MECHANISTIC STUDIES OF THE ANTICANCER ACTIVITY OF THE EXTRACTS OF *Eupatorium odoratum* L. AND *Euphorbia hirta* L.

FAIZAH HARUN

UNIVERSITI SAINS MALAYSIA

2012

MECHANISTIC STUDIES OF THE ANTICANCER ACTIVITY OF THE EXTRACTS OF *Eupatorium odoratum* L. AND *Euphorbia hirta* L.

By

FAIZAH HARUN

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

February 2012

ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Allah, the almighty, for giving me the opportunity, strength and patience to face obstacles along the way of completion of this dissertation. Without His kind blessings nothing would have been possible.

The first person whom I want to give my highest appreciation is my supervisor, Prof. Prabha Balaram for her encouragement, guidance and advice throughout this project. She has spent a lot of energy and time in helping me complete this project within the specific time frame. I am indebted to her for the patience and trust she had in me. I would like also to acknowledge my co-supervisor, late Prof Syed Mohsin Sahil Jamallullail for his advices and constant guidance throughout the project.

This ‘thank you’ remark also goes to my family members especially my mother, Mrs. Poziah Jab and my father, Mr.Harun Yaakob who gave me mental and financial support for completion of this project. Their endless love and motivation energized me to finish up the study.

Special thanks to INFORMM, which gave me opportunities to continue my Master study. I thank all INFORMM lab staff, especially En. Badrulsyam, Ms.Elis Rosliza and others whose names are not stated here profusely for their full cooperation and kind assistance during my labwork at INFORMM. My sincere appreciation goes to the administrative staff of INFORMM, Mrs. Noral Wiah, Mrs. Norazean, Mrs. Zalani, Mrs.Nor Lizaini, Ms. Nur Hazira, Mr. Nasir Mohamad, Mr. Mohd.Khairi, Mr. Hafis and others who were always there whenever I faced problems during my candidature as a student at INFORMM.

I would also like to thank School of Health Sciences (PPSK), Immunology Department of School of Medical Science (PPSP) which contributed in providing equipments, place for initial plant extraction procedures and to carry on with other experiments. My gratitude is extended to Prof. Ahmad Zakaria, Dean of PPSK, Prof. Mustaffa Musa, Head of the Department of Immunology PPSP, for granting me permission to use their available lab to do these experiments. My unconditional thanks go to Mr. Aminuddin, Mr. Zulkhairi, Mr. Hasbullah, Mr. Jamaruddin, Mr. Rusli and Mr.Shunmugan Vellosamy who gave full cooperation and helps in my laboratory work.

Deep from my heart with love and faith, I would like to thank all my supportive friends namely; Nor Fadhillah Kamaruzzaman, Wan NurFahizul Izzati, Siti Mariam Mawarni, Rasyada, Julia, Nuramalina, Wan Nurliana, Nur Eliyana, Iqbal, Mohd. Zafri, Wong Weng Kin, Yu Wei Chuen, Chin Kai Ling and B.Saatheeyavaane. My sincere thanks to USM for awarding me the USM Fellowship and financial support through Short-Term Grant No. 1001/CIPPM/811061 and chance for me to continue my MSc study at USM. Last but not least, my thanks are also extended to many other friends whose names are not stated here. Their direct and indirect continuous support have helped in many ways towards completion of this thesis.

TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS & SYMBOLS

MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
DNA	Deoxyribonucleic acid
µg/ml	Microgram per milliliter
mg/ml	Milligram per mililiter
B.C.	Before Christ
bp.	Base pair
IC_{50}	50 percent inhibitory concentration
DMSO	Dimethylsulfoxide
PVDF	Polivinylidene fluoride
RPMI	Roswell Park Memorial Institute
mM	Milimolar
ml	Milliliter
V	Voltage
DAB	Diaminobenzidine
HRP	Horseradish peroxidase
IgG	Immunoglobulin G
PI	Propidium iodide
PBS	Phosphate buffered saline
NaCl	Sodium chloride
KCl	Potassium chloride
Na_2HPO_4	Disodium hydrogen phosphate
$NaH_2PO_4.dH_20$	Sodium dihydrogen phosphate dihydrate
EDTA	Ethylenediaminetetraacetic acid
CO_2	Carbon dioxide
TBE	Tris Borate EDTA
pH	A measure of the acidity or alkalinity of a solution
A_{280}	Absorbance at 280nm
dH_2O	Distilled water
^oC	degree Celsius
nm	nanometer
g	gram
rpm	Revolutions per minute

KAJIAN MEKANISME ANTI KANSER TERHADAP EKSTRAK *Eupatorium odoratum* L. DAN *Euphorbia hirta* L.

ABSTRAK

Eupatorium odoratum L. (EO) dan *Euphorbia hirta* L. (EH) merupakan dua tumbuhan yang acapkali digunakan dalam rawatan tradisional untuk menyembuhkan luka, asma dan ketumbuhan. Walaupun terdapat laporan aktiviti anti-kanser dari genus tumbuhan ini, hanya sedikit maklumat yang diketahui berkaitan perihal mekanisme anti kanser daripada spesies ini. Dalam kajian ini, keupayaan ekstrak EO dan EH untuk membunuh sel kanser diuji menggunakan sel kanser payudara (MCF-7), kanser serviks (Hela), kanser ovari (CaOV-3) dan sel normal epithelial (Vero). Sel-sel kanser di rawat dengan ekstrak tumbuhan menggunakan sembilan kepekatan berbeza pada tiga tempoh masa yang berlainan (24, 48 72 jam) dan parameter berikut diperhatikan: (1) penentuan nilai IC_{50} dan kesan sitotoksik terhadap sel kanser melalui asai MTT dan perisian GraphPad Prism, (2) pemerhatian morfologi menggunakan mikroskop ‘inverted’ dan ‘fluorescent’, (3) kesan apoptosis menerusi kajian penanggaan DNA dan kadar pengekspresan protein (BCl-2 dan Bax), (4) kesan autofagi melalui pengekspresan BCl-2 dan LC3-A dan (5) kesan tehadap fasa kitaran sel menggunakan kaedah ‘flow cytometry’. Ekstrak etil asetat dari *E. odoratum* (EOea) menunjukkan kesan sitotoksik terhadap sel Hela, CaOV-3 dan MCF-7 pada nilai IC_{50} 40-95 µg/ml. Manakala, ekstrak aseton *E. odoratum* (EOace) hanya menunjukkan kesan sitotoksik terhadap sel MCF-7. Ekstrak etil asetat daripada *E. hirta* merekodkan nilai IC_{50} dalam lingkungan 40-55 µg/ml. Ekstrak tumbuhan yang digunakan dalam kajian ini menunjukkan kesan sitotoksik yang lemah terhadap sel Vero yang digunakan sebagai sel kawalan normal. Pemerhatian

