DNA FRAGMENTATION IN HeLa CANCER CELL LINES TREATED WITH *Syzygium polyanthum* EXTRACTS

by

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LIST OF ABBREVIATIONS

ELISA = enzyme-linked immunosorbent assay

S. polyanthum = *Syzygium Polyanthum*

DNA = Deoxyribonucleotide

Bcl-2 = B-cell lymphocyte-2

IAP = Inhibit of apoptosis

FSC = forward scatter

SSC = side scatter

OH = hydroxyl

TdT = terminal deoxynucleotidyl transferase

BrdUTP = 5-bromo-2'-deoxyuridine 5'-tri-phosphate

IC_{50} = 50% inhibitory concentration

MBA = methylene blue assay

DMEM = Medium Dulbecco's Modified Essential Medium

CO2 = carbon dioxide

PBS = phosphate buffer saline

EDTA = ethylene diamine tetraacetic acid

NaCl = sodium chloride

HCl = hydrochloric acid

NaH_2CO_3 = sodium bicarbonate

Na_2HPO_4 = sodium hydrogen phosphate

DMSO = dimethylsulfoxide

Rpm = rotation per minute

OD = optical density

LIST OF SYMBOL

Meter	m
Base pair	bp
Cell per milliliter	cells/ml
Centimeter cube	cm^2
Centimeter	cm
Degree celcius	°C
Mililiter	ml
Microliter	µl
Millimeter	mm
Nanometer	nm
Molar	M
Percent	%
Low or equal to	≤
Microgram per milliliter	µg/ml

ABSTRACT

Plants have a long history of use in the treatment of cancer. Plants have played an important role as a source of effective anti-cancer agents, and it is significant that over 60% of currently used anti-cancer agents are derived from natural sources, including plants, marine organisms and micro-organisms. Methylene blue assay is done to determine the IC_{50} value of *Syzygium Polyanthum*. IC_{50} is a concentration that needed to 50% of the cell population death. Plant extract with different concentrations are tested to cells depends on time. After treatment, live cell will take up methylene blue dye and the result will be analyzed by using ELISA reader at 660nm. The effectiveness of the plant extract will be determined based on IC_{50} value. TUNNEL assay is done to detect the rate of apoptosis in HeLa cells treated with *S.polyanthum*. TUNEL is actually stood for terminal deoxynucleotide transferase dUTP Nick End Labeling. It is use to detect DNA fragmentation that are produced by apoptotic cells. At the last of this experiment, we are able to detect DNA fragmentation event in HeLa cells treated with *S.polyanthum* extract.

ABSTRAK

Pengunaan tumbuhan untuk rawatan kanser telah digunakan dalam bidang perubatan sejak zaman dulu lagi. Tumbuhan memainkan peranan penting sebagai agen anti kanser, dan lebih daripada 60% anti kanser yang digunakan pada masa ini dihasilkan daripada alam semulajadi termasuk tumbuhan, organisma marin dan mikroorganisma. Asai Metalina biru dijalankan untuk menentukan nilai IC_{50} *Syzygium Polyanthum*. IC_{50} adalah kepekatan yang diperlukan untuk 50% daripada populasi sel mati. Ekstrak daripada tumbuhan dengan pelbagai kepekatan diuji ke atas sel berdasarkan masa yang ditetapkan. Selepas rawatan, sel hidup akan menyerap warna biru daripada metalina biru dan keputusan akan dianalisis mengunakan mesin pembaca ELISA pada jarak gelombang 660nm. Keberkesanan ekstrak tumbuhan akan diperoleh berdasarkan bacaan IC_{50}. Asai TUNNEL dijalankan untuk mengesan kadar apoptosis pada sel HeLa yang dirawat dengan *S.polyanthum.* TUNEL adalah merujuk kepada "terminal deoxynucleotide transferase dUTP Nick End Labeling". Ianya digunakan untuk mengesan fragmentasi pada DNA yang dihasilkan daripada sel apoptotic. Pada akhir eksperimen ini, kami mendapati bahawa kami dapat mengesan kejadian fragmentasi DNA pada sel HeLa yang telah dirawat dengan ekstrak *S. polyanthum.*

CHAPTER 1

PREFACE

1.1 Introduction

The use of plants, plant extracts and plant-derived chemicals in the treatment of diseases, in supplementing foods and in making cosmetics is firmly rooted in the past and still developing. Many drugs used in contemporary medicine have been derived from plants and were originally discovered through the traditional use by indigenous people. Progress in science and technology boosts the further development of medicinal plants as valuable sources of drugs and drug leads. Modern analytical methods, biotechnology approaches, genomics, proteomics and metabolomics are nowadays applied in medicinal plant research and contribute to the advancement of the field (Elfahmi *et al*, 2006). Plants have a long history of use in the treatment of cancer. More than 3000 plant species are listed that have reportedly been used in the treatment of cancer. (Hartwell, 1982).

Plants have played an important role as a source of effective anti-cancer agents, and it is significant that over 60% of currently used anti-cancer agents are derived in one way or another from natural sources, including plants, marine organisms and micro-organisms (Cragg *et al*, 2005).

