of eukaryotic cell death that is caused by aberrant mitosis (Erenpreisa & Roach, 1999; Ogawa *et al*., 2003). Mitotic catastrophe is characterized by the formation of micronuclei and accumulation of karyotypic abnormalities (Chang *et al*., 1999b). Although mitotic death may culminate in features of apoptosis, only apoptotic cell death is induced by wild-type p53 and inhibited by Bcl-2 (Merritt *et al*., 1997; Chang *et al*., 1999b). Nevertheless, mitotic catastrophe occurs in tumor following genotoxic insult or mitotic spindle and is selected as an alternative to rapid apoptosis (Erenpreisa *et al*., 2005). Agents that damage microtubules and disrupt the mitotic spindle also cause mitotic catastrophe in the treated-cells (Jordan *et al*., 1996).

1.4.9 Senescence

Senescence is an irreversible program of cell-cycle arrest that is contributed to the deletion of potentially cancerous cells in normal cells (Campisi, 2001). Terminal proliferation arrest may result from replicative senescence (Lowe & Lin, 2000). Replicative senescence is the progressive erosion of chromosome telomeres, which occurs with each cycle of cell division (Wynford, 1999). A senescent cell typically shows cellular changes, such as shortening of telomeres, flattened cytoplasm increased granularity and accumulation of karyotypic abnormalities (Lowe & Lin, 2000). At the biochemical level, senescence is accompanied by changes in metabolism and the induction of SA-β-gal (senescence-associated β-galactosidase) activity, which commonly used as a marker of senescence in human cells (Dimri *et al*., 1995). The senescence process is initiated by the activation of various tumor suppressor genes, including p53, p21, p16 and RB (Lin *et al*., 1998; Zhu *et al*., 1998). Therefore, one of the major roles of the senescence is to inhibit tumor development. Recent evidence has demonstrated that anticancer agent (doxorubicin) induced the

senescence-like phenotype in human tumor cells (Chang *et al*., 1999a). Thus, senescence may represent a non-apoptotic form of programmed cell death that may provide an important determinant of treatment response in tumor cells (Chang *et al*., 1999a).

1.5 Pathways of apoptotic & non-apoptotic cell death

Various mechanisms that involve apoptotic and non-apoptotic pathways of cell death have been well defined in normal physiology and during tumorigenesis (Okada & Mak, 2004). Defects in signaling cell death pathways are hallmarks of cancer. A recent study has summarized two major apoptotic pathways in mammalian cells: the death receptor induced extrinsic pathway and the mitochondria apoptosome mediated apoptotic intrinsic pathways (Putcha *et al*., 2002; Viktorsson *et al*., 2005). Both of the pathways lead to caspase activation and cleavage of cellular substrates (Strasser *et al*., 2000). At the molecular level, the signaling pathways that affect apoptotic sensitivity are activated by engagement of cell surface receptors (Rudin & Thompson, 1998; Viktorsson *et al*., 2005). The largest family of cell death receptors are members of tumor necrosis factor (TNF) receptor, including TNF-R1, Fas (Apo-1/CD95), TRAIL (Apo-2), D3 and D6 (Fulda & Debatin, 2004a) These death receptors are characterized by a cytoplasmic domain of 80 amino acids known as the death domain, which is essential in transmitting the death signal from the cell's surface to intracellular signaling pathways (Fulda & Debatin, 2004b).

The extrinsic pathway of apoptosis is triggered by various extracellular stimuli, such as UV or γ-irradiation, chemotherapeutic drugs and heat shock (Mor *et al*., 2002). This pathway is initiated by the engagement of death receptors on the cell surface (Kiechle & Zhang, 2002). Binding of ligands such as cytokines and TNF to TNFR1 (TNF receptor type 1), induces the recruitment of adaptor molecules TRADD (TNFR1 associated death domain) through association of death domain (Natoli *et al*., 1998). The TNFR1-TRADD complex engages with RIP and TRAF-2 (TNF receptor associated factor 2), leading to NFkB activation, which suppress apoptosis activation (Rudin & Thompson, 1998). The recruitment of FADD by the TNFR1-TRADD complex resulted in apoptosis through the activation of