morfologi sel MCF-7 yang dirawat dengan EOace menunjukkan kesan khusus kematian autofagi, dan ini dipastikan dengan pengekspresan protein LC3-A yang terlibat dalam proses tersebut. Pemerhatian yang sama turut direkodkan bagi sel CaOV-3 yang dirawat menggunakan EOea. Walau bagaimanapun, kesan pengekspressan adalah lebih rendah. Maka mekanisme kematian sel melalui mekanisme autofagi tidak dapat dipastikan. Bagi sel Hela yang dirawat dengan EHea, kesan autofagi dapat dilihat pada permulaan rawatan (24 jam). Namun demikian, pada jam ke 48, pengecutan sel dan pemampatan nukleus diperhatikan. Pada akhir jam ke 72, hampir kesemua sel menunjukkan kesan penanggalan dari permukaan kultur. Ini mencadangkan kematian sel melalui apoptosis. Mekanisme apoptosis dikuatkan dengan kesan penanggaan DNA dan peningkatan dalam pengekspresan protein Bax pada jam ke 48 dan ke 72. Analisis fasa kitaran sel menunjukkan peningkatan populasi sel Hela yang dirawat dengan ektrak EHea pada fasa G0/G1. Selain itu, corak penurunan populasi sel dalam fasa S dapat dilihat dalam rawatan EOea terhadap sel CaOV-3. Manakala, ekstrak-ekstrak lain tidak menunjukkan kesan terhadap fasa kitaran sel. Hasil kajian ini turut menujukkan kesan ekstrak adalah unik terhadap sesuatu sel kanser. Ekstrak aseton dan metanol mengakibatkan penambahan populasi sel. Pemerhatian ini mencadangkan agar penggunaan ekstrak ini hendaklah mengambil kira kadar pemberian dos pada sesuatu masa agar kesan sitotoksik dapat dikekalkan. Secara keseluruhan, keputusan kajian mencetuskan andaian awal terhadap dua mekanisma kematian sel yang berlainan oleh ekstrak tumbuhan terhadap sel yang berasal dari kawasan berbeza dan kepentingan untuk mengekalkan kepekatan ekstrak pada sesuatu tahap tertentu bagi mengekalkan kesan sitotoksiti ekstrak.

MECHANISTIC STUDIES OF THE ANTICANCER ACTIVITY OF THE EXTRACTS OF *Eupatorium odoratum* L. AND *Euphorbia hirta* L.

ABSTRACT

Eupatorium odoratum L. (EO) and *Euphorbia hirta* L. (EH) are two plants which are commonly used traditionally to treat wounds, asthma, warts and tumors. Even though the anti-cancer properties were described previously in the species, little was known on the activity of these plants specifically and on mechanism of cell death induced by these plant extracts. Hence, in this study, the ability of the plant extract to induce cell death was evaluated. Human breast carcinoma (MCF-7), human cervical carcinoma (Hela), human ovarian carcinoma (CaOV-3) and Vero cells were evaluated for response to treatment with EO and EH extracts. The cells were treated with nine different concentrations of respective extracts at three different time intervals (24, 48 and 72 hours) and assessed for the following parameters. (1) cell death using MTT assay and graph pad prism software to assess the cytotoxicity effects and IC_{50} values, (2) morphology of the cells using visible and fluorescent microscopy, (3) apoptosis using DNA ladder assay and expression of BCl-2 and Bax proteins as markers, (4) autophagy using BCl-2 and LC3-A protein expression as markers and (5) cell cycle effects using flow cytometry. Ethyl acetate extracts of *E. odoratum* (EOea) showed potent cytotoxicity against Hela, CaOV-3 and MCF-7 cells at IC_{50} values ranging from 40-95 µg/ml while EOace showed promising effects on MCF-7 cells only. Ethyl acetate extracts of *E. hirta* (EHea) showed effective cytotoxic effects on Hela and CaOV-3 cells with IC_{50} values in the range of 40-55 µg/ml. The extracts were non-toxic to Vero cells. Morphological observations on treatment with EOea and acetone extract (EOace), showed typical features of autophagic mechanism of cell

death in MCF-7 cells. This was confirmed by upregulated expression of LC3-A protein. This pattern was also observed with CaOV-3 cells even though at a lower degree. Lower expression of LC3-A and no DNA fragmentation were observed casting doubts over autophagy or apoptosis as the cell death mechanism. Hela cells treated with EHea showed initial autophagic signs with moderate LC3-A expression. At 24 hours, shrunken cells and nuclear condensation at 48 hours and detachment of the cells were seen by 72 hours suggesting apoptotic cell death. This was also supported by DNA ladder formation at 48 and 72 hours, reduced LC3-A protein expression and increased Bax protein expression at this time. Results of cell cycle analysis showed increase in G0/G1 cell population in Hela cells treated with EHea, trend towards S phase arrest in CaOV-3 cells treated with EOea, and cell cycle independent effects with other extracts. These results suggest the differential effects of the extracts on cell lines. The increased in IC_{50} values with time and observation of proliferative effects of the etracts at lower concentrations suggest the necessity of maintaining optimum bioavailable doses. However, this proliferation enhancing property can be made use of for formulation of wound healing agents. Thus, the observed results provide a description of the potential of the two plants evaluated in this study to induce cell death through different mechanisms in epithelial cells of different origin and also points to the need of maintaining required bioavailable doses when used for therapy.

CHAPTER 1 - INTRODUCTION

Cancer of the breast, uterine cervix and ovary are the leading female cancers and their incidence is on the increase. This fact prompted us to evaluate the effect of the plant extracts on breast adenocarcinoma (MCF-7), ovarian cancer (CaOV-3) and cervical cancer (Hela) cell lines and compared it with the normal epithelial cells in this study.

In spite of advances in treatment modalities such as surgery, radiotherapy, chemotherapy, and hormone therapy for the above cancers, the treatment response has not improved substantially. This calls for identification of novel effective chemotherapeutic agents which could have immense impact on the mortality and morbidity due to these cancers. The urge to seek for new chemotherapeutic drugs from plants is based on its application in traditional medicine to treat many kinds of disorders (Otsuki *et al.*, 2010). As an example, native people in Africa, use upto 4000 taxas of plant in their daily life for treatment of various kinds of diseases (Mulholland, 2005). The World Health Organisation (WHO) has assessed that >80% of the people in developing countries rely on herbal medicine for their medicinal uses (Gao & Ke, 2005) In modern treatment of cancer, plants have been used widely to enhance the immune response as well as to diminish side effects of conventional treatments (Kathleen N., 2001).

Anti-cancer activity by plant products is mostly brought about by induction of programmed cell death or by inhibition or stimulation of certain cellular pathways involved in cell proliferation or death. Programmed cell death (PCD) which is known to be induced by some of the plant extracts against some types of cells is an important mechanism by which cells maintain their homeostasis and remove dead cells (Schwartzman and Cidlowski, 1993). Three types of cell death mechanisms are reported. These include type I PCD; apoptosis, Type II PCD; autophagy and nonlysosomal cell disintegration or necrosis. These act by different mechanisms. Compared to necrosis, PCD has attracted more attention as the cell death mechanism does not cause inflammation to neighboring cells. Apoptosis is associated with characteristic changes such as membrane blebbing due to changes in membrane permeability, chromatin condensation and nuclear fragmentation, which are considered the hallmarks of apoptosis. The cell death caused by this mechanism only involves the particular cell (Bold *et al.*, 1997). The decision of the cells to undergo apoptosis or autophagy is decided mainly by the functional status of caspase-3 (Fazi *et al.*, 2008).