A recurring liability of natural products, at least in the area of cancer chemotherapy, is that while often very potent, they have limited solubility in aqueous solvents and exhibit narrow therapeutic indices. These factors have resulted in the demise of a number of pure natural products, but an alternative approach to utilizing such agents is to investigate their potential as "warheads" attached to monoclonal antibodies specifically targeted to epitopes on tumors of interest (Hartwell, 1982).

Up to the early 1990s, the discovery of novel anti-tumor agents from natural sources was largely based on testing for cytotoxic activity against cancer cell lines grown either in vitro or using in vivo models. Many of the naturally derived anti-cancer agents originally discovered using such assays, have been shown to exert their cytotoxic action through interaction with tubulin, (Cragg *et al*, 2005)

In cancer treatment, the drug that produced anti-proliferative characteristic with apoptosis is needed to inhibit uncontrolled proliferative activity of cancer cell without unnecessary inflammatory response (Richard *et al*, 2004). Apoptosis is a distinct mode of cell death that is responsible for deletion of cells in normal tissues; it also occurs in specific pathologic contexts. Morphologically, it involves rapid condensation, chromosomal DNA fragmentation and budding of the cell, with the formation of membrane-enclosed apoptotic bodies containing well-preserved organelles, which are phagocytosed and digested by nearby resident cells. There is no associated inflammation involved (Kerr *et al*, 1994). These changes involved pro-apoptotic (e.g p53, Bax) and anti-apoptotic (e.g Bcl-2) proteins that play an important role in mechanisms of apoptosis (Yau, 2004).

Complete removal of the cancer without damage to the rest of the body especially without effecting normal cell surrounding it is the goal of cancer treatment. Sometimes this can be accomplished by surgery, but the propensity of cancers to invade adjacent tissue or to spread to distant sites by microscopic metastasis often limits its effectiveness. The effectiveness of chemotherapy is often limited by toxicity to other tissues in the body. Radiation can also cause damage to normal tissue (Cragg *et al*, 2005). Due to many of side effect that being developed by these therapies, many researchers had encourage to find a new drug from plants that may be used into therapy so that several complications arise from the present treatment can be reduced.

1.2 Introduction of *Syzygium polyanthum*

S.polyanthum is a deciduous tropical tree with spreading branches and simple leaves, originally from Indonesia; it is also growing abundantly in Suriname. Common name is known as daun salam or serai kayu (Rukayah, 2000). Indonesian bay leaf can reach a height of 90 feet although 60 feet is more common. The flowers are pink and somewhat fragrant while the fruits are round; red at first, later brown. The seeds are small and brown (World Agroforestry Centre, 2008).

The dried brown leaves of daun salam are aromatic and somewhat sour; used as a spice in the Indonesian - as well the Surinam kitchen; they are applied to meat (Rukayah, 2000). The main phytochemicals in this plant are: eugenol, citral and methylchavicol. Salam is propagated by seed, cuttings or air layering (World Agroforestry Centre, 2008).

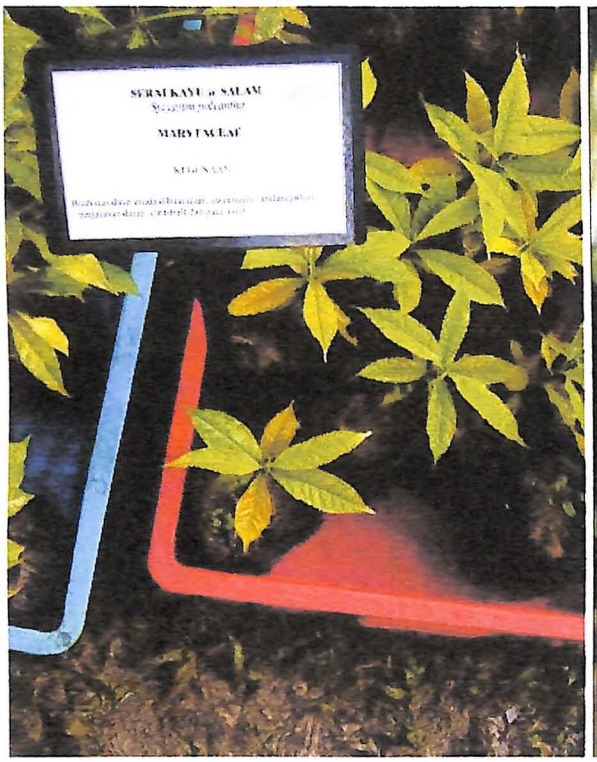

Figure 1.: Plantlet of *S.polyanthum*

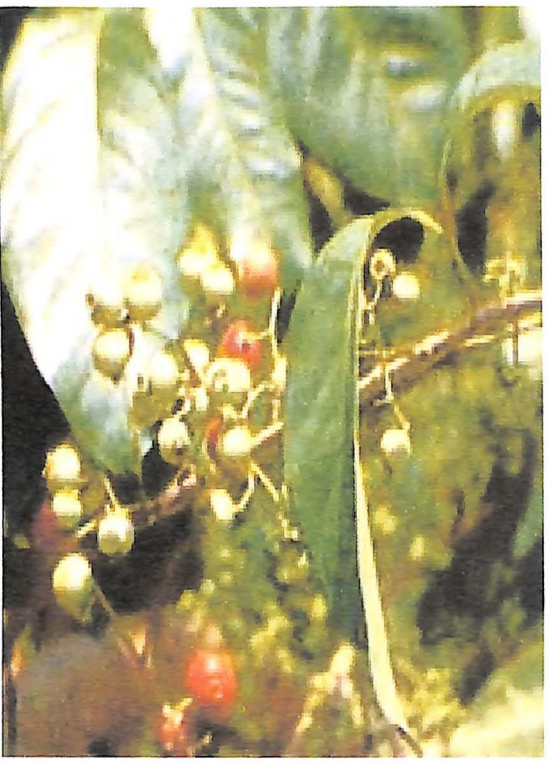

Figure 2: Fruits of *S.polyanthum*

(Source: http://www.melur.com/myherba.asp)

