ฤทธิ์ของสารสกัดจากหัวบอระเพ็ดพุงช้าง (*Stephania venosa* (Bl.) Spreng) ต่อการชักนำให้เกิดอะพอพโตสิสในเซลล์เม็ดเลือดขาว

นางสาวทิพย์สุดา ปลื้มใจ

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จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

INDUCTION OF APOPTOSIS BY THE EXTRACT FROM *STEPHANIA VENOSA* (BL.) SPRENG TUBER ON LYMPHOCYTE.

Miss Tipsuda Plumchai

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ทิพย์สุดา ปลื้มใจ : ฤทธิ์ของสารสกัดบอระเพ็ดพุงช้าง (*Stephania venosa* (Bl.) Spreng.) ต่อการชักนำให้เกิดอะพอพโตสิสในเซลล์เม็ดเลือดขาว. (INDUCTION OF APOPTOSIS BY THE EXTRACT FROM *STEPHANIA VENOSA* (BL.) SPRENG TUBER ON LYMPHOCYTE) อ. ที่ปรึกษา: รศ.พญ. ธาดา สืบหลินวงศ์, อ. ที่ปรึกษาร่วม: ผศ. ดร. วัชรี ลิมปนสิทธิกุล; 89 หน้า ISBN 974-17-2932-4

มีพืชสมุนไพรไทยหลายพันชนิดที่ได้รับการกล่าวอ้างสรรพคุณว่าสามารถใช้ในการรักษาโรคมะเร็ง แต่ขาดหลักฐานยืนยันทางวิทยาศาสตร์ ทั้งนี้รวมถึงบอระเพ็ดพุงช้าง (*Stephania venosa* (Bl.) Spreng) ซึ่งมีการนำส่วนหัวบนดินมาใช้ในการแพทย์แผนไทยสำหรับรักษาโรคต่างๆ เช่นมะเร็ง การศึกษาครั้งนี้มีวัตถุประสงค์จะนำการทดลองทางวิทยาศาสตร์ในระดับการเพาะเลี้ยงเซลล์มาใช้ในการทดสอบและพิสูจน์เพื่อยืนยันผลในการรักษาโรคมะเร็ง วิธีทดลองนำเซลล์เม็ดเลือดขาวที่ได้จากผู้บริจาคโลหิตมาทำการเพาะเลี้ยงในอาหารเลี้ยง RPMI ที่ความหนาแน่น $4x10^5$เซลล์/มล. มาศึกษาฤทธิ์ของสารสกัดบอระเพ็ดพุงช้างความเข้มข้นต่างๆ ที่สามารถยับยั้งการเจริญเติบโตของเซลล์ได้ 50 เปอร์เซ็นต์ (IC_{50}) ตรวจสอบความเป็นพิษต่อเซลล์ด้วยเทคนิค Trypan blue dye exclusion หลังจากเพาะเลี้ยงเซลล์ 48 ชั่วโมง ศึกษาความคงตัวของสารสกัดบอระเพ็ดพุงช้างที่เตรียมในรูปของสารละลายโดยตัวชี้วัด 2 ตัวได้แก่ การวัดค่า pH และการทดสอบความเป็นพิษต่อเซลล์เม็ดเลือดขาวของสารละลายที่แบ่งเก็บ ทำการศึกษาเป็นเวลา 12 สัปดาห์ ค่าความเข้มข้นของสารสกัดบอระเพ็ดพุงช้างที่ยับยั้งการเจริญเติบโตของเซลล์ได้ 50 เปอร์เซ็นต์ คือ 300 ไมโครกรัม/มล. ซึ่งเป็นค่าความเข้มข้นที่ถูกเลือกมาใช้ในการศึกษาฤทธิ์การเหนี่ยวนำให้เกิดอะพอพโตสิส ในเซลล์เม็ดเลือดขาวที่ได้จากผู้บริจาคโลหิต และผู้ป่วยมะเร็งปากมดลูก โดยทำการทดสอบเซลล์เม็ดเลือดขาวด้วยสารสกัดบอระเพ็ดพุงช้างที่ความเข้มข้น 100, 300 ไมโครกรัม/มล. และ การฉายรังสีโคบอลต์ 60 ที่ขนาด 0.5 Gy. รวมทั้งการให้สารสกัดบอระเพ็ดพุงช้างความเข้มข้น 300 ไมโครกรัม/มล. ร่วมกับการฉายรังสี ทำการตรวจสอบเซลล์ที่เกิดอะพอพโตสิสที่เวลา 48 ชั่วโมงหลังการให้สารทดสอบ ด้วยวิธี *in situ* terminal deoxynucleotidyl transferase assay (TdT assay) และทำการศึกษาฤทธิ์ในการยับยั้งการแบ่งตัวของเซลล์เม็ดเลือดขาวที่ถูกกระตุ้นด้วย Phytohhemagglutinin A (PHA)

ผลการทดลองพบว่าสารสกัดบอระเพ็ดพุงช้างมีฤทธิ์เป็นพิษต่อเซลล์โดยเหนี่ยวนำเซลล์ตายแบบอะพอพโตสิส ทั้งในเซลล์เม็ดเลือดขาวที่ได้จากผู้บริจาคโลหิต และเซลล์เม็ดเลือดขาวของผู้ป่วยมะเร็งปากมดลูก และมีฤทธิ์เพิ่มขึ้น (additive effect) เมื่อให้สารสกัดบอระเพ็ดพุงช้างร่วมกับการฉายรังสี อีกทั้งสามารถยับยั้งการแบ่งตัว ของเซลล์เม็ดเลือดขาวที่ถูกกระตุ้นด้วย PHA โดยมีค่า IC_{50} ของความเข้มข้นของบอระเพ็ดพุงช้างเท่ากับ 40 ไมโครกรัม/มล.

ผลการศึกษาเบื้องต้นนี้ อาจสามารถสรุปได้ว่าสารสกัดน้ำจากหัวบอระเพ็ดพุงช้างน่าจะได้รับการศึกษาเพื่อพัฒนาเป็นยาสมุนไพรสำหรับต้านมะเร็งต่อไป

ภาควิชา	สหสาขาเภสัชวิทยา	ลายมือชื่อนิสิต………………………………………....
สาขาวิชา	เภสัชวิทยา	ลายมือชื่ออาจารย์ที่ปรึกษา………………………………..
ปีการศึกษา	2545	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม…………………………..

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KEY WORDS: *STEPHANIA VENOSA* (BL.) SPRENG / APOPTOSIS / LYMPHOCYTE

TIPSUDA PLUMCHAI: INDUCTION OF APOPTOSIS BY THE EXTRACT FROM *STEPHANIA VENOSA* (BL.) SPRENG TUBER ON LYMPHOCYTE. THESIS ADVISOR: ASSOC. PROF. TADA SUEBLINVONG, M.D., THESIS COADVISOR: ASST. PROF. WACHAREE LIMPANASITTHIKUL, Ph.D. 89 pp. ISBN 974-17-2932-4

There are thousands of herbs in Thailand used in traditional medicine that are claimed to possess anticancer activity, but most of them have no scientific evidences to support their alleged medicinal quality. One among them is *Stephania venosa* (Bl.) Spreng or *S.venosa*. The tuber of *S.venosa* has been used in many Thai traditional medicines as remedy of various diseases including cancer. In this study, we aim to apply cell culture technique and design a scientific model for testing and proving the claimed anticancer activity of these Thai herbs. Lymphocytes obtained from healthy blood donors were cultured in RPMI medium with the density of $4x10^5$cells/ml and used in various tests throughout the study. The inhibitory concentration at 50% (IC_{50}) of water extract of *S.venosa* was determined by exposing $4x10^5$ lymphocytes to various concentrations of *S.venosa* for 48 hours, and trypan blue dye exclusion was the technique applied to detect the cytotoxic activity. Detection of pH change and the cytotoxic activity of aliquots of *S.venosa* were studied for a period of 12 weeks to monitor the stability of the water extract of the herb. Besides the study of cytotoxic activity, IC_{50} of water extract at 300 μg/ml was selected for the test of apoptotic activity, both in normal human lymphocytes and lymphocytes obtained from cervical cancer patients. Both sources of lymphocytes were exposed to various treatments including: 100 μg/ml *S.venosa*, 300 μg/ml *S.venosa*, 0.5 Gy ^{60}Co irradiation and a combination of 300 μg/ml *S.venosa* plus 0.5 Gy ^{60}Co irradiation. The apoptotic activity was detected at 48 hour after exposure by *in situ* terminal deoxynucleotidyl transferase assay (TdT assay). Furthermore, inhibitory effect of various concentrations of water extract of *S.venosa* was tested on PHA stimulated normal human lymphocytes.

Results from all studies indicated that, water extract of *S.venosa* tuber possessed cytotoxicity and apoptotic activities against both normal lymphocytes and lymphocytes obtained from cervical cancer patients. A combination of 0.5 Gy ^{60}Co irradiation with 300 μg/ml *S.venosa* exhibited additive effect when detected by TdT assay. Besides, water extract of *S.venosa* exhibited definite inhibitory activity on PHA stimulated normal human lymphocytes at IC_{50} of 40 μg/ml.

With the above data, it can then be primarily concluded that water extract of *S.venosa* should be further investigated and developed in line of other anticancer herbal drugs.

Inter-department Pharmacology — Student's signature……………………………..

Field of study Pharmacology — Advisor's signature……………………………..

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Tipsuda Plumchai

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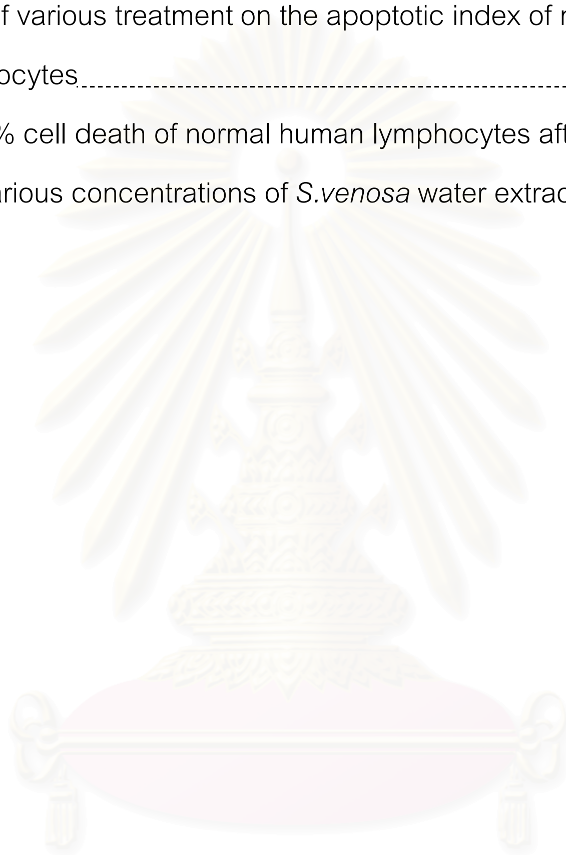
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List of Abbreviations

AchE	Acetylcholinesterase
APAF 1	Apoptosis protease activating factor1
BAD	BCL2 antagonist of cell death
BAX	BCL2 associated x protein
BCL2	B- cell CLL/ Lymphoma 2
BH3	BCL2 homology 3 domain
BID	BH3 interacting domain death agonist
BIM	BCL2 interacting mediator of cell death
bp	Base pair
^{o}C	Degree Celsius
Ca^{2+}	Calcium
c.p.m.	count per minute
CAD	caspase-activated DNase
Cytc	Cytochrome c
DAPI	4,6 -diamino-2-phenylindole
DNA	Deoxyribonucleic acid
DR	Death receptor
FADD	Fas associated death domain
FADU	Fluorescence analysis of DNA unwinding
FITC	Fluorescence isothiocyanate-conjugated
FIGO	The International Federation of Gynecology and Obstetrics
Flice	FADD - like interleukin 1 beta converting enzyme
FLIP	Flice-like inhibitory protein
Gy	Gray
HBSS	Hanks' Balanced Salts Solution
HPV	Human papilomavirus
IAP3	Inhibitors of apoptosis protein

List of Abbreviations (continued)

IC_{50}	50% inhibit concentration
ICAD	Inhibitor of caspase-activated DNase
IV	intravascular
K^+	Potassium
LD_{50}	Lethal dose 50%
μg	microgram
ml	millilitre
MW	molecular weight
NFκB	Nuclear factor κB
PBS	Phosphate buffer saline
pH	The negative logarithm of the concentration of hydrogen ions
PHA	Phytohemagglutinin A
PI3	Phosphatidylinositol- 3
PI3K	Phosphatidylinositol 3 kinase
r.p.m.	Revolution per minute
RNA	Ribonucleic acid
SODD	Silencer of death domain
STD	Sexually transmitted disease
TdT	Terminal deoxynucleotidyl transferase
TNF	Tumor necrosis factor
TUNEL	TdT – mediated dUTP nick end labeling