Autophagy (Greek auto=self; phagy=to eat) is recognized as the type II mechanism of programmed cell death in eukaryotes. Even though described as early as forty years ago (Ericsson, 1969), the mechanisms involved in this mode of cell death has attracted attention mainly in the last decade. It occurs at a small level in all cells to maintain cellular homeostasis (eg; cytoplasmic and organelle turnover). Autophagy involves formation of double membrane vesicles in the cytosol. These vesicles trap materials programmed for degradation (Uchiyama *et al.*, 2008; Levine and Yuan, 2005), damaged organelles and also other organelles in times of nutrient depletion. The nutrients recycled from the degrading organelles are used for survival of the cells for short periods of time. During nutrient deprivation, developmental transitions, oxidative stress, infection and other stresses, this process is up regulated, mainly as a pro-survival mechanism (Cuervo, 2004; Ravikumar *et al.*, 2009). The vesicles formed fuse with the lysosomes to degrade the trapped material. Autophagic regulation mainly consists of two mechanisms, namely, the mechanism to shut down autophagy during nutritional abundance and the mechanism to turn on autophagy during stress. In cancer cells, autophagy acts as a double edged sword. In the earlier stages of cancer cell growth, autophagic mechanisms are inhibited to allow the growth of the cells while in the later stages, the cells inside the hypoxic regions turn on autophagy for survival (Cuervo, 2004). In yeast, during nutrient deprivation, the cells adopt autophagy and eventually die because of the excessive self-consumption and bioenergetic failure. However, if the nutrients are available anytime before the death of the cell, the cells retain viability and return to its normal state (Levine and Yuan, 2005) suggesting that autophagy could be a mechanism cells use to hibernate during nutrient deprivation.

Autophagy is of importance in cancer stem cells and cancer cells resistant to chemotherapy. In cells that cannot die due to defects in the apoptotic pathway, as seen in many cancers, the autophagic mechanism is activated (Cuervo, 2004). Activation of autophagy suppresses apoptosis, thereby protecting some cells from the anti-cancer treatments while some cells, especially those in which the apoptotic mechanism is defective, undergo cell death following cancer therapy (Kondo *et al.*, 2005; Codogno and Meijer, 2005). Tamoxifen is reported to activate either autophagy or apoptosis or both in breast cancer cells (Bursch *et al.*, 1996). A number of compounds such as tamoxifen, Temozolomide, sodium butyrate and Suberoylanilide hydroxamic acid (SAHA), Arsenic trioxide, Rapamycin and treatment such as gamma radiation and hyperthermia induce autophagy in cancer cells (Brian *et al.,* 2001).

Eupatorium odoratum and *Euphorbia hirta* used in this study posses unique anti-microbial and other medicinal properties as reported in literature (Redo *et al.*, 2006). Traditionally, white latex of *E. hirta* plant is widely used to remove warts and tumors (Qing *et al.*, 2008). The crude extract from this plant has been reported to demonstrate potent cytotoxic effects towards several cell lines. For instance, crude methanolic extracts of *E. hirta* showed anti-proliferative effects towards human epithelioma of larynx (Hep-2) *in vitro* (Raja, 2011). Besides, aqueous extract also showed reduction in prostaglandins I, E and D, thereby increasing tumour growth (Wang & Dubois, 2006). As for *E. odoratum*, it is traditionally used as a wound healing agent. Compounds from this plant include those with strong anti-cancer properties such as quercetin, eupalinin and luteolin (Bose *et al.*, 1973, Suksamrarn *et al.*, 2004; Yuan *et al.*, 2005). *In vitro* study reports cytotoxic activity of extracts from leaves of this plant on lung cancer cells (NCI-H187) (Suksamrarn *et al.*, 2004). Eventhough anti cancer activities of these plants have been reported, very few reports are available on the mechanism of cell death induced by the crude extracts especially on caspase-3 independent cell lines. In addition, the relevance of these plants in treating various kinds of microbial diseases and cancers in traditional medicine provides evidence for these plants to be developed as new chemotherapeutic drugs. Thus, the results from this study can be used as preliminary data for development of new anti cancer drugs from these plants targeting on the specific cell death mechanism.

Aim and objectives of the study

This study was designed with the aim of evaluating the cytotoxic potential of *E. odoratum* and *E. hirta* on breast, cervix and ovarian cancer cells, as well as to identify the mechanism of cell death by the extracts.

The specific objectives of this study were:

1. To evaluate the cytotoxicity profile of the different extracts from *E. odoratum* and *E. hirta* on MCF-7, CaOV-3, Hela and Vero cell lines by MTT cytotoxicity assay.
2. To determine the mechanism of cell death induced by potent plant extracts by morphological observation, DNA laddering, and expression of proteins involved during apoptosis and autophagy by immunocytochemistry staining.
3. To determine the effect of the potent extract on cell cycle phases using flow cytometry analysis.
4. To assess if there is any pro-proliferative effects for these extracts which is of importance in their use as drugs.

CHAPTER 2 - LITERATURE REVIEW

This literature review encompasses descriptions of the (1) characteristics, and worldwide and Malaysian incidence of cancer in relation to the cell lines used in this study, (2) current treatment modalities and their drawbacks, (3) plants as anticancer agents, (5) modes of cell death by plant extracts and (6) characteristics and importance of the plants used in this study.

2.1 Cancer-A life threatening disease

The word 'cancer' refers to diseases that exhibit two characteristics in common: (1) an uncontrolled growth of cells and (2) the ability to invade and damage normal tissues locally or at distant sites in the body. The body is made up of large number of cells and has certain norms of behavior in order to maintain homeostasis (Jameson, 2002). The normal cells show the characteristics of polarity, adhesion to the basement membrane, definite nuclear cytoplasmic ratio, transition from basal cell to differentiated cells, cell-cell contact, display of territorial limits, controlled and regulated growth, and controlled death or senescence in a programmed manner. However, at times, there is a disruption of this balance when the cells show abnormal growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, loss of contact inhibition (Hanahan, 2000), ability to invade adjacent tissues and metastasize to distant tissues, immune escape, immortality, defects in DNA repair and sustained angiogenesis.