Figure 3: The leaves of *S. polyanthum*

(Source: http://www.flickr.com/search/?s=int&q=serai+kayu&m=text)

In herbal medication, *S.polyanthum* is used to treat many diseases such as diabetes mellitus, diarrhea and hypertension (Khatijah *et al*, 2006). Parts of this plant that usually use for treatment included leaves, root, bark and fruits. For diabetes mellitus and hypertension, 20 pieces of leaves will be boiled with 3 glasses of water until volume of water becomes 1 glass. The water will be drinking twice a day before breakfast and dinner. To treat diarrhea, boiled the leaves and then cooled it before added some salt and drink. Root and bark of *S.polyanthum* can be used to treat scabies by apply them at the area of affected. There also have some opinion said that *S.polyanthum* can be used to reduce amount of cholesterol by boiled the leaves and drink the water 3 times a day. It also can be administered as an astringent which can causes cells to shrink by precipitating proteins from their surfaces (Borneo Focus, 1998).

Leaf and bark extracts are used medicinally against diarrhoea. (Noorma Wati, 1995) Pounded leaves, bark and roots are applied as poultices against itches (Noorma Wati, 1995). These plants have been reported to have antioxidant properties (Eng *et al*, 2004)

This plant also have another name besides daun salam and serai kayu. The names are depends on place where it situated as example at Cambodia it known as pring sratoab, Filipino as Indonesian bay-leaf, Indonesian as manting (Javanese), Malay as kelat samak and Thai as daeng-kluai (central) (World Agroforestry Centre, 2008).

In scientific classification, *S.polyanthum* is in plant kingdom, *Magnoliophyta* phylum, *Magnoliopsida* class, *Myrtales* order, M*yrtaceae* family, *Syzygium* genus and *S.polyanthum* species. (ARS Systematic Botanists, 2004)

S.polyanthum is widely distributed and locally common as understorey tree in lowland primary and secondary forests, also in thickets, bamboo forest and teak plantations, in Java up to 1000 m, in Sabah up to 1200 m, and in Thailand up to 1300 m altitude. *S.polyanthum* is also widely distributed in Burma (Myanmar), Indo-China, Thailand, Malaysia, and Indonesia (Java, Sumatra, Kalimantan) (World Agroforestry Centre, 2008).

1.3 Rationale of the study

Besides from interest that been exposed by previous studies in searching of anticancer agent that exist in natural, usage of plant as natural medicine with widely distributed also contribute to done an experiment to *S.polyanthum* species. Furthermore, limited source that related with *S.polyanthum* plant also induces me to choose this species as a part of my final year research.

1.4 Research objective

The purposes of conducting the research are:

- To determine the IC_{50} value of *S.polyanthum* extract

- To identify apoptosis rate of HeLa cancer cell lines treated with *S.polyanthum* via tunnel assay.