CHAPTER I

INTRODUCTION

1. Background and Rationale

Cancer was estimated to be accountable for about 7 million deaths (12 % of all deaths) worldwide in 2000; it was only preceded by cardiovascular diseases (30% of all deaths), and infectious and parasitic diseases (19%). Cancer was also estimated to be accountable for almost 6% of the entire global burden of diseases of the same year. More than 70% of all cancer deaths occurred in low – and middle –income countries and, although the risk of developing/dying from it is still higher in the developed regions of the world, the control of communicable diseases as well as aging of the population in developing countries, point to an increasing burden of cancer worldwide.(1) Cancer is also one of the major causes of death in Thai population only next to cardiovascular disease and accident. The Report on Cancer in Thailand vol. II published by the Ministry of Public Health in 1999 showed that cancer of the cervix was the most prevalent cancer in Thai women with estimated new cases of 5,462 in 1993.(2) In 2000, the Annual Report of Cancer Registry of the National Cancer Institute, Department of Medical Service, presented the incidence of cervical cancer as reported by records and statistics was the second most common cancer out of the leading cancers of the females (Table 1). The age specific incidence showed that there was an early increase before age 20, then a steep rise around the age of 40 – 50 (Figure 1). (3) While the incidence of cervical cancer at King Chulalongkorn Memorial Hospital, as reported by records and statistics was the third most common cancer (11.3%, 294/2602) out of ten leading cancers for in – patients in 2001.(4)

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Table 1. The leading sites of cancer in female [3]

Site	No.	%
All sites	2,246	100
Breast	717	31.9
Cervix	595	26.5
Colon and rectum	97	4.3
Ovary	87	3.9
Thyroid	84	3.7
Lung	75	3.3
Oral cavity	71	3.2
Liver	65	2.9
Lymphoma all types	57	2.5
Corpus uteri	56	2.5

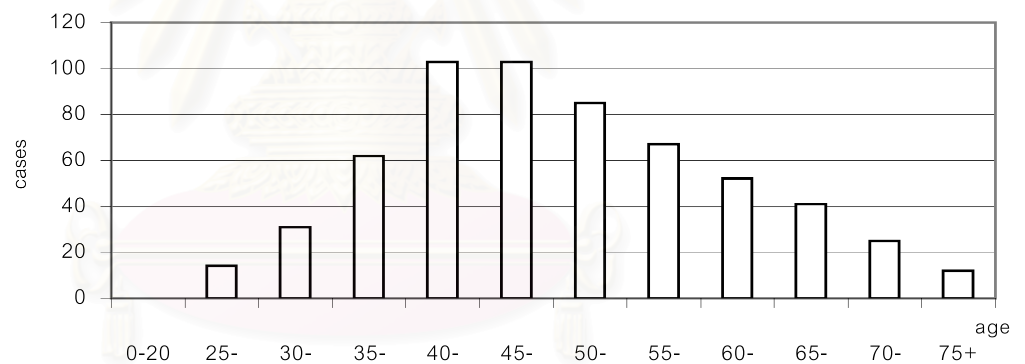

Figure 1. The age specific incidence of cervical cancer [3]

Although, radiation is still the most important therapy in cervical cancer, it has been reported that tumors, which have not received prior irradiation, seem more susceptible to chemotherapy and capable to downstage to surgery or radiotherapy for their treatment. The role of chemotherapy as a treatment in the management of cervical cancer is still limited. Investigations are underway throughout the world looking for neoadjuvant therapy for advanced squamous cell carcinoma of the cervix. Now, many drugs have been engaged in the treatment, to mention a few : metrotrexate, 5-fluouracil,

cyclophosphamide, vincristine, vinblastine, adriamycin, bleomycin, mytomicin c, cisplatin. [5-8]

A variety of anticancer drugs have been demonstrated to inhibit the growth of carcinoma cell by induction of apoptotic cell death. Apoptosis or programmed cell death is the most common form of physiological cell death that occurs under a variety of physiological and pathological conditions. The dysregulation of apoptosis pathway can play a significant role in the growth and therapeutic responsiveness of cancer cells. In addition, recent studies have shown that taxol and vinblastine, plant alkaloid derivatives, can induce apoptotic cell death in several human culture cell lines; many agents from plants are modulating through apoptotic process in the treatment of cancer. Furthermore, the process of apoptosis in many cell types is short-lived, but lymphocytes in cell culture remain in this process for many days and appear to be a useful assessment of individual sensitivity to radiation. Its apoptotic activity can possibly represent that of other cells in the body. Therefore, lymphocytes apoptosis assay may be used as a model to study the efficacy of a cytotoxic agent. [9-14]

Recently, great deals of attentions has been given to the therapeutic use of herbal remedies, based on their safety, efficacy and economy.[15-16] There are many medicinal plants in Thai traditional medicine used for the prevention and treatment of cancer, among them is *Stephnia venosa* (Bl.) Spreng (*S.venosa*) which is a Thai folk medicinal plant. Its tuber contains over 30 alkaloid compounds; it has been used in the treatment of various diseases including cancer. However, at present, there are some scientific data about the efficiency or toxicity of *S.venosa* in cancer remedy. Whereas drug resistant problem seems to increase in cancer patients who are receiving chemotherapy. Thus, a need to develop new drugs is required. In this study, we aims to confirm the cytotoxic effect and investigate apoptotic activities of water extract of *S.venosa* tuber on both normal human lymphocytes and lymphocytes from cervical cancer patients.

2. Objectives

The purposes of the study are as follows:

1. To examine whether water extract of *S.venosa* tuber possess any cytotoxic, apoptotic or antiproliferative activities on human lymphocyte
   - 1.1 The cytotoxic effect of the extract on normal human lymphocytes was determined by trypan blue dye exclusion assay.
   - 1.2 The apoptotic activity of the extract on normal human lymphocytes was estimated by ApopTag *In situ* Apoptosis detection kit.
   - 1.3 The apoptotic activity of the extract on lymphocytes from cervical cancer patients was also estimated by ApopTag *In situ* Apoptosis detection kit.
   - 1.4 The antiproliferative effect of *S. venosa* was assayed by ^{3}H- thymidine incorporation methods.
2. to investigate the stability of water extract of *S.venosa* tuber
   - 2.1 The pH change of the extract stock solution was measured weekly for 12 weeks.
   - 2.2 A weekly evaluation the cytotoxic effect of the extract stock solution on normal human lymphocyte was weekly evaluated by trypan blue dye exclusion method for 12 weeks.

3. Expected Benefit and Application

The knowledge gained from the work may corroborate to Thai traditional medicine report which may eventually lead to the development of a new anticancer agent from this tropical herb. In addition, the model employed in this study can be used as a model for studying herbal medicinal plants claimed to contain anticancer property.

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4. Keywords:

Stephania venosa (Bl.) Spreng

Apoptosis

Lymphocyte

5. Review of Related Literatures

5.1 cancer

In normal cell growth there is a finely controlled balance between growth promoting and growth-restraining signals such that proliferation occurs only when required. The balance is tilted when increased cell numbers are required, for example during wound healing and during normal tissue turnover. Differentiation of cells during this process occurs in ordered manner and proliferation ceases when no longer required.[17]

Cancer cells are defined by two heritable properties: first, reproducibility in defiance of the normal restrains. The continued cell proliferation occurs and loss of differentiation may be found. Second, the process of metastasis will occur when cells retain the ability to undergo repeated cycles of proliferation as well as migrate to other sites in the body and express clonogenic or colony forming capability.

The derangement of the mechanisms that control cells replication and in the synthesis of nucleotides and protein are common features of malignant transformation. There is increase evidence to implicate the insertion of foreign nucleic acid sequence in the host's genome (oncogenes) in the genesis of cancer. It may be that more than one defect is required to produce.

Tumorigenesis is possibly started with stimulations from chemical agents, heat and radiation etc. causing changes of both biological and cytoplasmic material in the cell leading to the generation of free radicals and activated caspases enzyme inducing DNA damage. When DNA damage occurred, there are 3 possible fates: 1) DNA damage is restored back to normal via DNA repairing mechanism, 2) when the DNA damage seems irrepairable, the affected cell will engage in a suicidal process (apoptosis). Apoptosis can then be considered as a protective mechanism against tumorigenesis since death cell can not turn into cancer cell, 3) If the cell does not die nor repairing itself to normal then it or its progeny may live long enough to accumulate mutation that become to the cancer cells.[18] (Figure 2)

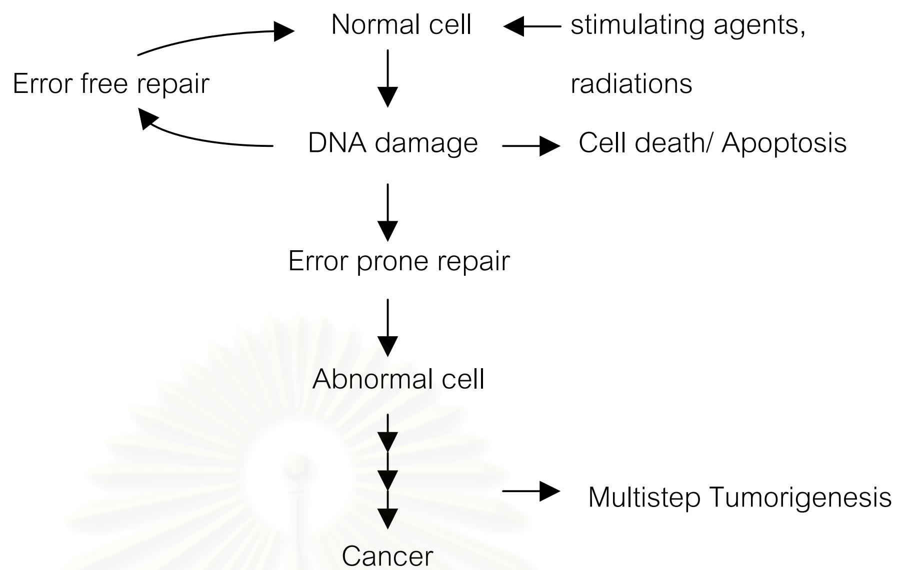

Figure 2. The fates of cell with damaged DNA.

The etiologies of cancer are related to various factors including sex, age, race, genetic predisposition and exposure to environmental carcinogens. Chemical carcinogens, such as tobacco smoke, aflatoxins and benzene have been listed in cancer induction in human. However, certain herpes and papilloma groups DNA viruses and type C RNA virus have also been implicated as causative agent responsible for human cancer. For genetic factor, there are two main types of cell death regulatory molecules: inducers and repressors. Cell death inducers facilitate apoptosis, they include, amongst others, the transcription factor Myc, the DNA repair- associated protein p53, certain cyclin- dependent kinases, and a subset of the BCL2 family. Cell death repressors block or delay apoptosis are included the death repressor protein BCL2, the Bcr-Abl chimeric protein found in chronic myeloid leukemia, etc.

Clearly, two types of situation can result in too little cell death and lead to cancer development. A cell death repressor protein may become expressed or its function enhanced such that the cell acquires resistance to conditions that would have normally killed it. Alternatively, loss of function mutation in cell, again with the result that the cell survives under conditions where it would have otherwise died.

5.1.1 Incidence of Cervical cancer

Besides cardiovascular disease and accident, cancer is one of the major causes of death in Thailand as well as in the developed countries, such as the United State. The Report on Cancer in Thailand volume II, published by the Ministry of Public Health in 1999 showed that cancer of the cervix was the most common cancer found in Thai women.[(2)] The aged specific incidence showed that there is an early increase before age 20, steep rise to about age 45-50 years old and followed by a plateau then declined. The strong risk factors in cervical cancer include early age at first intercourse, a history of multiple sexual partners, genital human papillomavirus infection (HPV) or other sexually transmitted disease (STD), and the presence or history of other genital tract abnormalities (Figure3). Women infected with HPV-16 and HPV-18 exhibit a 60 –folds greater risk of developing cervical cancer than uninfected women; both types have been identified in approximately 84 % of cervical cancer. Other risk factors include active or passive ("second-hand") smoking, poor nutrition, taking steroid oral contraceptives and a current or past sexual partner with risk factors for STDs, or HIV/AIDS has been shown to associate with an increasing risk of cervical cancer in many studies. In addition, carotenoids, vitamin C and vitamin E found in foods of plant origin have possibly protective effect against cancer. Survival rates for cervical cancer are generally high, most of the early cases have higher five year survival after initial diagnosis. The survival rates are considerably better for cancer cases diagnosed at early stage.