Cancer is considered a genetic disease. It occurs when the deoxyribonucleic acid (DNA) present in a gene is altered by external or internal agents. In such a situation, the gene can no longer instruct the cells to behave in the normal manner (Hanahan,

2000) and often results in cancer growth. The causes for DNA within a gene to be altered, recombine incorrectly or to mutate are multifold. The causative factor may be environmental as in the case of UV radiation from sunlight, exposure to chemicals used in industries and agriculture or infectious agents such as viruses and bacteria. Endogenous factors such as free radicals produced during metabolism or due to inheritance of susceptible mutations also cause mutations. Cell division and cell death are regulated by a network of proteins which are products of cellular genes. Alterations in these proteins by the cellular environment or damaging agents can lead to the disruption of the balance and cause uncontrolled division and accumulation of cells (Hanahan, 2000)

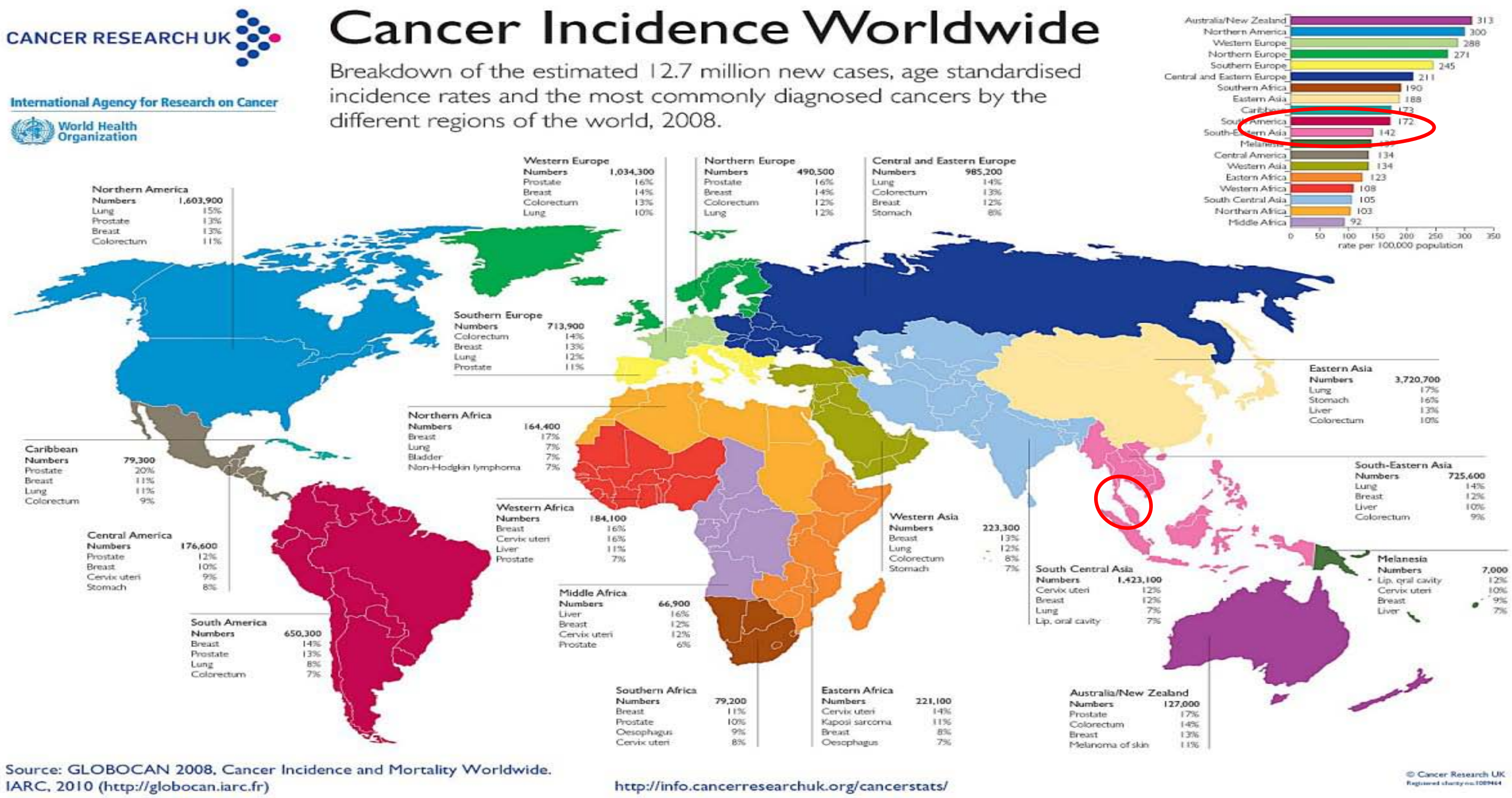
Malignant transformation is thought to be the result of the accumulation of genetic defects (Pavan *et al.,* 2008) and involve a multistep progression in involving oncogenes, tumor suppressor genes, DNA damage repairing genes and genes involved in cell death. Damage in any of these can lead to cancer formation. Literature show that plants and their products are capable of reducing or eliminating many of these effects and hence are worth evaluating for their efficacy as drugs (Sultana *et al.,* 1995).

2.1.1 The cancer burden

Cancer occupies number one position as the leading cause of death worldwide. It accounted for 12.7 million cancer cases and 7.6 million deaths reported in 2008 (Figure 1.1) (Ferlay *et al.,* 2008). Of these, 56% of the cases and 64% of the deaths were reported from the developing countries. The Asian countries contributed to 45% of the cases and 12% of the deaths. The deaths due to cancer are projected to increase to over 11 million deaths by 2030 (Colin & Dejan, 2006).

According to the Globocan report 2008, South-Eastern Asia contributed for about 142 cancer cases per 100, 000 populations in 2008. Lung cancer was reported to be the leading cancer (14%), followed by breast (12%), liver (10%) and colorectal cancers (9%) (Globocan, 2008).

In peninsular Malaysia 70, 000 new cancer cases were diagnosed among Malaysians between 2003-2005. According to National Cancer Registry (NCR) Malaysia, 43.7% (29, 596 cases) cases were among males and 56.3% (38, 196 cases) among females. The most common cancers reported during this period were breast cancers (18%), large bowel cancers (11.9%) and lung cancers (7.4%) (Ferlay *et al.*, 2008). Hence it is imperative that novel chemotherapeutics are discovered in order to reduce the burden of this disease both in Malaysia and worldwide.



Picture source : http://info.cancerresearchuk.org/cancerstats/

Figure 2.1: Figure showing the prevalence of cancer cases worldwide in 2008.

2.1.2 Current treatment modalities and drawbacks

Managing and treating of cancer can be done in various modalities targeted to cure, control the growth and prevent it from metastasizing. The treatment may be curative or palliative. The conventional therapies used to treat cancer that we are all familiar with are chemotherapy, radiation therapy and surgery depending on the location, grade of the tumor and stage of the disease. Modern therapy using mediators, complimentary medicine and plant products are gaining momentum.

Chemotherapy is a systemic treatment using chemicals as drugs (Giani, 1976). The main goal of chemotherapy includes the total destruction of the cancerous cells and controls the disease from progressing. In advanced stages, it is also used to relieve the symptoms caused by cancer. Cisplatin is a commercially available anti-cancer drug used for treatment of cancers of epithelial origin including those of lung, stomach, ovary, bladder (MacMillan, 2010). Tamoxifen is commonly used for treatment of estrogen receptor positive breast cancers.