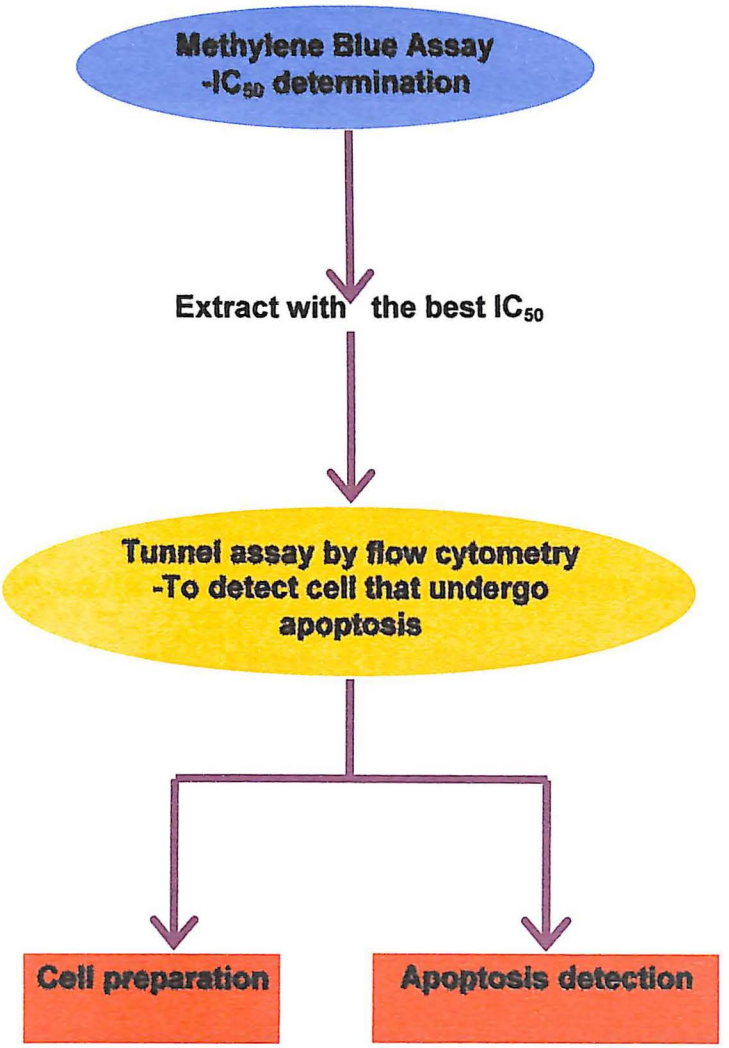

Figure 4 Flow chart of the antipoliferative activity of *S.polyanthum* extract towards HeLa cancer cell

CHAPTER 2

LITERATURE REVIEW

2.1 Cancers

Cancer is a leading cause of death worldwide. The disease accounted for 7.9 million deaths (or around 13% of all deaths worldwide) in 2007. Deaths from cancer worldwide are projected to continue rising, with an estimated 12 million deaths in 2030 (WHO, 2008). A total of 21,464 cancer cases were diagnosed among Malaysians in Peninsular Malaysia in the year 2003, comprising 9,400 males and 12,064 females (Gerard *et al*, 2003).

The most important things in determining the choice of therapy and in assessing prognosis are the type and location of the cancer, the stage of the disease (the extent to which the cancer has metastasized, or spread), and its grade (how abnormal the cancer cells look and how quickly the cancer is likely to grow and spread) (National Cancer Institute, 2008).

Cancer is a progressive medical problem in Malaysia. Currently, its mortality is the third leading cause of death in Malaysia. The five most frequent cancer reported in Malaysia (1996) are men – lung, colon/rectum, stomach, liver followed by nasopharynx. For women the five most frequent cancers are breast, colon/rectum, cervix, lung and ovary. Up to 90%

of cancers in Malaysia are associated with the environment and individual life styles (Gerard *et al*, 2003).

2.2 Cervical cancer

Cervical cancer is the second leading cause of cancer deaths among women around the world. The World Health Organisation (WHO) reported nearly 500,000 new cases of cervical cancer in 2002 alone (Malaysian oncology society, 2008). It constituted 12.9% of total female cancers. There were a total of 1,557 cases of cervical cancer, with an ASR of 19.7 per 100,000 populations. Cervical cancer incidence rate increased with age after 30 years. It has a peak incidence rate at ages 60-69 years, and declined thereafter. These features were very similar to data in 2002. Chinese women had the highest ASR of 28.8 per 100,000 populations, followed by Indians with ASR of 22.4 per 100,000 population and Malays with ASR of 10.5 per 100,000 populations (Gerard *et al*, 2003).

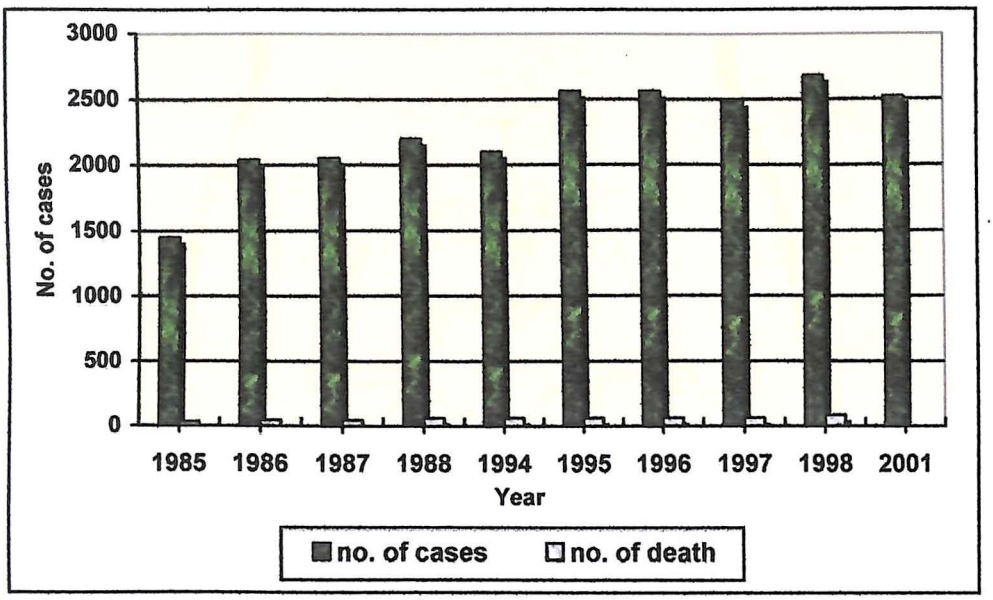

Figure 5: The prevalence of cervical cancer in Malaysia from 1985 to 2001

(Source: Sistem Maklumat dan Dokumentasi Kementerian Kesihatan Malaysia)

The cervix is the lower part of the uterus (womb). It is sometimes called the uterine cervix. The body (upper part) of the uterus is where a fetus grows. The cervix connects the body of the uterus to the vagina (birth canal). The part of the cervix closest to the body of the uterus is called the *endocervix*. The part next to the vagina is the *exocervix* (or *ectocervix*). The place where these 2 parts meet is called the *transformation zone*. Most cervical cancers start in the transformation zone (American Cancer Society, 2008).