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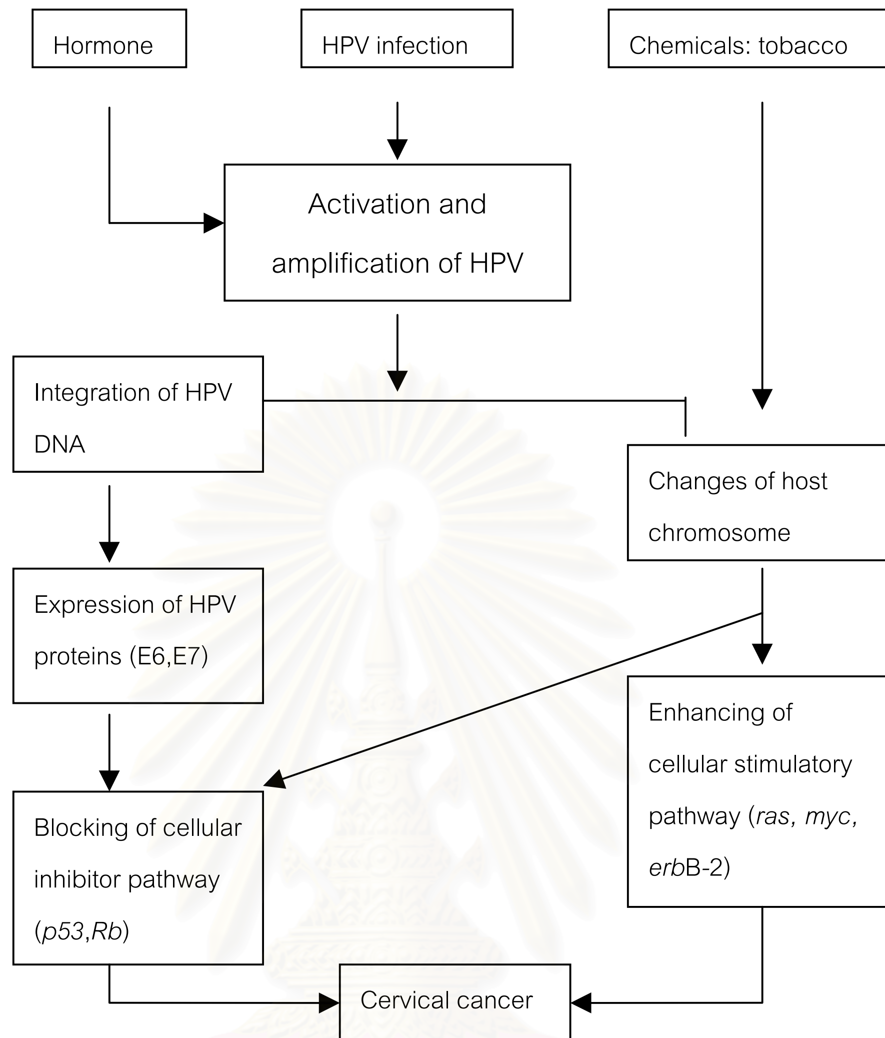

Figure 3. Etiology of cervical cancer [7]

Identification of staging is important before institution of therapy. The initial staging system proposed in 1929 by Subcommittees of the League of Nations was later revised in 1937 and 1950. This function was taken over by FIGO (the International Federation of Gynecology and Obstetrics) in collaboration with the World Health Organization and the International Union against Cancer. The staging recommendations were last revised in 1995, as shown in Table 2.

Table 2. FIGO staging of carcinoma of cervix uteri, 1995

Stage	Description
Stage I	Carcinoma is strictly confined to the cervix (extension to the corpus should be disregarded) IA Invasive cancer identified only microscopically IA1 Measured invasion of stroma no greater than 3 mm in depth and no wider than 7 mm IA2 Measured invasion of stroma greater than 3 mm and no greater than 5 mm and no wider than 7 mm IB Clinical lesion confined to the cervix or preclinical lesion greater than IA IB1 Clinical lesion no greater than 4 cm in size IB2 Clinical lesion greater than 4 cm in size
Stage II	Carcinoma extends beyond the cervix but has not extended on to the pelvic wall; the carcinoma involves the vagina but not as far as the lower third IIA No obvious parametrial involvement IIB Obvious parametrial involvement
Stage III	Carcinoma has extended to the pelvic well, on rectal examination, there is no cancer-free space between the tumor and pelvic wall; tumor involves the lower third of the vagina, all cases with a hydronephrosis or non functioning kidney should be included unless they are known to be due to other cause IIIA Involvement of the lower third of the vagina IIIB Extension onto the pelvic wall or hydronephrosis or non functioning kidney
Stage IV	Carcinoma has extended beyond the true pelvis or has clinically involved the mucosa of the bladder or rectum IVA Spread of the growth to adjacent organs IVB Spread to distant organs

Currently, treatment of cervical cancer consists of surgery, chemotherapy and radiotherapy. The treatment regimen for each patient is based on staging, invasiveness, and the response to treatment. The regimen may be a combination between irradiation and chemotherapy or chemotherapy and surgery etc. Although, radiation is the most important therapy, there has been reported that tumors, which have not received prior irradiation, seem more susceptible to chemotherapy and capable to downstage to use surgery or radiotherapy in the treatment. Now, many drugs have been recommended in the treatment, including metrotrexate, 5-fluouracil, cyclophosphamide, vincristine, vinblastine, adriamycin, bleomycin, mytomicin c, and cisplatin. The previous studies

showed that chemotherapy that included the drug cisplatin, when given simultaneously with radiation therapy, prolonged the survival in women with this disease. Furthermore, drug resistant problem seems to increase in cancer patients treated with chemotherapy. Thus, development of new drugs is required for cancer treatment.[5-7]

5.2 Cancer chemotherapeutic agents

With present methods of treatment, surgery or radiation therapy, which are quite effective when the tumor has not metastasized by the time of treatment. Earlier diagnosis might lead to increase cured of patients with such local treatment; however, in the remaining cases, early micrometastasis is a characteristic feature of the neoplasm, indicating that a systemic approach such as chemotherapy will be required for the effective cancer management. Cancer chemotherapy as currently employed can be a curative treatment in certain disseminated neoplasms that have undergone gross or microscopic spread by the time of diagnosis. Of major importance are the demonstrations that, the use of chemotherapy along with initial surgery can increase the cure rate in relatively early- stage breast cancer and osteogenic sarcoma.[9-11,21-22]

At present, chemotherapy provides palliative rather than curative therapy for many other forms of disseminated cancer. Effective results in temporary clearing of the symptoms and signs of cancer and prolongation of life. In the past decade, advances in cancer chemotherapy have also begun to provide evidence that chemical control of neoplasia may become a reality for many forms of cancer. This will probably be achieved first through combined therapy, in which optimal combinations of surgery, radiotherapy, and chemotherapy are used to eradicate both the primary neoplasm and its occult micrometastases before gross spread can be detected on physical or X-ray examination. A major effort to develop anticancer drugs through both empirical screening and rational design of new compounds has now been under way for over three decades. The drug development program has employed testing in a few well-characterized transplantable animal tumor systems. Simple *in vitro* assays for measuring drug sensitivity of a attack of human tumor cells augment and shorten the testing program and are used currently as the primary screening test for new agents by the National Cancer Institute and many pharmaceutical firms. After new drugs with potential

anticancer activity are identified, they are subjected to preclinical toxicologic and limited pharmacologic studies in animals. Promising agents that do not have excessive toxicity are then advanced to clinical trails. The remainder of clinical testing is similar to that for other drugs but may be accelerated. Ideal anticancer drugs should have the following characteristics: little or no adverse effects, high efficacy against multiple sites, effectiveness at achievable dose levels, a known mechanism of action, low cost, history of use by the human population and general human acceptance.[12,14,19-20,23]

Classes of drugs that have entered development include inducers of differentiation, intended to force neoplastic cells past a maturation block to form end stage cells with little or no protective potential, can be divided into 7 groups.[9]

1. Alkylating Agent

   Alkylating agents exert cytotoxic effects via transfer of their alkyl groups to various cellular constituents. Alkylation within DNA is the N7 position of guanine, probably represent the interactions, that can result in miscoding through abnormal base pairing with thymine and lead to cell death. The major toxicity of alkylating agents is on the bone marrow and results in dose-related suppression of myelopoiesis. Most alkylating agent used widely in clinic are nitrosoureas, procarbazine, and cisplatine.

2. Antimetabolites

   The biochemical pathways of antimetabolites have been related to nucleotide and nucleic acid synthesis. When an enzyme is known to have a major effect on cell replication, inhibitors of the reaction have proved to be useful anticancer drugs. The major classes of antimetabolites are folic acid analogue (e.g., methotrexate), purine antagonists (e.g., 6-mercaptopurine, 6-thioguanine and hypoxanthine) and pyrimidine antagonists (e.g.,fluorouracil, cytarabine and gemcitabine). Toxic effects are observed in the bone marrow, skin and gastrointestinal mucosa.

3. Antimitotic agents

    Their mechanisms of action involve depolymerization of microtubules, which are an important part of the cytoskeleton and the mitotic spindle. They bind specifically to the microtubular protein tubulin in dimeric form. The drug-tubulin complex is added to the forming end of the microtubles to terminate assembly, and depolymerization of microtubules. This results in mitotic arrest at metaphase, dissolution of the mitotic spindle, and interference with chromosome segregation. Several agents are used in clinic, such as vinblastine, vincristine, both alkaloid derived from *Vinca rosea* and taxanes, which is an alkaloid ester, derived from yew tree. The major toxicities are bone marrow suppression, especially in patients with pre-existing hepatic impairment.

4. DNA Topoisomerase II Inhibitors

    DNA Topoisomerase II is a homodimeric protein and major component of the nuclear matrix. At present, two types of topoisomerases II have been described in human cells, Topoisomerase IIα (MW $\approx$ 170 kDa) and Topoisomerase IIβ (MW $\approx$ 180kDa). They may have important roles in DNA packaging, replication and transcription. Thus, the normal regulation of topoisomerase II inhibitor is probably linked to the ability of cell to enter a G_0 period, malignant cells. Topoisomerase II inhibitors are commonly divided into two groups: 1) DNA intercalators such as the anthracyclins, anthracenediones, acridines and ellipticines. 2) Non intercalators such as the epipodophyllotoxins, teniposide and etoposide.

5. Antibiotics

    All of clinically useful antibiotics now available are products of various strains of the soil fungus *Streptomyces*. Machanisms of action of these agents included: 1) high-affinity binding to DNA through intercalation, with consequent blockade of the synthesis of DNA and RNA, and DNA strand scission through effects of Topoisomerase II; 2) binding to membranes to alter fluidity and ion transport; and 3) generation of the semiquinone free radical and oxygen radicals through an enzyme- mediated reductive

process. These include the anthracyclines, actinomycin, bleomycin, mitomycin and plicamycin. Bone marrow depression is the major toxicity of these agents.

6. Hormone agents

Hormone therapy relies on the presence of receptors for endogenous hormones needed for the cell proliferation. Unlike agents in the other classes of antineoplastic drugs, members of this class generally do not cause severe toxicity. Sex hormones or their antagonists are most effective in tumors arising from cells that are normally hormones dependent, namely breast and prostate. There are several ways in which hormones can affect malignant cells: A) A hormone may have a direct cytotoxic action on the malignant cell. B) A hormone may suppress production of other hormones by a feedback mechanism.

7. Miscellaneous agents

Hydroxyurea
- Therapeutic use: treatment of leukemia
- Mechanism: inhibit ribonucleotide reductase, which is essential for DNA synthesis.
- Adverse effect: bone marrow depression

Mitotane
- Therapeutic use: treatment of adrenal carcinoma.
- Mechanism: produces tumor regression and relief of the excessive adrenal steroid secretion
- Adverse effect: gastrointestinal disturbance and dermatitis.

Interleukin –2 (IL-2)
- Therapeutic use: treatment of metastatic renal cancer or melanoma.
- Mechanism: activate peripheral T lymphocyte that lyses several types of cancer cells.
- Adverse effect: fever, fluid retension leading to pulmonary edema, hypotension and cardiac arrhythmias.