Modern cancer therapies include use of bio-mediators such as angiogenesis inhibitors, targeted therapy using monoclonal antibodies, small molecules such as receptor inhibitors, small peptides used for homing of drugs, immunotherapy using immune mediators and other therapies such as photodynamic therapy, bone marrow transplantation, gene therapy, laser therapy, and also bacterial preparations and plant products.

In spite of millions of dollars spent on improving cancer therapeutics, it is disheartening to note that the overall survival rate for all cancers combined increased slightly from 49.3% in 1974-76 to 53.9% in 1983-1990. The old adage about cancer which says "hopefully the cancer won't kill you before the cancer cure does" is true to an extent, because, in conventional cancer therapy, the body is bombarded with toxic agents such as drugs and radiation. Even though the attack is directed against the cancer cells, the normal cells are also affected adversely, especially the immune system. This makes the elimination of the cancer cells less efficient. Besides this, the conventional treatments speak in terms of 5 year and 10 year survival rates which are transient improvements in the life span, often compromising on the quality of life (Conventional Cancer Treatments, 2011). Uncomfortable episodes of nausea, gastrointestinal problems, anemia, leucopenia, bleeding, general malaise, mental impairment ("chemo-brain"), hair loss, depression, somnolence, cognitive abnormalities, fatigue, loss of platelets, pain, irritation, fibrosis, secondary tumours, morbidity, increased risk of heart disease etc. are usually observed in the patients on conventional therapy (Giordano & Hortobagyi, 2007; Harbeck *et al.*, 2011; Jones, 2011; Wefel *et al.*, 2001; Soussain *et al.*, 2009; Sood *et al.*, 2006; Abramowski, 2010). Furthermore, it is not possible to kill every cancer cell by the conventional treatments, a fact known by all oncologists. The importance of a fully functional immune system is very well recognized in the modern times and cancer is very often referred to as "failure of the immune system". Drug resistance is another major hurdle in the removal of cancer cells by chemotherapy (Chung & Pascale, 2004). So the need of the moment is a therapeutic system which makes sure that there is no uncomfortable side effects to the patients and retains the integrity of the immune system to see that all the cancer cells in the host are killed following their reduction by conventional therapeutic modalities. Plants are reported to act by targeting multiple pathways and increasing the immune status of the body (Christiane *et al.*, 1993). Hence it is expected that plants will be able to control cancers in a better way when compared to the conventional treatments.

2.2 Anti-cancer substances from natural products

Nature is a rich source of therapeutic compounds with millions of species of plants, animals, marine organisms and microorganisms and natural chemical structure types resulting from nature's combinational chemistry efforts. In the words of Rudyard Kipling (1910), British Nobel Laureate "Anything green that grew out of the mould was an excellent herb to our fathers of old." Kipling and Aristotle (1910) have righty put it as "Nature does nothing without purpose or uselessly."

A study on crude extracts is crucial in order to screen anti cancer properties of the plant in the initial stages of new chemotherapeutic development. This is based on evidence from studies on crude extracts from many plants. As for example, the study done by Bibi *et al.* (2011) observed anti-tumor effects of crude methanolic extract from *Aster thomsonii* plant against H157 and HT144 cancer cell lines *in-vitro*. In addition, methanol and *n*-hexane crude aqueous extracts of *Inula viscosa* showed cytotoxic effects towards MCF-7 and Hep-2 cancer cell lines *in-vitro* (Wamidh *et al.*, 2009). Crude extracts of seeds and leaves of neem plant have showed potent anti-tumour activity against Ehrlich ascites carcinoma cells (EACC) (Hassan *et al.*, 2010).

Cragg and Newman (2006) has listed down compounds that originated from plants which are currently used as anti-cancer agents. These include camptothecin, tototecan, innotecan, taxol, docetaxel, vinblastine (VLB), vincristine (VCR), teniposide, etoposide, podophyllotoxin, elliptinium and homoharringtonine. VLB and VCR are vinca alkaloids which are isolated from *Catharanthus roseus*. Clinically, it is used to treat leukemias, lymphomas, cancers of the breast and lung, and Kaposi's sarcoma. VCR is effective in treating lymphomas and particularly effective in acute lymphocytic leukemia during childhood (Sriram & Yogeeswari, 2010). Etoposide and etinoposide which are isolated from *Podophyllum peltatum* (American mandrakes) are now commonly used clinically for treatment of lymphomas and bronchial cancers and also testicular cancers (Doo *et al.*, 2010). Paclitaxel or taxol isolated from *Taxus brevifolia* is widely used for treatment of cancers of the breast, ovary and non-small lung cancer (NSCLC) besides showing effectiveness against Kaposi's sarcoma (Ettinger, 1993). Camphothecin, another compound from *Camptotheca acuminate* is used widely in small cell lung cancer (SCLC) treatment. Homoharringtonine, isolated from *Cephalotaxus harringtonia*, is successfully used in China for acute myelogenous leukemia and chronic myelogenous leukemia treatment. All these compounds have been discovered after the initial crude extracts being evaluated for their activities which stresses on the importance of evaluation of crude extracts.

Many other compounds form plants are still in research as anti-cancer drug candidates. These include Calanolide A and B, isolated from *C.lanigerum* and *C.teysmannii* which are indigenous to Sarawak, Malaysia (Kaysmann *et al.*, 1992).

2.3 Traditional medicines in cancer therapy

Humans began using plant alkaloids as pharmaceuticals as early as 3000 B.C. (Roberts and Wink, 1998). The medicinal benefits of herbs are extensively quoted throughout history in the Vedas, Bible, Koran and other texts and the treatment using these herbs have come to be known as herbalism or herbal medicine. Herbalism is a traditional folk medicine based on the use of plant components (Anon, 2011).

Indigenous people in Africa use up to 4000 taxas of plant for medicinal purposes (Mulholland, 2005). The medicines from plants are used as tinctures, teas, poultices, powders and other herbal formulations by these people (Samuelsson, 1992). The specific combination of the plants and the methods of preparation and application for the particular ailments were mostly passed down to the next generations orally. No written documents were maintained in most instances. In the more recent times, some documentation became available in the form of the Hindu scriptures (6000 years ago) and Chinese text books (2700 B.C.). The Sumerians practiced herbal medicine around 5000 years back and early Egyptian medicine practices were documented in 1000 B.C. It was the Greek physician, Pedanius Dioscorides, in the first century A.D, who first compiled the European treatise on the properties and uses of medicinal plants, De Materia Medica. The Greek contributed in a big way to the documentation of the medicinal practices, as preserved in the writings of Hippocrates and the Greek book that founded the science of Botany, 'Theophrastus Historia Plantarur' written in the fourth century B.C. (Michael, 2010).

However, it is only in the near recent periods that the importance of natural products was recognized and the use of plants involving isolation of active compounds from plants was attempted. The isolation of morphine from opium in the early 19th century was one of the first examples (Kinghorn *et al.*, 2003; Samuelsson, 1992).