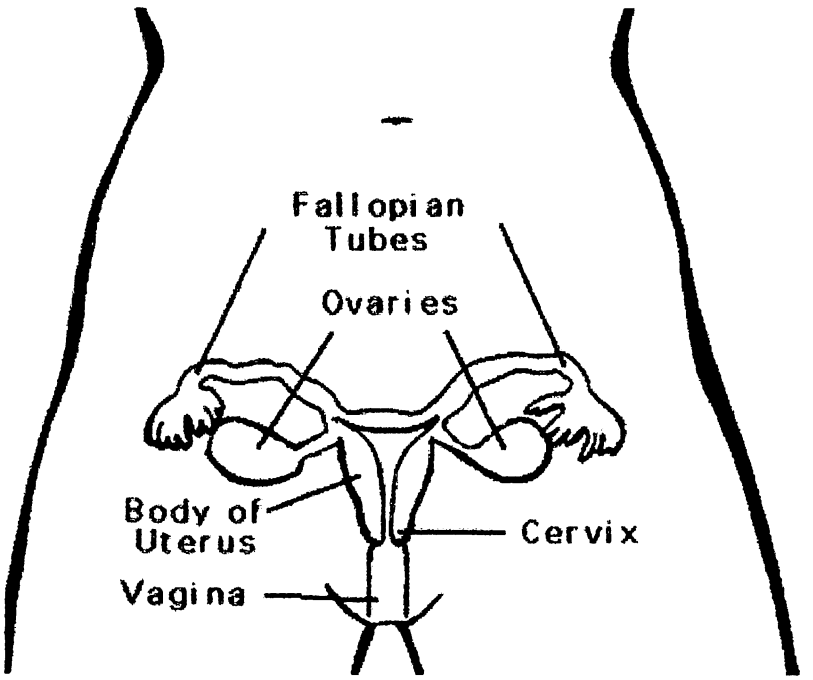

Figure 6: Picture shows location of the cervix in human body.

(Source: http://www.cancer.org/docroot/CRI/content/CRI_2_4_1X_

What_is_cervical_cancer_8.asp)

Cervical cancer is caused by several types of a virus called human papillomaviruses (HPV). The virus spreads through sexual contact. Most women's bodies are able to fight HPV infection. But sometimes the virus leads to cancer. Cervical cancer may not cause any symptoms at first, but later, may have pelvic pain or bleeding from the vagina. It usually takes several years for normal cells in the cervix to turn into cancer cells.

The process used to find out if cancer has spread within the cervix or to other parts of the body is called staging. The information gathered from the staging process determines the stage of the disease. It is important to know the stage in order to plan treatment. Before staging, pelvic examination and certain investigation test like CT-scan and chest X-ray procedure should be done. The results of these tests are viewed together with the results of the original tumor biopsy to determine the cervical cancer stage. Staging system that developed by the International Federation of Gynaecology and Obstetrics (FIGO) and Tumor, Node, Metastasis (TNM) system is the most widely used as shown in table 1.

Table 1. Staging system for cervical cancer by FIGO and TNM

TNM stage	*FIGO stage*	
Primary tumor (T)		
TX	-	Primary tumor cannot be assessed
T0	-	No evidence of primary tumor
Tis	0	Carcinoma in situ
T1	I	Cervical carcinoma confined to uterus (extension to corpus should be disregarded)
T1a	IA	Invasive carcinoma diagnosed only by microscopy. All macroscopically visible lesions - even with superficial invasion - are T1b/1B. Stromal invasion with amaximal depth of 5.0 mm measured from the base of the epithelium and a horizontal spread of 7.0 mm or less. Vascular space involvement, venous or lymphatic, does not affect classification
T1a1	IA1	Measured stromal invasion 3 mm or less in depth and 7 mm or less in lateral spread
T1a2	IA2	Measured stromal invasion more than 3.0 mm and not more than 5.0 mm with a horizontal spread 7.0 mm or less
T1b	IB	Clinically visible lesion confined to the cervix or microscopic lesion greater than IA2
T1b1	IB1	Clinically visible lesion 4.0 cm or less in greatest dimension
	IB2	Clinically visible lesion more than 4.0 cm
T2	II	Cervical carcinoma invades beyond uterus but not to pelvic wall or to the lower third of vagina
T2a	IIA	Tumor without parametrial invasion
T2b	IIB	Tumor with parametrial invasion
T3	III	Tumor extends to the pelvic wall, and/or involves the lower third of the vagina, and/or causes hydronephrosis or nonfunctioning kidney
T3a	IIIA	Tumor involves lower third of the vagina, no extension to pelvic wall
T3b	IIIB	Tumor extends to pelvic wall and/or causes hydronephrosis or

		nonfunctioning kidney
	IV	Cervical carcinoma has extended beyond the true pelvis or has involved (biopsy proven) the bladder mucosa or rectal mucosa. Bullous edema does not qualify as a criteria for stage IV disease.
T4	IVA	Spread to adjacent organs (bladder, rectum, or both)
M1	IVB	Distant metastasis