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5.3 Apoptosis

The survival of living beings depends on the correct and co- ordinated functioning of various cell types. During the initial stage of development, the viability of the organism depends on the selection and differentiation of adequate cells in the various tissues. At later stages, the maintenance of the organism requires a specific cellular adaptability. As an example, blood cells are constantly renewed from haematologic precursors. Lymphocytes or reproductive cells show rapid expansion in response to immediate requirements. On the other hand, neural cells evidence a limited capability for renewal, and many neurons survive and persist throughout the life of the individual. For each cell line, control of the number of cells is the result of a dynamic balance between cell proliferation and cell death. Cell death can occur through two different processes, termed necrosis and apoptosis. For these two modalities of cell death there are differentiating functional and morphologic characteristics that are also definitely for each modality of cell death. (Figure 4.) [27,30-31,33]

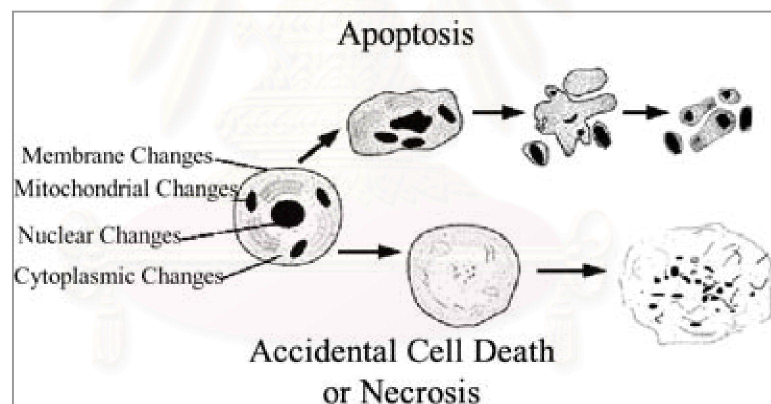

Figure 4. Schematic representation of the morphologic differences between cell death through necrosis and cell death through apoptosis.[32]

Necrosis, also known as a pathological cell death that are damaged by injury, such as by mechanical damage and exposure to toxic chemicals. Cell necrosis ensues when the aggression exceeds the regenerative capability of the cell, and is accompanied by a number of characteristics, morphologic and metabolic changes. The earliest changes occur in mitochondria, with minimal changes in the nucleus. These changes are followed by dissolution of the organelles and loss of the control over the

selective permeability of the cell membrane, with entrance of fluid into the cell. This causes cell edema and vesiculation, finally, leading to the disruption of the cell membrane and the exit of intracellular contents into the extracellular space. The release of hydrolases by the lysosome disruption gives rise to accelerated cell disintegration, which affects neighboring groups of cells and is accompanied by an inflammatory reaction in the surrounding tissue in response to the release of cell debris.

In contrast, apoptosis is a genetically programmed process for cells to commit suicide in certain cases, which is often called programmed cell death or PCD. The phenomenon of apoptosis in the cells was originally defined by Kerr in 1972 as the orderly and characteristic sequence of structural changes resulting in the programmed death of cell. The temporal sequences of eventual fragmentation of the dying cell into a cluster of membrane-bound segments (apoptotic bodies) which is digested by macrophages. Apoptosis can be triggered by noxious agent, and often appear spontaneously or in response to physiological stimuli. They suggest that it should be called “apoptosis” which is used in Greek to described the “dropping off” or “falling off” of petals from flowers or leaves from trees. (There is no consensus yet on how to pronounce its; some say *APE oh TOE sis*; some say *uh POP tuh sis*).

Apoptosis is an essential process in the developments of multicellular organisms which depend on the elimination of selected cells. It controls the number of cells to balance among growth, proliferation and death. There are five main functions for apoptosis, most of which involves the elimination of unwanted cells: 1) sculpting structures: Mouse paw, for example, apoptosis eliminates the cells between developing digits (Figure 5A.). Similary, apoptosis is involved in hollowing out solid structures to create lumina (Figure 5B.). 2) deleting unneeded structures: In the course of animal development, cells die by apoptosis when the structure they formed is no longer needed. As a tadpole becomes a frog, it deletes its tail cells (Figure 5C.). The structure that are required in one sex but not in the other, for example, the Mullerian duct forms the uterus and oviducts in female mammals but it is not needed in male and is thought to be lost by apoptosis. Conversely, the Wolffian duct forms the vas deferens, epididymis, and seminal vesicle in males, but it is not needed in females and is eliminated by apoptosis (Figure 5D.). 3) controlling cell numbers: In many organs,

apoptosis helps regulate cell numbers (Figure 5E.). In the developing nervous system, for example, cell death adjusts the number of nerve cells to match the number of target cells that require innervation. 4) eliminating abnormal, misplaced, non-functional, or harmful cells, examples are seen in the vertebrate immune system, where developing T and B lymphocytes that either fail to produce self – reactive receptors that make the cells potentially dangerous are eliminated by apoptosis (Figure 5F.). When the DNA damage seems irrepairable, the affected cell will engage in apoptotic process (Figure 5G.). 5) the death program may be involved in producing specialized differentiated cells without organelles. Certain cell types, including skin keratinocytes, lens epithelial cells and mammalian red blood cells, lose their nucleus and other organelles in the process of terminal differentiation. The differentiated lens cells and red blood cells continue to live in the sense that they continue to metabolize, where as differentiated keratinocytes die and form a layer of corpses on the surface of skin.

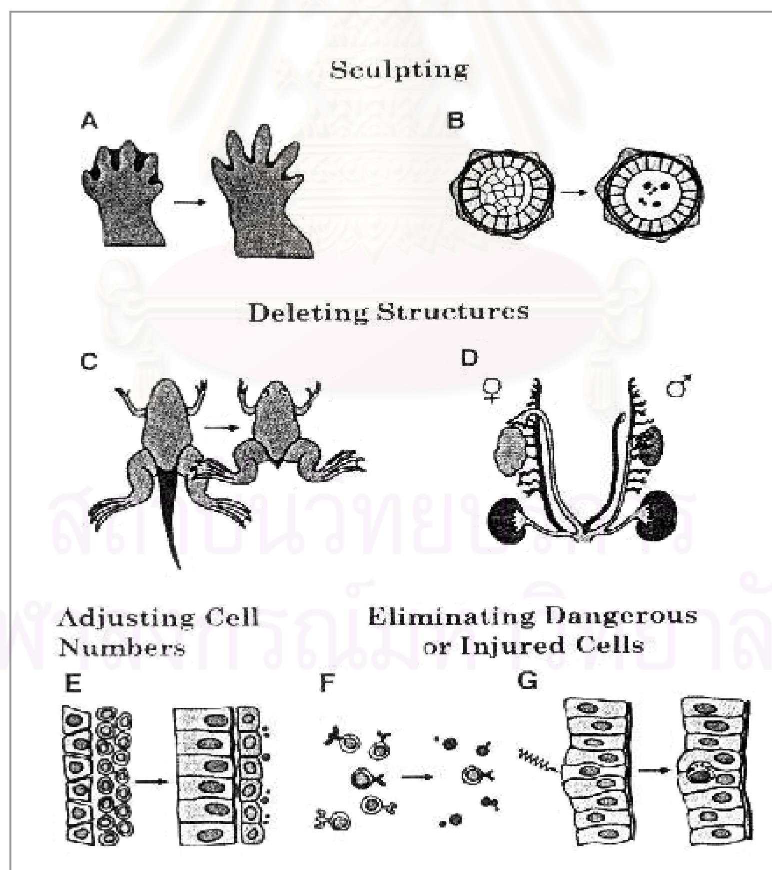

Figure 5. Some functions of apoptosis in animal development [26]

Cell undergoing apoptosis displays profound morphological changes, which can be divided into 3 phase: [25,28-29,34-35]

Phase I. Cells rapidly shrink and lose their normal intercellular contacts subsequently exhibit dense chromatin condensation. Cytoplasmic organelles are compacted. Dilatation of endoplasmic reticulum is observed but the mitocondria remain morphologically normal.

Phase II. There is blebbing at the cell surface and crenation of the nuclear outline, leading to controlled fragmentation of both nucleus and cytoplasm that subsequently split up into particles of various sizes called "apoptotic bodies". Some apoptotic bodies contain variably size, spherical, nuclear fragments of condensed chromatin. These bodies may be phagocytosed by neighboring cells or macrophages.

Phase III. The residual nuclear and cytoplasmic structures undergo progressive degradation. In this phase, several apoptotic cells and numerous apoptotic bodies are appeared. Subsequently, cell develop, "secondary necrosis" where membranes surrounding condensed chromatin disappear and the appearance is that of a residual lysosomal body. The different features of apoptosis and necrosis are summarized in Table 3.

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Table 3. Cardinal features of apoptosis and necrosis [36]

Features	Apoptosis	Necrosis
Stimuli	Physiological and pathological conditions without ATP depletion	Toxins, severe hypoxia, massive insult and conditions of ATP depletion
Energy requirement	ATP- dependent	None
Histology	Chromatin condensation, apoptotic bodies, death of single isolated cells	Cellular swelling, disruption of organelles, death of patches of tissue
DNA breakdown pattern	Ladder of fragments in internucleosomal multiples of 185 base pairs.	Randomly sized fragments
Plasma membrane	Intact, blebed, with molecular alterations	Lysed
Phagocytosis of dead cells	Neighboring cells	Immigrant phagocytes
Tissue reaction	No inflammation	Inflammation

The events in apoptosis are shown in Figure 6. in their chronological order. Apoptosis induction, in e.g. caused by TNF$\alpha$and Actinomycin D, is transmitted via receptor activation and cytochrome c release from mitochondria. The mitochondrial potential decreases, which is the point of no return for the cells to undergo apoptosis. The activation of caspases is currently the hot research field as major differences are found in different cell types and tissues. Phosphatidylserine exposure in the outer leaflet of the cell membrane is the first evidence of morphological changes, which are later seen as shrinkage, and blebbing of the cell. DNA fragmentation finally is the last step in apoptosis, before the cell is phagocytosed.

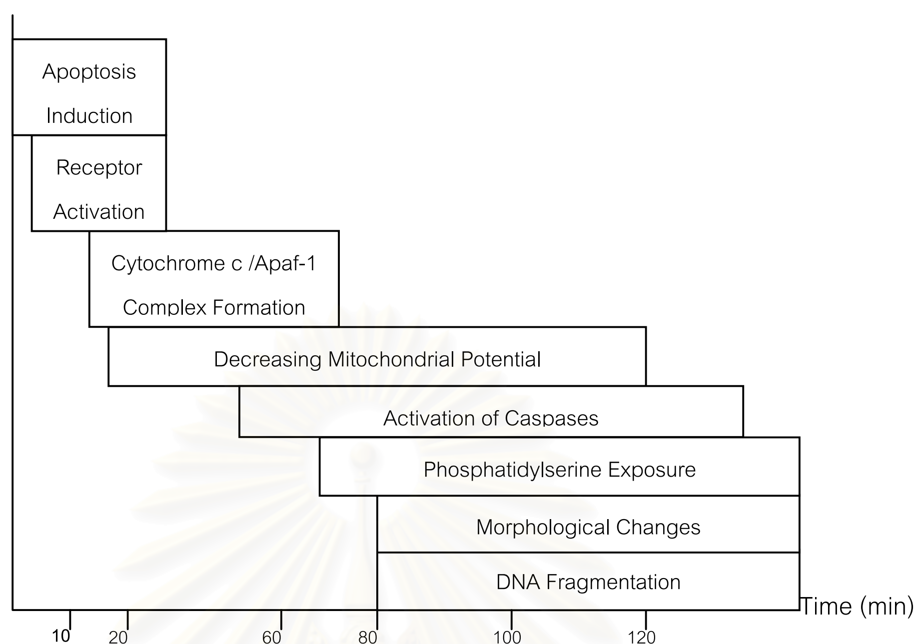

Figure 6. Hela apoptosis induction with TNFα/Actinomycin D [40]

5.3.1. Mechanisms of apoptosis

Apoptotic death can be triggered by a variety of stimuli, and not all cells necessarily will die in response to the same stimulus. Any agent or set of conditions that stresses the metabolism or normal response mechanisms of a cell is likely to trigger the process of apoptosis. However, the level of stress is crucial. At high stress levels, cell die by necrosis, because they have no time to respond to the stimulus and die instantly. Examples include high level of toxins, sharp change in pH and high agitation rates. At intermediate level of cell stress, the cell is injured but not killed. The cell has time to activate its own death programs. Thus the cell dies in a controlled way, by apoptosis. At low levels of environmental stress, cell can switch on the production of heat shock proteins, which enable them to survive until the stress is removed. However, once a certain stress threshold is passed and survival is deemed impossible. Cell died by apoptosis. [37-40]

Many changes are observed as cells undergo apoptosis. Internucleosomal DNA degradation, a biochemical hallmark of apoptosis, because of the protease - mediated

cleavage of a nuclease inhibitor called ICAD (inhibitor of caspase-activated DNase), which releases a unique endonuclease called CAD (caspase-activated Dnase)

Caspases are the cysteine - dependent proteases, which asssociated to the proteolytic cleavages during apoptosis. These protease family differs in primary structure and substrate specificity but shares several common features. Of the twelve known human caspases, six (caspases-3, -6, -7, -8, -9, and –10) are definitely involved in apoptosis in various model systems. One current classification scheme divides these apoptotic caspases into two classes, effector (or downstream) caspase, which are responsible for most the cleavages that disassemble the cell, and initiator (or upstream) caspases, which initiate the proteolytic cascade. Caspases-3, -6, and –7 are the major effector caspases. Once activated, these enzymes are capable of cleaving the vast majority of polypeptides that undergo proteolysis in apoptotic cells. Caspases-8 and –9 are the major initiator caspases. Upon activation, caspase-8 and –9 acquire the ability to cleave and activate effector caspases.