2.4 Biological activities of natural products: Anti-tumor point of view

Darwin's theory of evolution states 'survival of the fittest' as one of the main strategies of evolution. In accordance with this, plant species that have evolved the capacity to defend themselves from potential predators and to inhibit other plants competing for space have been selected for (natural selection) survival (Mans *et al.*, 2000). To survive in nature, in the face of the stiff competition, the plant has evolved sophisticated defense mechanisms including an arsenal of toxic substances, such as terpenes and alkaloids. A survival mechanism adopted by the plants is production of toxic substances that can kill the predator organisms or inhibit the growth of other plants. This compound could also be effective in killing human cells and hence act as anti cancer compounds. This is usually brought about by secondary metabolites known as anti-herbivory that can influence the behaviour, growth and survival of organisms and plants. The anti-herbivory compounds can be classified into three sub-groups: The alkaloids, terpenoids and phenolics (Carmona *et al.*, 2011). Alkaloids, activate or inhibit enzymes, bind to nucleic acids and inhibit protein synthesis and affect a diversity of metabolic systems in humans and animals (Roberts and Wink, 1998). Terpenoids and phenolic compounds have a diversity of functions in humans and animals. Similar to these compounds, a number of compounds have been identified in the recent times which are of help in combating cancer cells using diverse mechanisms. Traditional practitioners exploited these natural defense mechanisms of the plants to treat various kinds of diseases including cancer. Taking cues from these practices, modern medicine has developed several medicines for cancer treatment from natural products. The most significant examples are the development of vinca alkaloid family isolated from the periwinkle *C. roseus*, found in the rain forests of Madagascar and taxols from the bark and leaves of *Taxus brevifolia* (Noble, 1990).

Acronychia baueri, a plant of Australian origin which is used for blood cancer treatment contains alkaloid acronycine which is responsible for its activity. *Allium sativum* was used by the Japanese to increase immunity in cancer patients which indirectly prevents cancer progression. Similar effects were discovered in *Astragalus membranaceus* root. *Aloe vera* juice and *Althea officinalis* were used for treatment of gastro-intestinal tract and skin cancers. In Malaysia, *Andrographis paniculata* was used in diabetic patient as well as in cancer treatment. A study by Ajaya *et al.* (2004) revealed the anticancer activity of this plant against HT-29 (colon cancer) cells, attributed to the presence of andrographolide present in the extract. Researchers also observed control of growth of ovarian cancers when treated with *Curcuma zedoaria* as well as *Cratageus cuneata* (Ajaya *et al.*, 2004). Besides being used directly to reduce cancer cell progression, plants are also used as complementary medicine in conjunction with standard drugs. This was found to be helpful in reducing side effects of these drugs. For example, Chinese herbal medicine (CHM) when combined with chemotherapy was observed to raise the efficacy level as well as reduce chemotherapy toxicity (Zhou *et al.*, 2008).

Apart from giving promising effects when combined with commercially available drugs, practitioners also combine several types of plants and mix it to produce tonics, ready for consumption. Triphala (Ayurvedic formulation), used in India for cancer treatment was shown to have anti-cancer effects against breast adenocarcinoma cells (MCF-7) *in vitro* (Sandhya *et al.*, 2006).

Active compounds which demostrate anti-cancer activity have been screened by many researchers all over the world. For instance, studies on *C.roseus* revealed that its alkaloid compound, vincristine and vinblastine can retard childhood leukemia and Hodgkin's disease progression. Recent studies of extracts from *Trailliaedoxa gracilis* showed anti-proliferative effects against small intestine (SI) neuroendocrine tumors (NETs) without affecting normal fibroblasts *in vitro.* The mechanism of cell death suggested was apoptotic cell death (Svejda *et al.*, 2010). A study by Kan (2008) demonstrated anti-proliferative effects of phthalides isolated from *Angelica sinensis* on colon cancer. In addition, the extracts also showed synergistic effects of this compound against colon cancer cell lines when combined with chemotherapy. Results from South-western Nigeria studies in plants commonly used in cancer treatment by old folks proved the cytotoxic effects of some of the extracts against cancer cell lines tested (Ashidi *et al.*, 2010). A study conducted by Kong *et al.* (2010) revealed that G*lehnia littoralis* suppressed proliferation and MMP expression in HT1080 cells *in vitro* (Kong *et al.*, 2010). In the study, glucopyranosides, furanocoumarins and polyacetylenic alcohols were isolated and found to be the functional compounds. The polyacetylenic alcohols showed highest inhibitory activity against human cancer cell lines (Kong *et al.*, 2010).

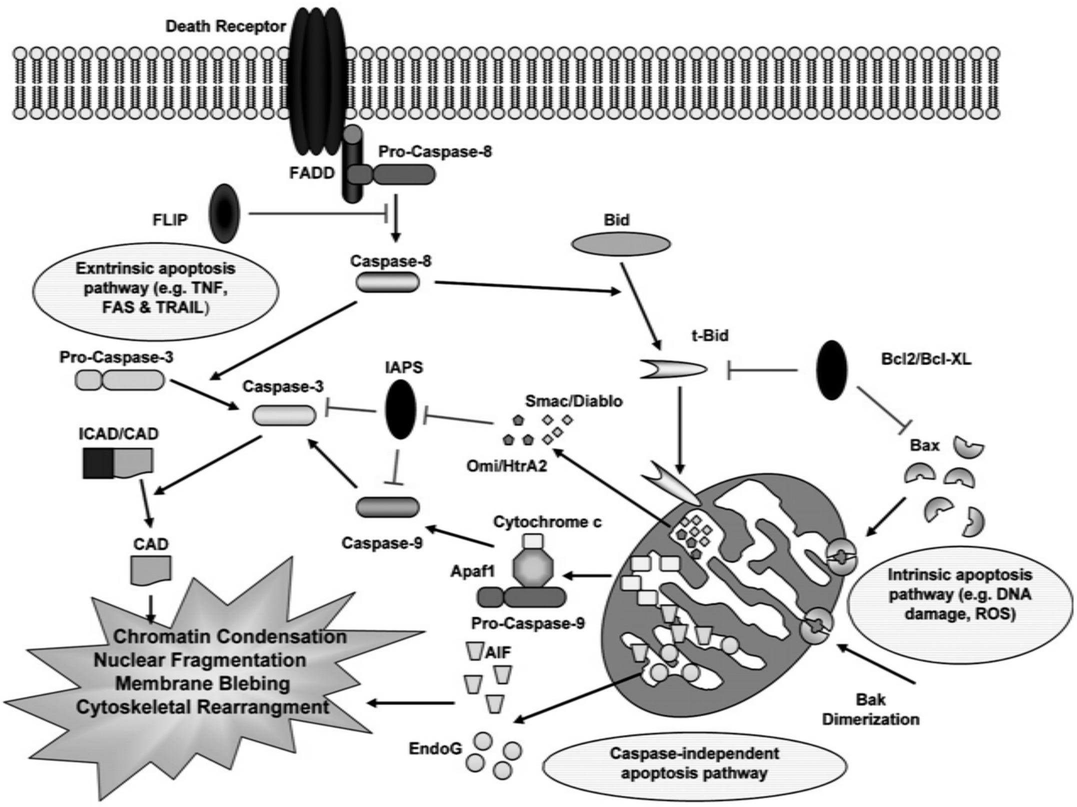
2.5 Mechanism of anti-cancer activity of natural products

Plant extracts are known to induce cell death through different mechanisms. The cell death mechanisms are activated, most often to remove unwanted, dead or senescent cells or cells damaged beyond repair. At times, this is also used as a survival mechanism in response to stress such as nutritional depletion or exposure to toxic materials. The persistence of the stress exhausts the cell of its survival mechanism and leads to cell death. Cytotoxic effects of plant extracts can induce different kinds of cell death mechanism namely; necrosis and programmed cell death (PCD). However, programmed cell death is more favorable to be studied as it does not cause damage the neighboring cells (Susan, 2007). PCD is a normal and basic biological process which has important roles during human development, tissue homeostasis and elimination of damaged cells. Two different types of PCD are (1) apoptosis and (2) autophagy.