(Source:http://www.uptodate.com/patients/content/image.do;jsessionid=781D6739590EBDB71C043E044D8DB276.1003;jsessionid=781D6739590EBDB71C043E044D8DB276.1003?imageKey=obst_pix/cervic24.htm&title=Cervical%20cancer%20staging)

Cervical cancer that invades deeper than the outside layer of cells on the cervix is referred to as invasive cancer and requires more extensive treatment. Treatment for cervical cancer depends on several factors, such as the stage of the cancer, other health problems you may have and your own preferences about treatment. Surgery (radical hysterectomy) is typically used to treat the early stages of cervical cancer and must be perform by trained, skilled and experienced surgeons. Although surgical treatment is effective treatment for early stage of cervical cancer, it may also give temporary side effects include pelvic pain and difficulty with bowel movements and urination (mayoclinic, 2008).

Besides, radiation therapy also is the one of the popular treatment that usually used to treat cervical cancer. It uses high-powered energy to kill cancer cells but it will give side effects of radiation to the pelvic area include upset stomach, nausea, diarrhea, bladder irritation and narrowing of your vagina, which can make intercourse difficult. Premenopausal women may stop menstruating as a result of radiation therapy and begin

menopause. Chemotherapy is done by used strong anti-cancer chemicals to kill cancer cells. Low doses of chemotherapy are often combined with radiation therapy, since chemotherapy may enhance the effects of the radiation. Higher doses of chemotherapy are used to control advanced cervical cancer that may not be curable. Side effects of chemotherapy depend on the drugs being administered, but generally include diarrhea, fatigue, nausea and hair loss. Certain chemotherapy drugs may cause infertility and early menopause in premenopausal women (mayoclinic, 2008)

2.3 Apoptosis and cancer

Apoptosis, or programmed cell death, is a highly regulated process that allows a cell to self-degrade in order for the body to eliminate unwanted or dysfunctional cells. During apoptosis, the genome of the cell will fracture, the cell will shrink and part of the cell will disintegrate into smaller apoptotic bodies. Unlike necrosis, where the cell dies by swelling and bursting its content in the area, which causes an inflammatory response, apoptosis is a very clean and controlled process where the content of the cell is kept strictly within the cell membrane as it is degraded. The apoptotic cell will be phagocytosed by macrophages before the cell's contents have a chance to leak into the neighbourhood. Therefore, apoptosis can prevent unnecessary inflammatory response (Yau, 2004). A characteristic biochemical feature of the process is double-strand cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments. In many, although not all of the circumstances in which apoptosis occurs, it is suppressed by inhibitors of messenger RNA and protein synthesis. (Kerr *et al*, 2004)

Apoptosis can be triggered in a cell through either the extrinsic pathway or the intrinsic pathway. The extrinsic pathway is initiated through the stimulation of the transmembrane death receptors, such as the Fas receptors, located on the cell membrane. In contrast, the intrinsic pathway is initiated through the release of signal factors by mitochondria within the cell (Yau, 2004).

Intrinsic pathway is initiated from within the cell. This is usually in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. This pathway involves release from the mitochondria of pro-apoptotic proteins that activate caspase enzymes, which ultimately trigger apoptosis (Coultas *et al*, 2003)

Apoptosis occurs spontaneously in malignant tumors, often markedly retarding their growth, and it is increased in tumors responding to irradiation, cytotoxic chemotherapy, heating and hormone ablation (Kerr *et al*, 2004). Cancer may arise from the dysfunction in the apoptotic pathway. Due to the sensitivity of the intrinsic pathway, tumors arise more often through the intrinsic pathway than the extrinsic pathway. In the intrinsic pathway, a very common cause of tumorgenesis is mutation of the p53 protein. Besides regulating apoptosis, p53 also regulates the check points in the cell cycle, DNA repair, senescence and genomic integrity.

2.4 DNA fragmentation

Some of the biochemical features of apoptosis such as loss of membrane phospholipid asymmetry and DNA fragmentation can also be used to identify apoptosis. Cell viability assays can be combined with apoptosis assays to provide more information about mechanisms of cell death through multiplexing assays on a single sample.

As the majority of the events that characterize apoptotic death can easily be revealed by multiparameter flow cytometry, a lot of methods have been proposed to analyze and quantify the apoptotic process by this type of analysis (Nicoletti *et al*, 2008). Cells undergoing apoptosis display typical changes in their morphological and physical properties (cell shrinkage, condensation of chromatin and cytoplasm) which are well measurable by flow cytometry. Thus, a series of methods have been proposed for measuring apoptotic cell death through evaluation of light scattering parameters of cells (Darzynkiewicz *et al*, 1992)

Interaction of a particle with the laser beam produces a light scatter in a forward direction (FSC, that correlates with cell size) and a lateral direction (SSC, that correlates with granularity and/or cell density). While necrotic death is characterized by a reduction in both FSC and SSC (probably due to a rupture of plasma membrane and leakage of the cell's content), during apoptosis there is an initial increase in SSC (probably due to the chromatin condensation) with a reduction in FSC (due to the cell shrinkage). This pattern can be easily observed in some models of apoptosis, such as the apoptotic death induced by

glucocorticoids in murine thymocytes (Figure 7) but it is much less clear in other cell models (i.e. the majority of tumor cell lines). (Nicoletti *et al*, 2008)