There are 2 signalling mechanisms by which a cell commits suicide by apoptosis: apoptosis triggered by internal signals, generated by signals arising within the cell and apoptosis triggered by external signals, which triggered by death activators (TNFα, Fas ligand (FasL) and Lymphotoxin) binding to receptors at the cell surface. (Figure 7) [10-11]

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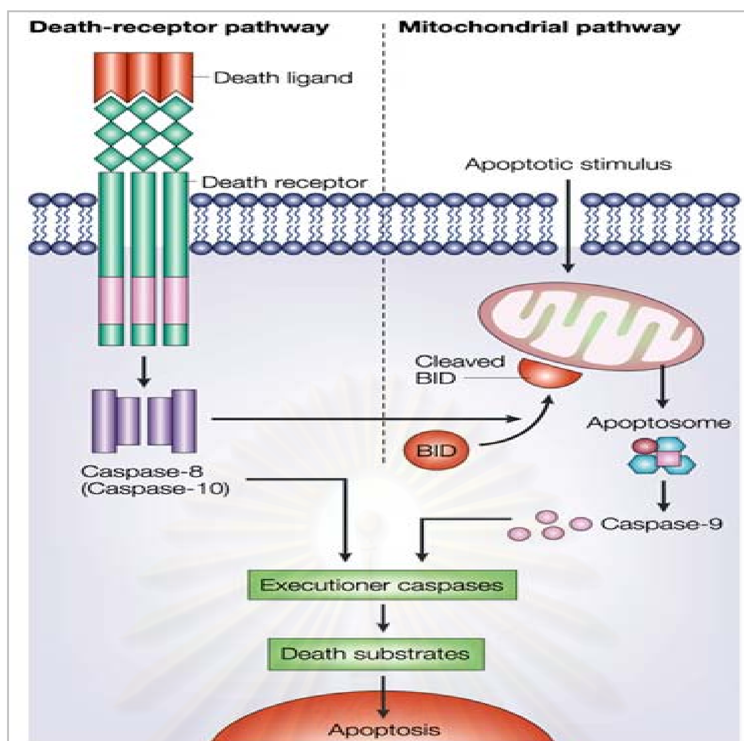


Figure 7. The two main apoptotic signalling pathways [11]

External signals; ligation of Fas (CD95) by FasL or a cross-linking antibody results in receptor trimerization followed by binding of the adaptor molecule FADD (Fas associated death domain) to the cytoplasmic domain of the receptor. FADD in turn recruits procaspase-8. Because FADD is a multimeric protein, it can act as a molecular scaffold that juxtaposes multiple procaspase-8 molecules. The end result is the liberation of the cleaved and fully active caspase-8 within seconds to minutes. Caspase-8 then cleaves procaspases-3 and –7; subsequently, the activated caspase-3 then, cleaves procaspase-6. Although the ligands and adaptor molecules are different for other death receptors (DR) (e.g., tumor necrosis factor -α receptor1 [TNFR1], DR3, DR4, DR5, and DR6) similar pathways appear to ultimately be activated. (Figure 8)

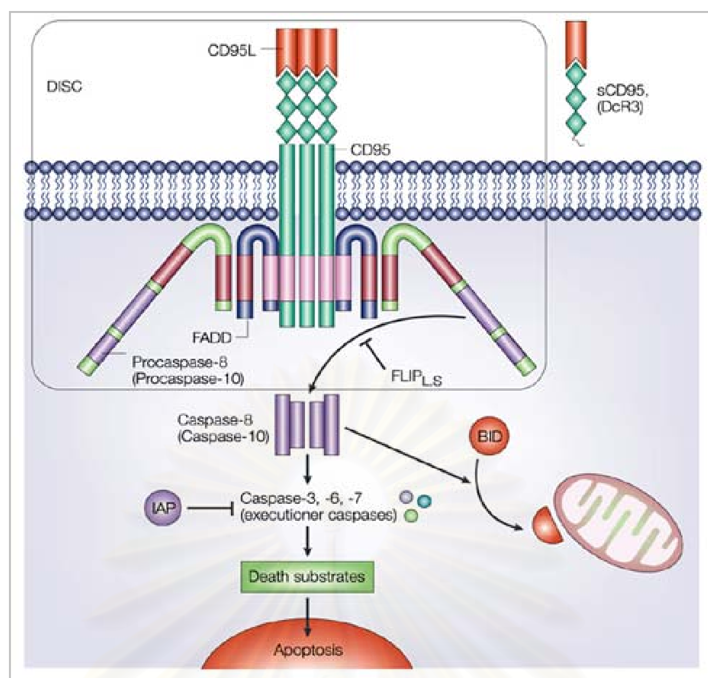


Figure 8. Apoptotic signalling through death receptor (External signal) [(11)]

Internal signal; chemotherapy, irradiation and other stimuli can initiate apoptosis through the mitochondrial (intrinsic) pathway. Pro-apoptotic BCL2 (B-cell CLL/lymphoma 2) family proteins, for example, BAX (BCL2- associated x protein), BID (BH3 interacting domain death agonist), BAD (BCL2 antagonist of cell death) and BIM (BCL2 interacting mediator of cell death) are important mediators of these signals. Activation of mitochondria leads to the release of cytochrome c (Cyt c) into the cytosol, where it binds apoptotic protease activating factor 1 (APAF1) to form the apoptosome. At the apoptosome, the initiator caspase-9 is activated. Apoptosis through mitochondria can be inhibited on different levels by anti-apoptotic proteins, including the anti-apoptotic BCL2 family members BCL2 and BCL-X_L and inhibitors of apoptosis proteins (IAPs), which are regulated by SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP binding protein with low pI). Another way is through survival signals, such as growth factors and cytokines, that activate the phosphatidylinositol 3-kinase (PI3K) pathway. PI3K activates a serine/threonine kinase (AKT also called protein kinase B), which then phosphorylates and inactivates the pro-apoptotic BCL2-family member BAD. (Figure 9)

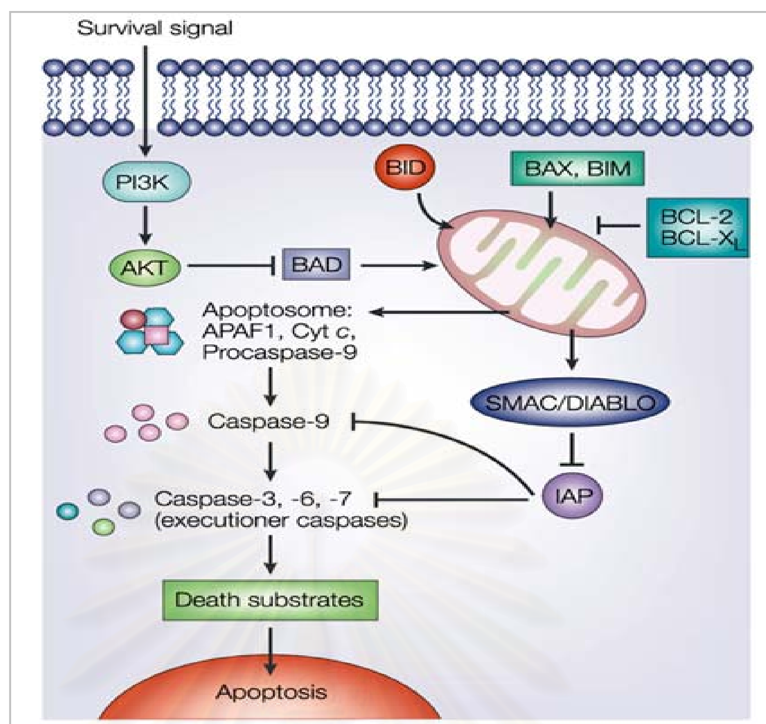


Figure 9. Apoptosis signalling through mitochondria (internal signal) [11]

Signaling through both the death receptor and mitochondrial pathways can be modulated by IAPs (inhibitor of apoptosis proteins), highly conserved polypeptides that selectively inhibit the activity and activation of various caspases. The expression of these polypeptides is regulated by NFκB (nuclear factor κB), a transcription factor whose activation is regulated in part by the activity of the protein kinase Akt. In addition, each of the two pathways is regulated in different ways. The death receptor pathway is regulated at a variety of levels. First, the levels of death receptor expression vary from cell type to cell type. Second, certain cell types express truncated or mutated receptors (decoy receptors) that compete for ligand but do not activate the cell death machinery. It is thought that the ability of death receptor ligands to induce apoptosis reflects the balance between death receptors and decoys. Third, polypeptides associated with the cytoplasmic domains of death receptors appear to modulate their function. In particular, the SODD (silencer of death domain) polypeptide binds to an intracellular domain of TNFR1 and prevents its spontaneous oligomerization in the absence of ligand. Finally, the interactions between FADD and procaspase-8 are regulated by FLIP (Flice(FADD-

like interleukin1β converting enzyme)- like inhibitory protein), a molecule that contains a prodomain similar to that of procaspase-8 but lacks a caspase active site. Because binding of FLIP to FADD prevents binding and activation of procaspase-8 and –10, FLIP overexpression cause resistance to death, that mediated by various death receptors. The mitochondrial pathway is also regulated at several different levels. Phosphorylation plays a major role in regulating this pathway. First, the activities of some of the BCL2 homologues, including BAD and BCL2 itself, can be modulated by phosphorylation. Second, Akt has been reported to phosphorylate procaspase –9, thereby diminishing its capacity for activation. Third, Akt has been observed to inhibit cytochrome c release from mitochondria by the process that does not involve phosphorylation of BAD or procaspase- 9. All of these antiapoptotic effects of Akt are the end result of signaling through a pathway that starts at cell surface receptor tyrosine kinases and proceeds sequentially through ras and phosphatidylinositol-3 (PI3) kinase to 3-phosphoinositol-dependent protein kinases that phosphorylate Akt.

There is crosstalk between these two pathways. For example, cleavage of the BCL2-family member BID by caspase-8 activates the mitochondrial pathway after apoptosis induction through death receptors, and can be used to amplify the apoptotic signal. Additional cross-talk can also occur downstream of the effector caspases. In particular, active caspase-6 can cleave and activate procaspase-8.