2.5.1 Programmed cell death Type I- Apoptosis

Apoptosis is an active form of cell death. During apoptosis, the decision to undergo cell death is made when the cells receive signal from outside (external) or from inside the cells (internal). Examples of external or extracellular signals include toxins, hormones, growth factor, nitric oxide or cytokines. Extrinsic pathway is activated by binding of tumor necrosis factor (TNF) to the death receptor embedded in the lipid bilayer of the cell. The binding induces recruitment of Fas-associated death domain (FADD) adaptor proteins which have death effect domain (DED). DED then recruits similar DED which is expressed by pro-caspase-8/10 and convert it to the active caspase-8/10. Active caspase-8 then activates pro-caspase-3 into active caspase-3. Activation of caspase-3 lead to the release of caspase activated DNase (CAD) from its inactive ICAD. Subsequently, DNA fragmentation result in chopping of DNA strand into multiple fragments ranging from 180-200 bp and multiples thereof in size.

Compared to extrinsic pathways of cell death, intrinsic pathways for cell death are activated in response to free radicals, hypoxia, radiation, DNA damage or activated oncogenes. Normally, BCl-2, an anti-apoptotic protein, exists as a homodimer on the mitochondrial surface while Bad exist as freeform inside the cytosol which prevents cell death. Upon receiving apoptotic signals, Bad is phosphorylated and translocated to the mitochondrial membrane and interact with BCl-2 protein. This results in translocation of Bax protein which binds with the mitochondrial surface. Interactions of this pro-apoptotic protein with the surface of mitochondrial membrane eventually lead to the formation of pores which allow cytochrome-c and pro-caspase-3 from intermembrane space to be released into cytoplasm. Free cytochrome-c interacts with Apaf-1 protein which recruits pro-caspase-9 to form a complex with cytochrome-c and apaf-1 called apoptosome. Activation of caspase-9 will induce activation of caspase-3, finally causing cell death. Figure 2.2 shows intrinsic and extrinsic pathways of cell death.



Source: (Ghavami *et al.*, 2009)

Figure 2.2: Figure showing intrinsic and extrinsic pathways of apoptotic cell death induced in cells in response to external and internal signals.

Apoptosis is characterized by distinctive hallmarks such as cell shrinkage, plasma and nuclear membrane blebbing, organelle relocalization and compaction, chromatin condensation and finally the formation of particles containing intracellular material also known as apoptotic bodies (Bold *et al.*, 1997).

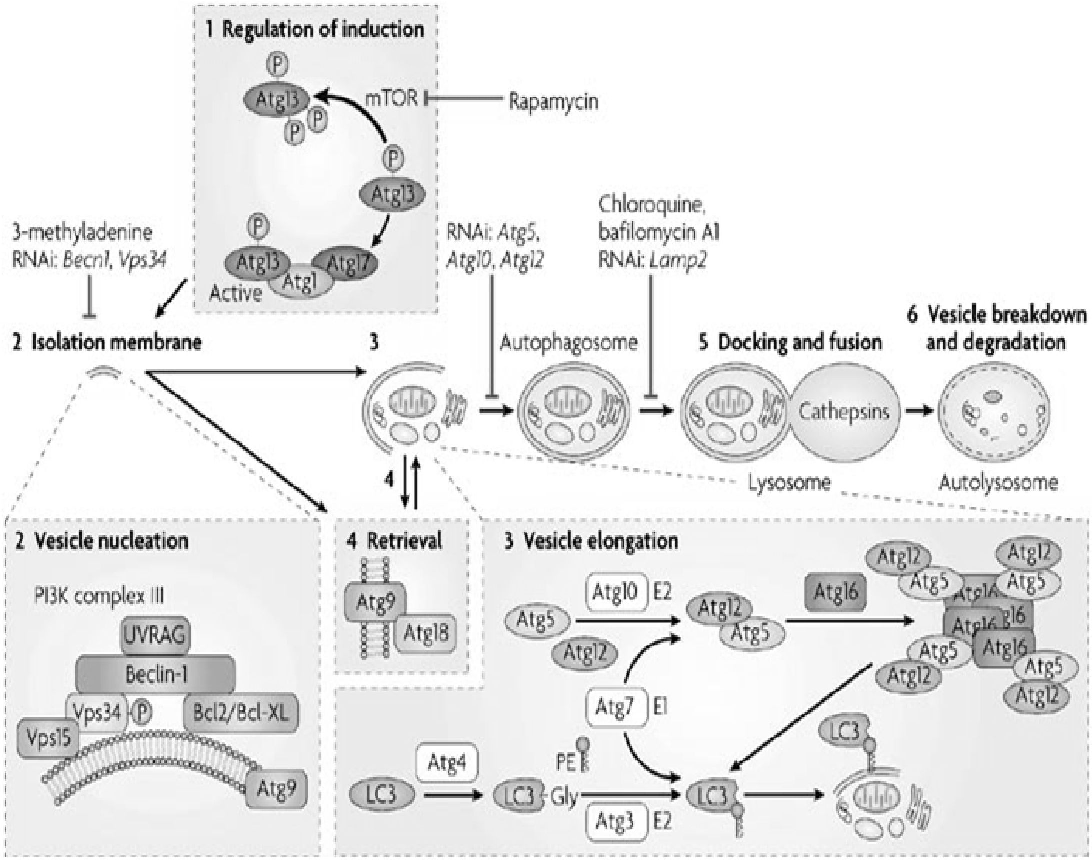
Apoptosis induction by extracts from plants has been reported. Japanese traditional medicine; Sho-saiko, which is used in treatment of chronic liver disease showed inhibition of growth of hepatocellular carcinoma cell lines *in vitro* and killed cells by promoting apoptosis and causing cell cycle arrest (Yano *et al.*, 1994). Mistletoe extracts induced apoptosis in B cell hybridomas, human leukemic monocyte lymphoma (U937) and mouse lymphoblast-like mastocytoma cell line (P815) *in vitro*. The extracts caused nucleosomal fragmentation which is evidenced as DNA fragments with approximate size of 180-200 bp (Janssen *et al.*, 1993). The procyanidins extracted from grapes was instrumental in releasing cytochrome-c from intermembrane space of mitochondria leading to caspase pathway activation (Agarwal *et al.*, 2002). The extracts from *Bituminaria bituminosa* and *Toona sinensis* are reported to induce apoptosis in human colon cancer (Maurich *et al.*, 2006) and human pre-myelocytic leukemia (HL-60) cells respectively.