Caspase-8 (a cell death protease) (Kiechle & Zhang, 2002). Besides the TNF system, Fas and TRAIL signaling pathways have been characterized extensively (Hu & Kavanagh, 2003). The Fas signaling pathway plays a crucial role in immune surveillance of transformed cells, in tissue remodeling and in the removal of self-reactive lymphocytes (Mor *et al*., 2002). TRAIL signaling pathway has attracted interest for cancer therapy because TRAIL seems to exert selective toxicity toward cancer cells but not in normal cells (Hu & Kavanagh, 2003). Both pathways begin with the ligation of death receptors such as Fas/CD95 or TRAIL (TRAIL-R1 & TRAIL-R2) by their cognate ligand (FASL) or agonistic antibodies resulting in receptor trimerization and recruitment of adaptor protein FADD via the death domain (Fulda & Debatin, 2004b). In turn, FADD binds to other cellular proteins to form the death-inducing signaling complex (DISC) (Cartee *et al*., 2003). Once the DISC is assembled, proCaspase-8 or proCaspase-10 oligomerization triggers its autoactivation by self-cleavage. Following activation, Caspase-8 or Caspase-10 initiates the downstream caspase cascade, which eventually ends the apoptosis process (Ghobrial *et al*., 2005). For the Fas signaling pathway, two distinct prototypic cell types have been identified, termed type I and type II (Cartee *et al*., 2003). In type I cells, activation of Caspase-8 is sufficient to directly activate downstream effector Caspases and lead in turn to apoptosis (Dai *et al.*, 2003). For type II cells, the amount of Caspase-8 generated at the DISC is insufficient to trigger apoptosis and the mitochondrial pathway is required for full activation of Caspases (Kim *et al*., 2002). Thus, this pathway involves Bid, which triggers the release of cytochrome c from mitochondria in order to translate the death signal (Kim *et al*., 2002).

The intrinsic pathway relies on mitochondria as initiators of cell death (Chen *et al*., 2004). Various signals converge on mitochondrial, including growth factor withdrawal, hypoxia, DNA damage and oncogene induction (Reed & Pellecchia, 2004). The mitochondrial pathway is initiated by the proapoptotic protein from the bcl-2 family such as Bax, Bak or Bid, which trigger the release of apoptogenic factors such as cytochrome c, Smac/DIABLO, Omi/Htr A2 and apoptosis- inducing factor (AIF) (Beere, 2004). Once released into the cytosol, cytochrome c binds to an adaptor protein, Apaf-1 (apoptotic

protease-activating factor 1) and recruits proCaspase-9 to form the apoptosome complex (Beere, 2004). In turn, Caspase-9 is activated and promotes activation of Caspase-3, Caspase-6 and Caspase-7, which induce DNA fragmentation and intracellular protein degradation (Haupt *et al*., 2003). Meanwhile, Omi/HtrA2 and Smac/DIABLO are able to promote Caspase activation by counteracting to inhibitor of apoptosis protein (IAP) (Okada & Mak, 2004).

Recent studies have identified an association between apoptosis and non-apoptosis cell death, especially autophagic cell death (Castro-Obregon, 2002). Both apoptosis and autophagy cell death are involved in complementary death pathways in cells (Tanabe *et al.*, 1999). If Caspase-8 is inhibited during extrinsic signaling pathway, RIP, JNK (Jun N-terminal kinase) and Atg genes directly activate autophagic cell death (Baehrecke, 2005). While active Caspase-8 cleaves RIP and prevents it from activating autophagic cell death (Baehrecke, 2005). The autophagy cell death is regulated by the class I and class III phosphatidylinositol-3-kinase (PI3K) signaling pathways in animal cells (Yuan *et al*., 2003). Class I PI3K is a negative regulator of autophagy that inhibits autophagosome formation in response to insulin-like and other growth factor signals (Levine & Yuan, 2005). Activation of class I PI3K leads to the phosphorylation of plasma membrane lipids, which recruit and active downstream autophagy inhibitors, including Akt/PKB (protein kinase B), Rhed and Tor (Lum *et al*., 2005). Consequently, inhibition of Tor by rapamyein blocks cell cycle progression and thereby results in autophagy (Yoshimori, 2004). By contrast, class III PI3K activity is important for early step of autophagic vesicle formation and involves the autophagy positive regulator Atg 6 (also known as Beclin 1 in mammalian cells) (Lum *et al*., 2005). Autophagic vacuole expansion also involves two ubiquitin-like conjugation pathways (the Atg 8 and Atg 12 system) (Ohsumi, 2001). However, the other non-apoptotic signaling pathways are not fully understood and remain to be elucidated.

Further understanding of these diverse signaling pathways, especially the regulations of apoptosis and autophagy in response to anticancer therapy, will provide a molecular basis in the discovery of novel targeted agents (Ghobrial *et al*., 2005). Thus, the novel agents may

overcome some resistant forms of cancer and may enhance the efficacy of conventional chemotherapy regimens.

1.6 Objectives of the present study

The specific aims of the present study are:

1. To evaluate the growth inhibitory and cytotoxic properties of some of the locally available medicinal plants traditionally used for cancer-related disease, by using a variety of cancer cell lines.
2. To identify the mechanisms of cell death elicited by the most potent plant extract on a variety of cancer cell lines.
3. To elucidate the pattern of apoptotic-related gene expression on different cancer cell lines following treatment with the plant extract.
4. To determine the cytotoxic activity of the fractions from the most potent extract.
5. To elucidate and determine the cell death mechanism exerted by the potent fractions.
6. To identify major compounds in the potent fractions.

Chapter 2
Materials and methods