5.3.2. Apoptosis and cancer

In a simplified manner, the diseases in which apoptosis has been involved can be divided into two groups. Increased apoptosis is characteristic of AIDS; neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis; ischaemic injury after myocardial infarction, stroke, and reperfusion; and in autoimmune diseases such as hepatitis and graft versus host disease. Decreased or inhibited apoptosis is a feature of many malignancies, autoimmune disorders such as systemic lupus erythematosus, and some viral infections. Cancers arise when changes in DNA cause the abnormal accumulation of cells. Many carcinogens damage DNA or interfere with enzymes necessary for accurate DNA replication. A cell can respond to this sort of injury in several ways: it can delay cell

division until the damage is repaired, it can undergo apoptosis, or it can progress without interruption through the cell growth cycle. Abnormal apoptosis can promote cancer development, both by allowing accumulation of dividing cells and by obstructing removal of genetic variants with enhanced malignant potential. High concentrations of BCL-2 also protect cells from apoptosis induced by *c-myc*. The *bcl-2* oncogene may be a generalised “cell death suppressor” gene that directly regulates apoptosis. All haemopoietic and lymphoid cells, many epithelial cells, and neurons contain *bcl-2* protein, found forming pores in the outer membrane of the mitochondrial, nucleus, and endoplasmic reticulum. Follicular B-cell lymphomas have high concentrations of *bcl-2* protein.Epstein-Barr virus proteins increase the expression of *bcl-2* in Burkitt’s lymphoma cells. The protein product of the p53 tumour suppressor gene can delay cell cycle progression before initiation of replicative DNA synthesis. Many human cancers have mutations or deletions in the p53 gene. Proteins encoded by oncogenic viruses can also bind and activate the p53 protein (e.g. Human papilloma viruses (HPV) have been implicated in causing cervical cancer. This is thought to be due to an increased expression of HPV oncogenes (E6) when HPV is inserted into the host genome in addition to higher binding affinities of E6 for tumor suppressor gene products, p53. Malfunction of p53 may promote cancer development by permitting cells with secondary mutations to duplicate their DNA before repair is complete.[43-49]

5.3.3. Apoptosis and chemotherapy

Although a few chemotherapeutic regimens have yielded lasting remissions or cures, it is clear that new therapeutic options are necesssary. In the development of new chemotherapeutic agents, several issues need to be addressed, including improved and durable antitumor efficacy, reduction of toxicities, which can prevent effective dosing of potentially efficacious drugs, and prevention of drug resistance caused by the inherent genomic instability of tumors. Advances in molecular biology now allow us to identify gene that go awry in cancer, and offer the opportunity to dissect the molecular mechanism underlying the disease. Now, many genes are known to affect tumorigenesis and tumor growth, and the key is to decide which ones have exploited in the areas of

signal transduction, cell-cycle regulation, apoptosis, telomere biology, and angiogenesis.

Since apoptosis programs can be manipulated to produce massive changes in cell death, the genes and proteins controlling apoptosis are potential drug targets. The agents that directly induce apoptosis may provide less opportunity for acquired drug resistance, decrease mutagenesis and reduce toxicity. Collectively, these observations indicate that cell can interprete a drug- induced insult in the same way as those physiological insults, such as hypoxia or growth factor deprivation. Mechanisms of drug resistance includes drug target amplification (methotrexate), enhanced repair of DNA damage (alkyllating agents), increased drug metabolism, or altered drug accumulation. It is becoming increasingly clear that the most important determinant of tumor resistance may be a generalized resistance to induction of apoptosis, rather than resistance based on specific alterations in the drug/ target interaction. Alterations in a variety of oncogenes and tumor suppressor genes have been shown to modulate responsiveness to apoptotic induction by chemotherapeutic agents and radiation. Susceptibility to activation of apoptosis pathway varies among cell types. The threshold for activation of apoptosis has been shown to be modulated by a variety of genetic changes. For instance, loss of gene function such as p53 or over expression of other genes such as *bcl-2* would increase the threshold for activation of apoptosis. New therapeutic strategies aimed at decreasing the threshold for activation of apoptosis (e.g., by inhibiting the *bcl-2* protein function) are currently under development, as well as agents capable of directly triggering the apoptotic cascade. It still remains to be seen whether these new drugs will offer lasting survival advantages to the patients. It is also apparent that not all of the approaches are performing as well as anticipated. There is clearly a learning curve with respect to the best ways to use these new agents, just as has been the case in the development of traditional cytotoxic drugs.[10-11,19,21-23,43]

5.3.4. Assessment of apoptosis

The assessment of apoptosis applies to the biology of tumors including clinical diagnosis and oncologic therapeutic. There are a number of methods for identifying apoptosis cells. Most methods currently in use rely chiefly on two phenomena: the condensation of nuclear chromatin and the DNA fragmentation. The most simple of all is the study of the morphological pattern by phase contrast microscopy which in the visible evidence of apoptotic process is of sudden onset and that is why its early stages appear to occur swiftly. Identification of the apoptotic cells by light microscope can then be confirmed by other techniques such as electron microscopy and histochemical or biochemical studies. The electron microscope is a tool in the study of the complex morphological changes that occur in apoptosis. The cardinal feature of apoptosis cells is DNA fragmentation. It is believed that the linker region between nucleosomes are the targets of endonuclease attack, resulting in fragments of 180 – 200 base pair (bp) and multiples of this unit length. The DNA extracted from a cell population holding these types of cleavage can be assessed by the appearance of a ladder of bands on a conventional agarose gel electrophoresis. Although this type of analysis is essentially qualitative, but it is the most common biochemical method used for the detection of apoptosis and is often considered the hallmark of apoptosis. Recently, the application of *in situ* end labeling of DNA at sites of cleavage has proved to be a useful tool in quantifying the percentage of apoptosis in a cell population and in identifying the individual apoptotic cells. The easiest technique relies on the use of digoxigenin labelled nucleotides by TdT reaction at the 3' hydroxyl termini of DNA strand breaks. The incorporated digoxigenin-nucleotides within the cells are detected with fluorescein labeled antidigoxigenin antibodies. The antidigoxigenin antibodies fragment carries a fluorescein to the reaction site, which generates an intense signal at 520 nm after excited by light of 490 nm wavelength. Finally, the cells were counter stained with the blue fluorescent dye 4,6-diamino-2-phenylindole (DAPI). Which stains the nuclei. These fluoresceins labeled apoptotic nuclei are detected by fluorescence microscope (Figure 10).These techniques are also termed the TdT – mediated dUTP nick end labeling or TUNEL, and can also be used in flow cytometric analysis. A number of flow cytometric techniques to discriminate live cell from dead cells has been designed. Another

potentially useful method to explore DNA strand breaks is the single-cell gel electrophoresis or comet assay. This method, is originally designed to study single-strand DNA oxidative damage, it has also gained a general acceptance for assessing DNA mutagenic damage. The fluorescence analysis of DNA unwinding (FADU) assay is a cellular bioassay based on time – dependent alkaline denaturation of DNA under moderate denaturing conditions (pH 12.2-12.4) starting from ends as well as from all DNA break points (single strand breaks, double strand breaks, alkali- labled lesion and DNA fragmentation which associated with apoptotic nuclei). DNA which remained double- straned after alkaline treatment was detected after neutralisation and immediate fragmentation followed by binding to the Hoechst 33258 dye (bisbenzimide) and measuring its fluorescence.[50-54]

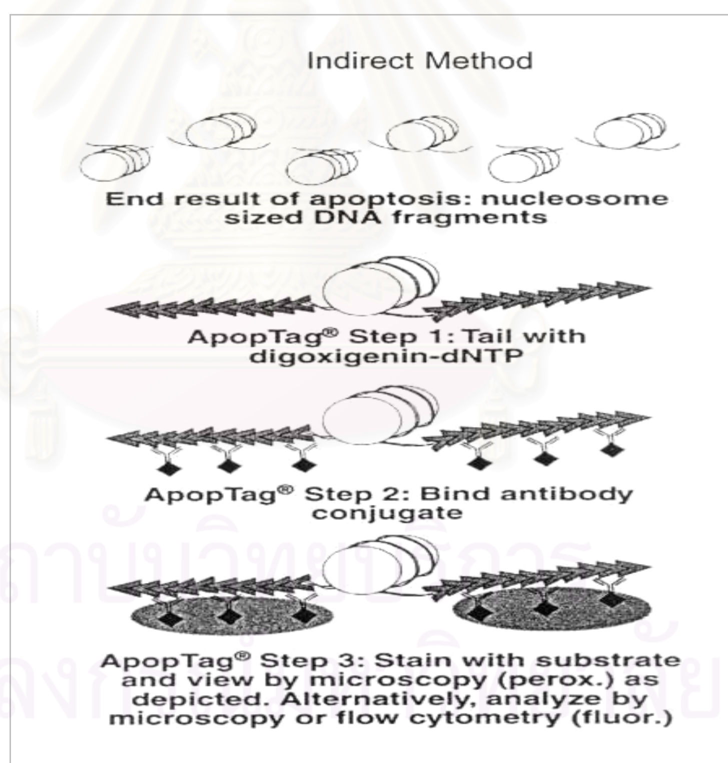


Figure 10. Process of *in situ* terminal deoxynucleotidyl transferase (TdT) assay.[54]

5.4 Botanical aspects of *Stephania venosa* (Bl.) Spreng

Stephania venosa (Bl.) Spreng or Boraphet phung chang (Southwestern); Plao lueat khruea (Northern); Cho koe tho (Karen / Northern); Kratom lueat (Northeastern); Kling klang dong (Southwestern); Borapent yang daeng (Peninsular) in Thai, also belong to the family of Menispermaceae.

Figure 11. *Stephania venosa* (Bl.) Spreng tuber

These plants are also distributed in Vietnam, Malaysia, Sumatra, Java, Sabah, Celebes and Philippines. The morphological descriptions were given: *S.venosa* is slender climber, containing red sap, entirely glabrous; leafy stems herbaceous, annual, arising from a large exposed rhizome, up to 40 cm diameter; perennial stems less than 1 m long and up to 4 cm diameter. Leaves are broad triangular-ovate, margin often slightly lobed (6-11 by 7-12 cm),base truncate or slightly cordate, apex obtuse and mucronulate, lower surface minutely papillose, glaucous, with reticulation usually drying reddish - brown; submembranous; petioles 5-15 cm. Male inflorescence is an axillary umbelliform cyme, 4-16 cm long. Male flowers are on pedicels (1-2 mm) which have 6 greenish sepals (2-2.5 mm long), usually unequal, outer 3 oblaneolate, inner 3 obovate, unguiculate. Petal 3, orange, obdetoid-obovate, 1.25 mm long. Synandrium is 1-1.75 mm long. Female inflorescences are much more condensed than the male, sometimes subcapitate. Female flowers very shortly pedicellate, asymmetrical. *Sepal* 1, w/wo

elliptic, 0.75 mm long. *Petals* 2, ± suborbicular, 0.75 mm long. *Carpels* subellipsoidal, 1.5 mm long. *Drupes* Obovate in outline, 7 mm long. Endocarp perforates, dorsally bearing 4 rows of 12-16 subcapitate projections.[55-57,60,62]

The tuber of this plant contains a wide variety of isoquinoline alkaloids, for example; Anonaine, Apoglaziovine, Ayutianine, N- carboxamidostepharine, Crebanine, 4- α-hydroxycrebanine, 7- oxocrebanine, Dehydrocreanine, Kamaline, Kikemanine, Nuciferoline, Mecambroline, Tetrahydroplamatine, β-n-oxide-stephadiolamine, 4-α-hydroxy N- carboxamidostepharine, Stepharinosine, o-methy Stepharinosine, Stesakine, Sukhodianine, Acetylsukhodianine, β-n-oxide-sukhodianine, Thailandine, Thairugosamine, β-n oxide ushinsunine, β-n oxide , 4- α- hydroxy ushinsunine, Uthongine and etc. (Figure12) [58,59,63,66,72,75]

A variety of plants that contain isoquinoline alkaloid compounds are use as traditional medicine in various cultures. In traditional medicine reputation, the tubers of *S. venosa* are used as nerve tonic, aphrodisiac, appetizer, antiasthmatic, antimicrobial, hypoglycemic and anticancer agents in some recipe of Thai folk medicine.[55,56,60] Eight isoquinoline alkaloids in *S. venosa* tuber; stephanine, crebanine, sukhodianine, dehydrocrebanine, tetrahydropalmatine, jatrorhizine and stephatine have showed antimalarial potential by *in vitro* growth inhibition of *Plasmodium falciparum* with the IC_{50} of 12, 278, 310, 40, 70, 7,800, 500 and 6,100 ng/ml, respectively. The comparison of these alkaloids to their analogs indicated that the 6α, -7- dehydro derivatives were about 3-4 times more potent than their parent compounds for antimalarial activity. Therefore, the antimalarial potential of these natural products can be augmented by introducing unsaturation to the C-6α andC-7 carbons of their molecules.[69] It has been reported that the methanol extract of *S. venosa* roots at the concentration of 0.1 mg/ml inhibited more than 90% of acetylcholinesterase (AchE) activity.[65] The cytotoxic activity of the water and ethanol extracts from the tuber of *S.venosa* against brine shrimp was exhibited with LC_{50} value of 184.9 and 90.8 μg/ml, respectively. The ethanol extract was tested against human cancer cell line MCF-7 (breast adenocarcinoma) which was found to be cytotoxic with an IC_{50} value of 11.6 μg/ml. The Palmatine and Crebanine are the most active fraction isolated from the ethanol extract, they are found to possess pronounced activity against MCF-7 with IC_{50} value in the range of 5 – 6 μg/ml. The