Current chemotherapeutic drugs which induce apoptosis include staurosporine and bleomycin. Both of them execute different pathways of cell death. The apoptotic pathway is often deregulated in cancers which result in the cancer cell developing resistance to therapy.

2.5.2 Programmed cell death Type II- Autophagy

Autophagy is classified as Type II programmed cell death to aid in the removal of damaged organelles. Autophagy acts as a survival mechanism in response to starvation, especially in vital organs, where depletion of nutrient is not permissible. However in disease such as cancer, expression of proteins related to autophagic mechanism is expressed to aid survivorship of tumor cells. Inducers of autophagy include starvation, hypoxia and microbial infection. In situations where apoptosis is down regulated, autophagy is induced (Buytaert *et al.*, 2007; Hetz, 2007; Lefranc *et al.*, 2007; Moretti *et al.*, 2007; Hu and Xuan, 2008).

Based on the mechanism used to deliver the organelles to lysosomes, autophagic cell death can be divided into three types; (1) macroautophagy, (2) microautophagy and (3) chaperon mediated autophagy. The most well explained mechanism of autophagy is macroautophagy. Isolated membrane engulfs old or nearly dead organelles to form autophagosomes which later fuse with late endosome or multivesicular body to form another complex named as amphisome. With the aid of proteins encoded by genes such as Atg12, Atg7, Atg10, Atg5, Atg6, Atg16, Atg4, Atg8, Atg7 and Atg3, amphisomes fuse with lysosomes to form autolysosomes. The fusion of amphisomes to form autolysosomes creates double membrane vacuoles which are visible under microscopic observation. It appears as orange punctuated particles in fluorescence microscopy. Generation of autophagic vacuoles have been suggested to be indicators of the strength of toxic insult (Bursch *et al.*, 2008). The cells try to survive in the face of the toxic insults by removal of the damaged organelles and toxic agents. However, in situation of excessive damage, the effort of the cells to survive gets exhausted and they undergo autophagic cell death (Bursch *et al.*, 2008). This process is also controlled by light-chain microtubule associated protein (LC3-A/B). Besides, Beclin proteins are involved during the formation and maturation of autophagosomes. The initiation of autophagy is also controlled by mammalian target of rapamycin (mTOR). Autophagic mechanism of cell death and genes involved are shown in Figure 2.3.



Source: (Maiuri *et al.*, 2007)

Figure 2.3: Figure showing different stages, regulatory genes and proteins during autophagic mechanism of cell death.
During induction, mTOR is activated and isolation of membrane is controlled by Beclin-1 protein. During organelles retrieval, the process is controlled by Atg family protein. Formation of autophagosome is preceded by fusion with lysosomes which lead to breakdown and degradation of organelles.

Studies on the mechanism of cell death by drugs show that many of the chemotherapy drugs use autophagy as the cell killing mechanism. Research by Ertmer *et al.* (2007) demonstrated the induction of autophagy by imatinib in chronic myelogenous leukemia (CML) (Ertmer *et al.*, 2007). Resveratrol has been reported to induce autophagocytosis in ovarian cancer cells (Opipari *et al.*, 2004), temozolomide and camptothecin (CPT) induce autophagy in glioblastoma (Lefranc and Kiss, 2006) and breast cancer cells (Motyl *et al.*, 2006). In addition, tamoxifen and epirubicin induce autophagy in breast tumor cells (Bilir *et al.*, 2000). Plant extracts also have shown potential to induce autophagic cell death. Treatment with tamoxifen induces autophagy in MCF-7 breast cancer cells in a caspase-3 independent manner. Table 2.1 show examples of plants which induce autophagy and their target pathways.

Table 2.1:Example of plants that induce autophagic cell death.

Plant name	**Cancer cells affected**	**Target pathway**
Phillyrea latifolia	Hepatocellular carcinoma	Up-regulation of eIF2-α, down-regulation of mTOR and BCl-2 (Longo *et al.*, 2008)
Solanum nigrum	HepG2 cells	Increase expression of p-JNK and Bax, cytochrome-c, caspase activation (Lin *et al.*, 2007)
Areca nut	Oral cancer cells	Activation of p38, MKP-1, HIF-1α (Lu *et al.*, 2010)
Glycyrrhiza glabra	Human LNCaP prostate cancer cells	Suppression of BCL-2 and mTOR pathway (Yo *et al.*, 2009)
Sutherlandia frutescens	Breast cancer cells	Activation of LC3 protein (Stander *et al.*, 2009)

2.5.3 Necrosis

Another mechanism of cell death by plant products which differs from Type I and Type II PCD is necrosis. It is defined as a type of cell death which lacks the features of apoptosis and autophagy. Necrotic cell death often induces local inflammation via induction of innate immune system (Golstein and Kroemer, 2007). As necrosis induce inflammation in the neighborhood, the mechanism has not gained importance as an indicator in screening of potential anticancer agents. Screening for anticancer activity of plants necessitates that the plant products do not induce necrosis.

In necrosis, organelles such as mitochondria dilate and ribosomes dissociate from endoplasmic reticulum. Later, nucleus disintegrates and in some cells, chromatin condensation occurs (not all cells). The nucleus is digested by enzymes, resulting in digested DNA which appears as DNA smear in agarose gel electrophoresis.

Necrotic cells can be detected easily as membrane of necrotic cells become more permeable and allows small charged molecules to enter into the cell that does not normally enter the cells. Common method of detection is via morphological analysis and via propidium iodide staining. In this method, binding of the dye to RNA or DNA result in increase of the PI fluorescence making them suitable for imaging analysis. Other methods of detection involve using of Hoechst stains, using N-hydroxysuccinimidyl biotin (NHS-Biotin) which is a permeable dye that stain dead cells, but not viable cells which have intact impermeable membrane (Ziegler and Groscurth, 2004).

2.6 Plants used in this study

2.6.1 *E. odoratum* (EO)

Eupatorium odoratum L. is a member of the asteraceae family and is locally known as bitter bush, Siam weed, baby tea, cariaquillo, Santa Maria, and pokok kapal terbang. Located mostly at the roadside, riverbank or wet area, it is a common native shrub in Florida and South Africa and in tropical countries including Malaysia. This free standing shrub can reach up to 1m tall. Its root is fine lateral and yellowish in color. The leaves are positioned opposite each other and have deltoid to oval-lanciolate shape. The leaf margins are dentate with a long pointed tip. Flowers of this plant are lavender, pink, white or blue in color. Figure 2.4 shows *E. odoratum* plant in its natural environment.

Figure 2.4: *E. odoratum* L. plant.