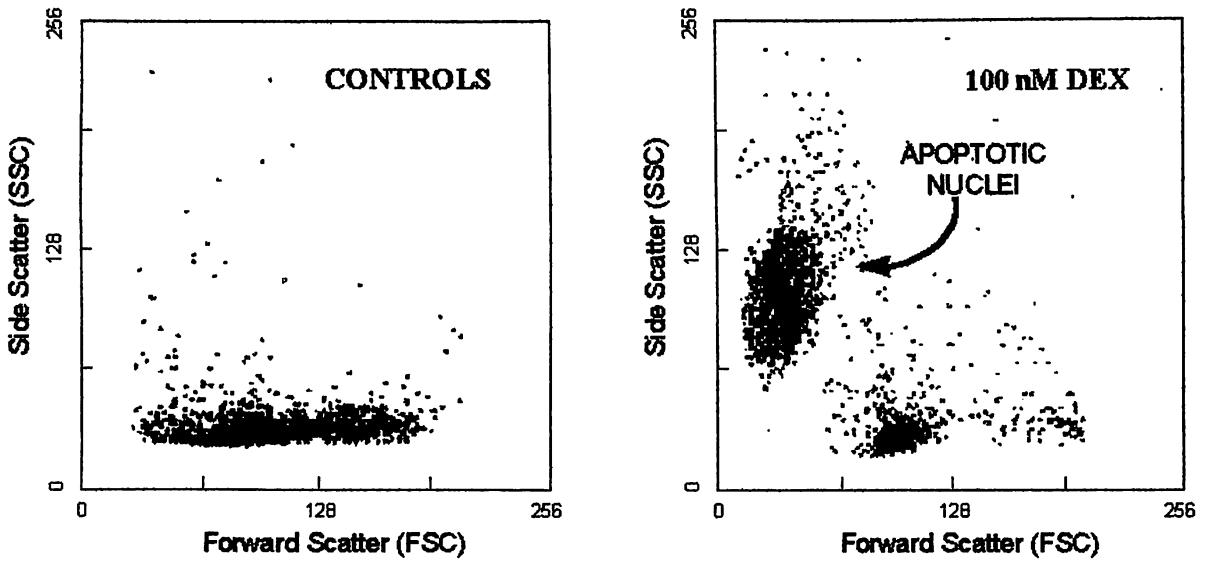

Figure 7: Apoptotic cells are clearly distinguishable from normal thymocytes for the reduced cell size (low FSC) and enhanced density (high SSC). (Source:http://www.cyto.purdue.edu/flowcyt/research/cytotech/amfc/data/page3.htm)

The products of DNA degradation are nucleosomal and oligonucleosomal DNA fragments (180 bp and multiplicity of 180 bp) which generate a characteristic "ladder" pattern during agarose gel electrophoresis. Because the DNA in apoptotic cells is partially degraded, the fraction of low molecular weight DNA can be extracted whereas the nondegraded DNA remains in the cell (Gong *et al*, 1994)

Changes in morphology of the dying cell can be detected by analysis of a light scatter signal by flow cytometry. The cell traversing through focus of a laser beam in flow

cytometer scatters the laser light. Analysis of the scattered light provides information about the cell size and structure (Salzman *et al*, 1990). While the intensity of light scattered at a forward direction correlates with cell size, the intensity of scattered light measured at a 900 angle to the laser beam (side scatter) correlates with granularity, refractiveness and the presence of intracellular structures that can reflect the light. The cell's ability to scatter light is altered during cell death, reflecting the morphological changes such as cell swelling or shrinkage, breakage of plasma membrane and in the case of apoptosis, chromatin condensation, nuclear fragmentation and shedding of apoptotic bodies. (Salzman *et al*, 1990)

Cell necrosis is associated with an initial increase and then rapid decrease in the cell's ability to scatter light simultaneously in the forward and right angle direction (Darzynkiewicz *et al*, 1996). This is a reflection of an initial cell swelling followed by plasma membrane rupture and leakage of the cell's content. During apoptosis, on the other hand, the decrease in forward light scatter (which is a result of the cell shrinkage) is not initially paralleled by a decrease in side scatter. Actually, a transient increase in right angle scatter can be seen during apoptosis in some cell systems (Swat *et al*, 1991). This may reflect an increased light reflectiveness by condensed chromatin and fragmented nuclei. In later stages of apoptosis, however, the intensity of light scattered at both, forward and right angle directions, is decreased (Ormerod, 1995).

It should be stressed, however, that the light scatter changes are not specific to apoptosis. Mechanically broken cells, isolated cell nuclei and necrotic cells also have diminished ability to scatter light. Identification of apoptosis or necrosis by light scatter,

therefore, requires additional controls, and should be accompanied by another, more specific assay (Nicoletti *et al*, 2008)

There have presence of DNA Strand Breaks in apoptosis cell. Endonucleolytic DNA cleavage results in extensive DNA breakage. The 3' OH ends in DNA breaks are detected by attaching to them biotin or digoxygenin conjugated nucleotides, in a reaction catalyzed by exogenous TdT ("end-labeling", "tailing", "TUNEL") or DNA polymerase (nick translation) (Gorczyca *et al*, 1992). Fluorochrome conjugated avidin or digoxygenin antibody are then used in the second step of the reaction to label DNA strand breaks. A simplified, single step procedure has been developed, using fluorochromes directly conjugated to deoxynucleotides (Li *et al*, 1995). More recently, a new method was introduced in which BrdUTP, incorporated by TdT, is used as the marker of DNA strand breaks (Li *et al*, 1996). The method based on BrdUTP incorporation (Li *et al*, 1995) is simpler, more sensitive and costs less compared with the digoxygenin or biotin labeling.