Palmatine and Crebanine also exhibited cytotoxic activity against brine shrimp with LC_{50} value of 74.4 and 29.6 μg/ml, respectively.[78] In addition,there are several plants that also compose of isoquinoline alkaloids like in *S.venosa* and some of these alkaloids have the various evidences of effective. The isoquinoline alkaloids from the tubers of *Stephania pierrei* Diels (Menispermaceae), which has synonym common name with *S.venosa*, have diverse pharmacological properties, for instance, the immunosuppressive activity of sinomenine, the anticancer potential of dicentrine, and the sedative effect of tetrahydropalmatine. The cytotoxicity of *S. pierrei* is mainly due to the presence of the aporphine alkaloid containing the 1,2-methylenedioxy group such as anonine, where as the antimalarial activity is attributed to the aporphine alkaloid and the tetrahydroprotoberberines possessing a phenolic functionality.[68] Anonaine is also the most potent alkaloid isolate from the roots of *Annona cherimolia*. It has the inhibiting effect to the KCl - induced contraction on isolated strips of rat thoracic aorta with an IC_{50} value of 1.20 $\pm$ 0.07 x 10^5 M.[61] Anonine alkaloid from *Artabotrys maingayi* is able to inhibit in vitro ^3H - dopamine uptake by rat striatal synaptosome.[73] Ushinsunine was found to have antiplasmodial activity against *P. faciparum* (IC_{50} = 5.99 μM) and is toxic to KB cells(IC_{50} = 42.5 μM).[76] Ushinsunine - β - N - oxide alkaloid from *Cananga odorata* fruits has cytotoxicity against two hepatocarcinoma cell lines with IC_{50} for HepG2 and Hep2,2,15 are 6.2 and 2.4 μg/ml respectively.[64] Crebanine (IV) 5mg/kg can convert $BaCl_2$ induced arrhythmia into sinus rhythm and increases the tolerant dose both of aconitine and $CaCl_2$ to produce ventricular fibrillation (VF) and cardiac arrest (CA) in rats. The drug can also decrease the incidence of VF and CA by chloroform in mice.[77] Crebanine is an antagonist of the contraction of biliary system of quinea pigs that is induced by histamine, Ca^{2+} and K^+.[75] In addition, crebanine and 1- tetrahydropalmatine has obvious blocking effects on alpha 1 and alpha 2 adrenoceptors.[71]

As part of the ongoing project to search for natural products that possess cancer chemotherapeutic compounds from Thai plants and the previous information suggest that the *S.venosa* can be a potential source of biologically active compounds which may be used as lead molecules for the development of new anticancer drug. Then we try to set up an in vitro experimental model, which can scientifically support the use of *S.venosa* tuber for cancer treatment in Thai folkloric medicine.

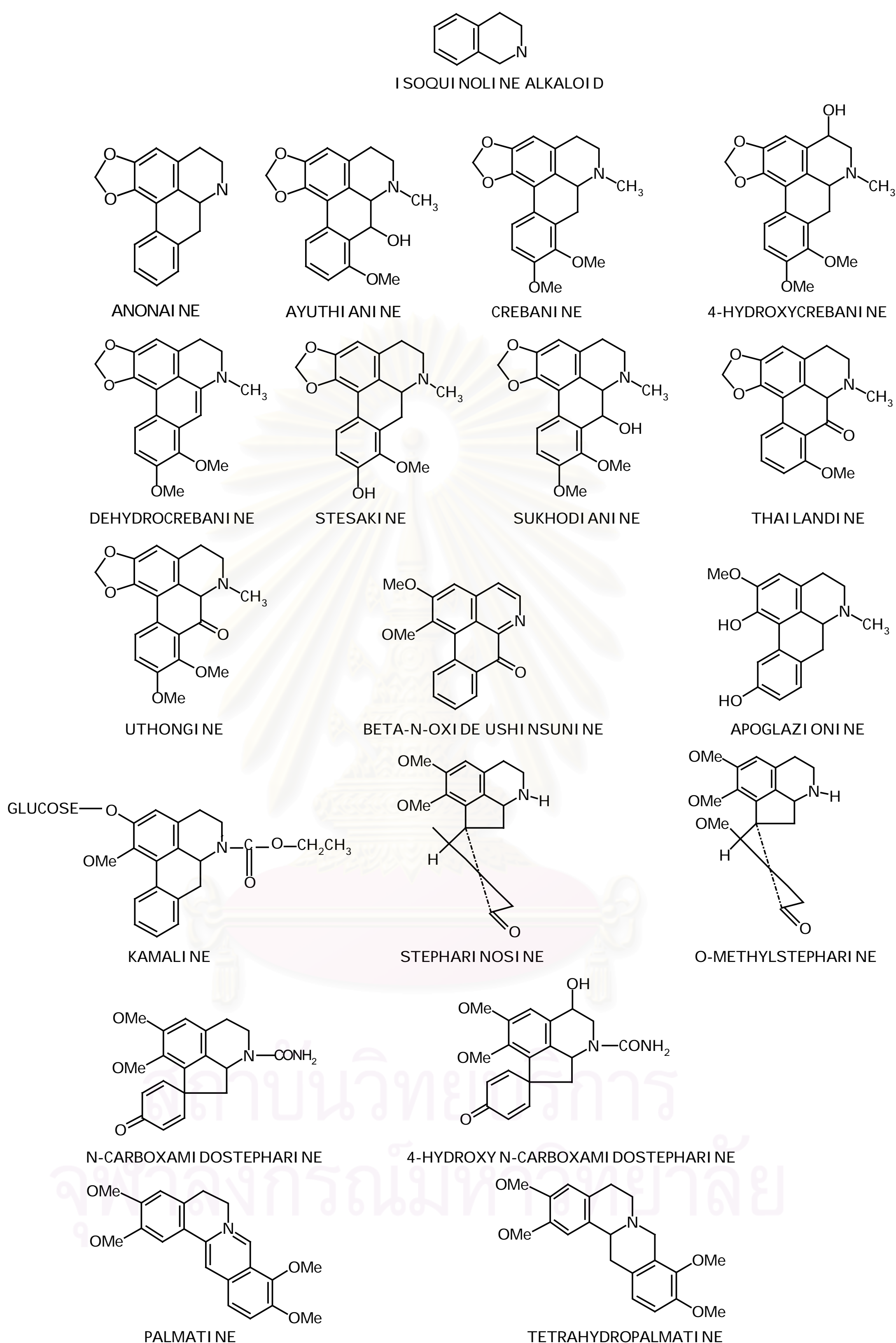


Figure 12. some structure of the isoquinoline alkaloids in the tuber of *S.venosa*. (58,59,63,66,72,75)

CHAPTER II

MATERIALS AND METHODS

Materials

1. *Stephania venosa* (Bl.) Spreng.

Water extract of *S.venosa* tuber was kindly provided by Assoc. Prof. Dr.Pathama Leewanich (Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University, Thailand). The lyophilized material was kept in a desiccator; an aliquot was dissolved in distilled water, filtered through a 0.22 μ membrane and stored at –20°C for further study. The required concentrations were prepared by diluting the concentrated stock solution which was 0.02 g/ml for stability test and proliferation assay. For cytotoxic assay, the water extract of *S.venosa* was freshly prepared at the concentration of 0.012 g/ml; and in the study of apoptotic activity, the stock solution was used -at the concentration of 0.03 g/ml.

2. Specimens

20 ml of heparinized whole blood sample was collected from each of nine cervical cancer patients who attended Cervical Cancer Clinic at the Division of Radiotherapy, Department of Radiology, King Chulalongkorn Memorial Hospital. Every patient gave her signed inform consent form. Six of the patients were diagnosed as staged IIB and three had stage IIIB cervical cancer; they were 41 - 76 years of age. Patients who were HIV positive or had either been through radiotherapy or chemotherapy were excluded.

In the mean time,20 ml of heparinized whole blood sample was taken with informed consent from each of thirty-eight healthy female blood donors aged between 22 - 54 years who attended the National Blood Bank, Thai Red Cross Society for blood donation. These volunteers should neither smoke nor drink alcohol and should not take any medications during 14 days prior to their blood donation.

The blood samples of patients and the controls were brought to laboratory, and lymphocytes were separated for further study.

3. Laboratory supplies

Aerosol resistance pipette tip: 200 μl (Molecular Bio- products, USA)

Aluminum foils (Diamond®, USA)

Autoclave tape (3M, USA)

Beakers: 50 ml, 1,000 ml (Pyrex®, USA)

Coplin staining jars

Cylinders (Pyrex®, England)

Disposable gloves

Glass pipettes: 1 ml, 5 ml, 1 ml (Witeg, Germany)

Heparinized Vacutainer 10 ml (Vacutanier®, USA)

Humidified chamber

Microscope glass cover slips (Chance, England)

96 multiwell plates (Nunc, USA)

Needle (Vacutainer System PrecisionGlide™, UK)

Parafilm (American National Can™, USA)

Pasteur pipettes

Plastic cover slips (ApopTag®)

Reagent bottles: 250 ml, 1000 ml (Duran®, Germany)

Slide (Super Frost, Germany)

Slide box

Slide film (Eritchrome 400, Kodak)

Slotted microscope slide staining dish

Sterile membrane filters (Whatman®, Japan)

Sterile polypropylene centrifuge tube: 15 ml, 50 ml. (Nunc™, USA)

T 25 Tissue Culture flasks (Nunc™, USA)

Tube rack

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

4. Equipments

Autoclave (HICLAVE™, HIRAYAMA)

Autopipette (Gilson, France)

Biohazard Lamina- flow hood (Gelman Science)

CO_2- Incubator (REVCOULTIMA)

^{60}Co Teletherapy machines (Eldorado-78, Canada), Radiotherapy Unit, Department of Radiology, Faculty of Medicine, Chulalongkorn University.

Differential counter

Fluorescent microscope (Olympus), Andrology Unit, Department of Obstetrics and Gynecology, Faculty of Medicine, Chulalongkorn University.

Freezer – 20° C

Fume hood (Model 252, NEWLAB®)

Hemocytometer (Boeco, Germany)

Incubator (Heraeus)

Light microscope (Olypus, Japan)

Liquid scintillation counter 1219 Rackbeta (LKB-Wallad, Finland)

Low- speed centrifuge (Beckman)

pH meter SA 520 (Orian, USA)

Refrigerator 4°C, - 20°C (SANYO)

Timer

Thermometer

5. Reagents

5.1 General reagent

Absolute ethanol (Merk, Germany)

Acetic acid (Merk, Germany)

Actinomycin D (Cosmegen,Lyovac,USA)

Clorox (Clorox, USA)

Fetal Bovine Serum (Gibco BRT, Germany)

Formaldehyde (Sigma,USA)

Gentamycin (GOH, Thailand)

[^{3}H]- thymidine

Hanks' Balanced Salts Solution (HBSS) Powder (Gibco BRL)

Heparin (LEO, Denmark)

Histopaque® -1077 (Sigma, Germany)

Hoeschts 33258 Dye Solution (Sigma, Germany)

Hydrochloric acid: (Merck, Germany)

L- Glutamine (Gibco BRL)

Paraformaldehyde powder (Sigma, Germany)

Phytohemagglutinin (PHA) (Sigma,USA)

Potassium chloride (Merck, Germany)

Potassium hydrogen phosphate (Merck, Germany)

RPMI 1640 (Gibco BRL)

Sodium chloride (Sigma, USA)

Sodium hydroxide (Merck, Germany)

di- Sodium hydrogen phosphate monobasic (Merck, Germany)

Sodium bicarbonate (Baker, USA)

5.2 Reagent kit

ApopTag® Fluorescein kits (Intergen)

Methods

A. The lymphocytes preparation

Using Histopaque – 1077, lymphocytes were isolated from whole blood specimen according to the following protocol:

1. Pipette 5 ml of Histopaque-1077 at room temperature into each 15 ml polypropylenes centrifuge tubes.

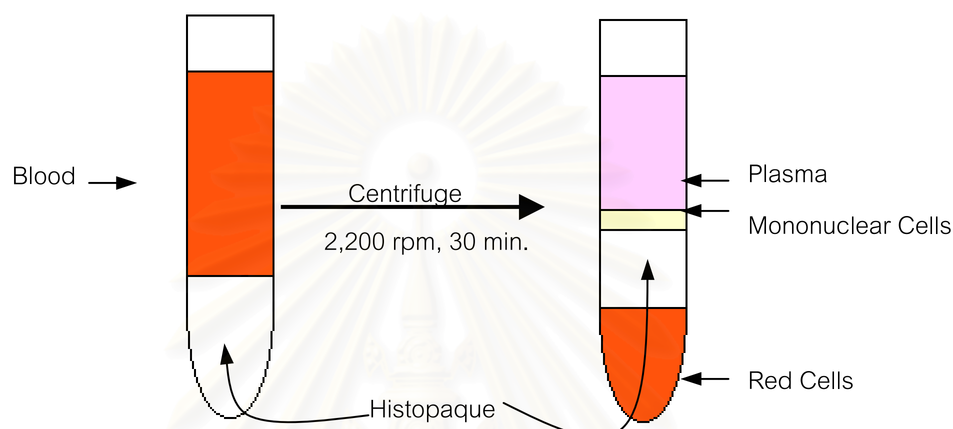
2. Mix the blood 1:1 with Hanks' Blanced Salts Solution (HBSS), adds 2 μl/ml Heparin at room temperature.