2.5 Cisplatin

Cisplatin, cisplatinum or *cis*-diamminedichloridoplatinum (II) (CDDP) is a platinum-based chemotherapy cytotoxic drug and acts as an alkylator agent. It was used widely to treat the cancer of bladder, head and neck, ovaries, germ cell, lung and testicles and may also be a useful treatment for cancers of the brain, adrenal cortex, breast, cervix, uterus, endometrium and osteosarcoma (David *et al*, 1997). At the centre of this drug is an atom of the metal platinum (Figure 8). This alkylating agent possesses positively charged alkyl groups that are able to bind negatively charged (electron-rich) sites on DNA

(Mayoclinic.com, 2008). Appear in clear liquid form, this drug contains an antitumor activity which is mediated via the formation of platinum adducts with DNA. After given intravenously, cisplatin exists in the bloodstream in its native dichloro form, which then enters cells in the target tissue via passive diffusion. It distorts the DNA double helix by bending it up to 40 degrees and thereby maintaining the planar stereochemistry diamineplatinum (II) complex. Thus, inhibits the DNA synthesis (Jakob *et al*, 2004).

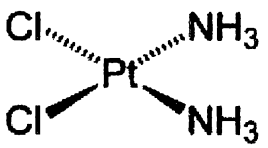

Figure 8: Chemical structure of cisplatin

(Source: http://pubs.acs.org/cen/coverstory/83/8325/8325cisplatin.html)

Following intravenous administration, cisplatin is rapidly and extensively protein bound. Over 90% is bound to plasma protein within 4 hours of administration while 90% of this free drug is cleared from the plasma during the first 2 hour injection. It is cleared almost exclusively by the kidneys, primarily as a result of glomerular filtration (Michael *et al*, 1999).

Cisplatin and their possible side effects can affect individual people in different ways. The following are some of the side effects that are known to be associated with this drug; high blood uric acid level (hyperuricaemia) which can cause kidney problems and gout, low blood calcium level (hypocalcaemia), disorder of the peripheral nerves causing weakness and numbness (peripheral neuropathy), low red blood cell count (anaemia),

decrease in the number of white blood cells in the blood (leucopenia), decrease in the number of platelets in the blood (thrombocytopenia), immunosuppression, nausea and vomiting which may last for up to a week after treatment, loss of taste or a metallic taste, hearing problems (ototoxicity), an extreme allergic reaction (anaphylaxis) and kidney damage (NetDoctor, 2004).

In 1999, the results of five randomized clinical trials showed that adding cisplatin-based chemotherapy to radiation therapy (chemoradiation) for the treatment of cervical cancer that has spread locally (in the cervix or its immediate vicinity) or regionally (within the pelvis) improves survival compared to treatment with radiation therapy alone. Cisplatin-based chemotherapy during pelvic radiation therapy improves long-term progression-free and overall survival among locally advanced cervical cancer patients collectively and for stage IIB and III disease, individually. There was no observed increase in late toxicity with cisplatin-based chemoradiotherapy but nowadays, resistant against cisplatin becomes clinical problems for the treatment of cancers. The mechanism of having resistance of cancer cells to platinum complexes such as cisplatin, may decrease in the uptake of platinum into cells, increase in glutathione and metallothionein level as intracellular detoxication, increase of DNA repair ability and increase of permissibility to DNA injury (Kishimoto *et al*, 2008). Concisely, other chemotherapeutic agent that give less or not resistance to the cancer cells need to be developed to improved treatment of cancer.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

Dulbecco's modified Eagle's medium (DMEM) and penicillin-streptomycin were provided from Gibco Invitrogen (Auckland, New Zealand). Dimethyl sulfoxide (DMSO), and ethylene diamine tetraacetic acid (EDTA) cell culture tested were bought from Sigma Co. (St. Louis, USA)

Phosphate-buffered saline (PBS) was purchased from Zymed Invitrogen (Carlsbad, USA). Falcon Flask (25 cm^3, 75 cm^3 and 125 cm^3), syringe 50 ml, polypropylene conical tube (15 ml and 50 ml) were provided from Falcon Becton Dickson, filter tip (Greiner bio-one), appendorf tube from appendorf and 0.4% trypan blue (Appendix A).

S. polyanthum were purchased from Pasar Siti Khadijah, Kota Bharu, Kelantan, Malaysia. The leaves of the plants were washed using tap water and dried in oven at 50°C. After completely dry, the leaves were then blended into powder form.

Sodium chloride (NaCl), methanol and sodium hydrogen carbonate ($NaHCO_3$) were obtained from Merck (Germany). Hydrochloric acid (HCl) 1 N solution, glutaraldehyde 25% aqueous solution, methylene blue certified were bought from Sigma Co. (St. Louis, USA) Sodium bicarbonate (NaH_2CO_3) and sodium hydrogen phosphate (Na_2HPO_4) were