3. Layer 9 ml of the blood/ HBSS mixture onto the top of Histopaque in each tube. Be careful not to mix the two parts together, and cap the tube tightly.

4. Centrifuge 2,200 rpm for 30 minutes at room temperature.

5. Use a pipette to remove carefully the top plasma layer from each tube without disturbing the interface. Discard this layer into a container of Clorox.

6. Use a swirling motion with the pipette, collect the interface (white ring of lymphocytes) from each tube. The next layer is the residual histopaque. Try not to collect much of this layer and do not collect the red cell layer, which sits at the bottom of the tube. (See the following diagram)



7. Immediately transfer the lymphocytes ring to a new sterile polypropylene 15 ml centrifuge tube. Add 12.5 ml HBSS (+2 μl/ml heparin + 1% fetal bovine serum) to each tube. Cap tightly, then invert the tubes 3 times to mix.

8. Centrifuge at 1,000 rpm for 10 minutes at room temperature.

9. Discard the supernatant from each centrifuge tube into a Clorox container.

10. Flip to softly disperse the lymphocyte pellet. Add 5 ml HBSS/ 2 μl/ml heparin/1% fetal bovine serum to the first tube. Pipette the mixture of HBSS and cells up and down 3 times to further disperse the cells. Transfer the cell suspension from the first tube to a second centrifuge tube and repeat the mixing process. Continue the procedure until all of the cells are pooled in the last tube.

11. Add an additional 5 ml of HBSS/2 μl/ml Heparin/1% Fetal Bovine Serum to the last tube to bring the volume to 10 ml. Mix by pipetting up and down 3 times.

12. Centrifuge at 1,000 rpm for 10 minutes at room temperature.

13. Decant the supernatant into the Clorox container and resuspend the pellet. Add complete RPMI medium (RPMI 1640 media + 5.2 % Fetal Bovine Serum

+ L- Glutamine 20.28 μl/ml + Gentamycin 0.97 μl/ml) to 10ml. Mix by pipetting up and down 3 times.

14. Put an aliquot of cell suspension on hemocytometer and count the cells on as described in Appendix I. Calculate the total cell counts and add complete RPMI medium to obtain the suspension of cells the concentration of 4×10^5 cells/ml for culture. Transfer cell suspensions into microplate or flask and incubate in CO_2 incubator at 37°C, 97% humidity, 0.35 % CO_2.

B. Cytotoxicity assay

The cytotoxic effect of water extract of *S.venosa* tuber on normal human lymphocytes was determined through the study of cell viability by the trypan blue dye exclusion assay. To begin the cytotoxic assay, an aliquot of 190 μl lymphocytes suspended in completed RPMI to a cell density of 4×10^5 cells/ml were pipetted into each well of a 96-well plate. Subsequently, 10 μl of the freshly prepared *S.venosa* from stock solution was add to give final concentration of 18.25, 37.5, 75, 150, 300 and 600 μg/ml. Aliquots of 10 μl of distilled water and 0.08 μM actinomycin D were added into separated the control and positive control well, respectively. The control, positive control and each concentration of *S.venosa* were triplicately performed. Then the plate was incubated in a CO_2 incubator at 37 °C, 97% humidity, 0.35%CO_2 for 48 h, the cell viability was evaluated by trypan blue dye exclusion test and the IC_{50} of *S.venosa* cytotoxicity was calculated.

C.Trypan blue dye exclusion test for cell viability

Trypan blue is one of several stains recommended to use in dye exclusion procedures for viable cell counting. The method is based on the principle that viable cells do not take up certain dye, where as dead cells do.

1. Take a clean hemocytometer slide and fix the coverslip in place.
2. Aliquot 50 μl of the suspended cell culture into an eppendorf test tube. Add 50 μl of 0.4 % trypan blue solution.
3. Leave the mixture for 1 minute. (Do not leave them for a longer period of time, or else viable cells will deteriorate and take up the stain.)

4. Load the counting chamber of the hemocytometer. Place the slide on the microscope. The number of unstained (viable) and stained (dead) cells were counted separately.

5. Calculate the percentage of cell viability and the concentration, which gave 50% inhibition of cell growth (IC_{50}), was calculated.

$$\% \text{ cell viability} = \frac{\text{total viability cells (unstained)}}{\text{total cells (stained and unstained)}} \times 100$$

D. Stability test of the water extract of *S.venosa*

To investigate the stability of the water extract of *S.venosa* tuber, a stock solution of the water extract of *S.venosa* tuber was prepared at a concentration of 0.02 g/ml. The stock solution was then aliquoted into 12 aliquots, and kept at $-20^{o}C$ for the study. To avoid the freeze -thaw effect which might deteriorate the active ingredient within the stock solution of *S.venosa*, each week, only one aliquot was taken out and thawed for the stability study. Two tests were used to evaluate the stability of *S.venosa* water extract stock solution. The first test was to measure the change in pH weekly for 12 weeks. The second test was a weekly evaluation of the cytotoxic effect of *S.venosa* on normal human lymphocytes using the trypan blue dye exclusion technique. The stock solution that had been thawed out was diluted and added into lymphocyte cultures in a 96-well plate to obtain the final concentration of 3, 10, 30, 100, 300 and 1000 μg/ml. After 48 hours incubation in CO_2 incubator, the cells were accessed for viability using trypan blue exclusion technique.

E. Apoptotic activity of the extract on normal human lymphocytes

From the cytotoxic study, the water extract of *S.venosa* exhibited a cytotoxic activity against normal lymphocytes with an IC_{50} of 300 μg/ml. To evaluate whether the cell death process was due to apoptosis, we used the ApopTag *In situ* apoptosis detection kit to confirm the death process, the kit detected apoptotic cells by direct fluorescence detection of the digoxigenin-labeled 3'-OH DNA ends generated by DNA fragmentation and typically localized in morphologically identifiable nuclei and apoptotic bodies.

The apoptosis detection kit contained 3 steps as described below:

- Cell preparation and treatment
- Harvest and fixing cells for ApopTag
- Slidemaking for detect apoptosis

- **Cell preparation and treatment**

1. The lymphocytes were prepared from whole blood taken from healthy volunteers using Histopaque-1077 as described in **A.**

2. Transfer 10 ml of 4 x 10^5 cells/ml cell suspension into the 25 ml T- tissue culture flasks.

3. The triplicates of lymphocyte culture flasks are divided into 5 groups as shown in the diagram of Figure 13

4. Incubate all the culture flasks in CO_2 incubator at 37°C, 97% humidity, 0.35 % CO_2, for 48 hour, then the cells are ready to be harvested.

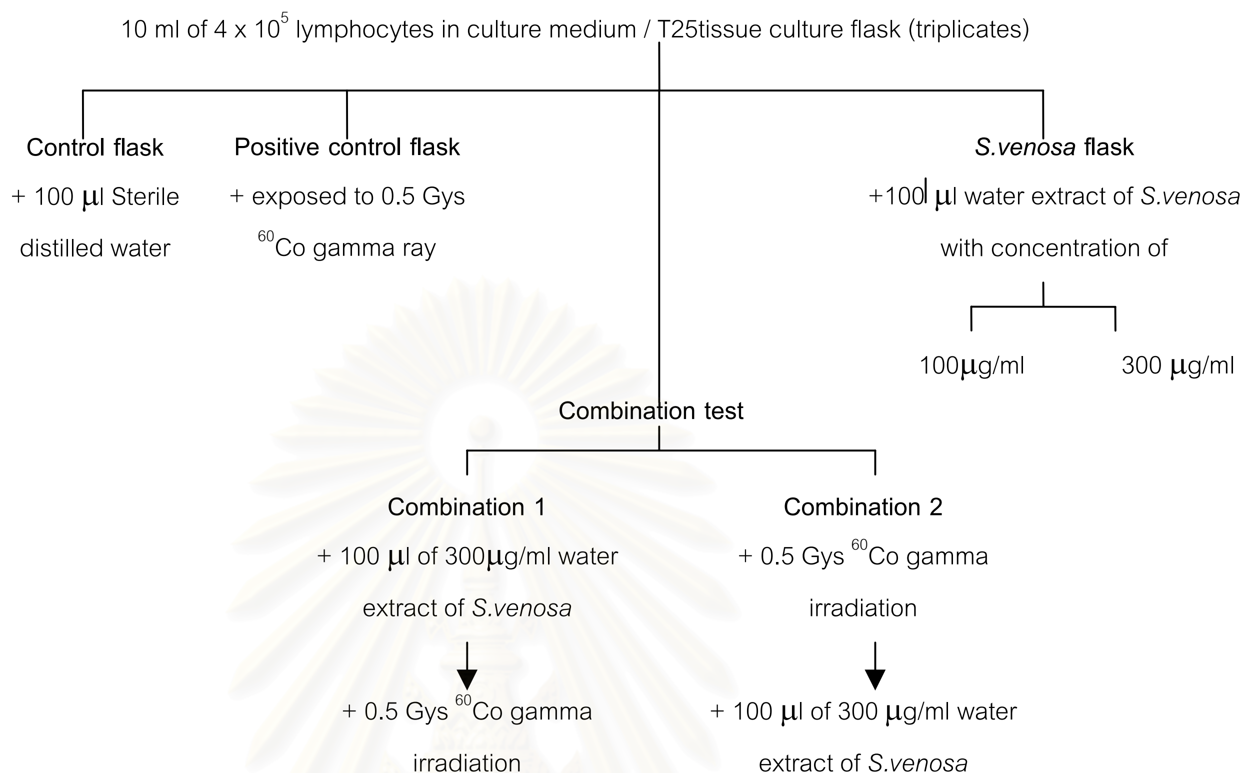


Figure 13. Diagram presentation designed for apoptosis study

- Harvest and fixing cells with ApopTag

1. Mix cells in the culture flask by pipetting up and down 3 times.
2. Transfer 3 ml of the 4 x 10^5 cells/ml into a new polyporpylene centrifuge tube. Centrifuge at 1,000 rpm for 10 minutes at 0° C.
3. Aspirate out the supernatant and flip to softly disperse the lymphocyte pellet.
4. Add 2 ml PBS at 0^oC to each tube. Mix cells by pipetting up and down 3 times. Centrifuge at 1,000 rpm for 10 minutes at 0° C.
5. Aspirate out the supernatant and flip to softly disperse the lymphocyte pellet.
6. Repeat step 4 to 5.

7. Resuspend the cells in 250 μl PBS /1% paraformaldehyde fixative. Let sit for at least 10 minutes at room temperature and proceed to the slidemaking protocol. For storing, add PBS/1% paraformaldehyde fixative to 10 ml volume and store in the refrigerator until use (within 2 weeks).

- Slidemaking for the detection of apoptosis

1. Clean microscope slides by rinsing with double distilled, deionized water and dry.
2. Centrifuge the paraformaldehyde fixed sample at 1,000 rpm for 10 minutes at room temperature.
3. Carefully aspirate the supernatant out without disturbing cell pellets. Flip to softly disperse the lymphocyte pellets.
4. Add 100 μl PBS/1 % Paraformaldehyde fixative to resuspend cells. Flip to softly disperse the lymphocyte pellets.
5. Drop 20 μl of the cells suspension onto a clean slide.
6. Allow the slides to air dry for 1 hour in a fume hood or laminar flow cabinet.
7. Fix the cells by placing the slides in absolute ethanol overnight at –20°C.
8. Transfer the slides from absolute ethanol. Allow the slides to air dry for 1 hour in a fume hood or laminar flow cabinet, and mark around the area of the specimens.
9. Wash the slides for 5 minutes in PBS at room temperature for 3 times.
10. Allow the slides to air dry, put one drop (13.5 μl) of Equilibration buffer (ApopTag Kit) to the area marked on the slides. Cover the marked area with plastic coverslip (Figure 14), and incubate the cells at room temperature for 5 minutes on bench top at room temperature.

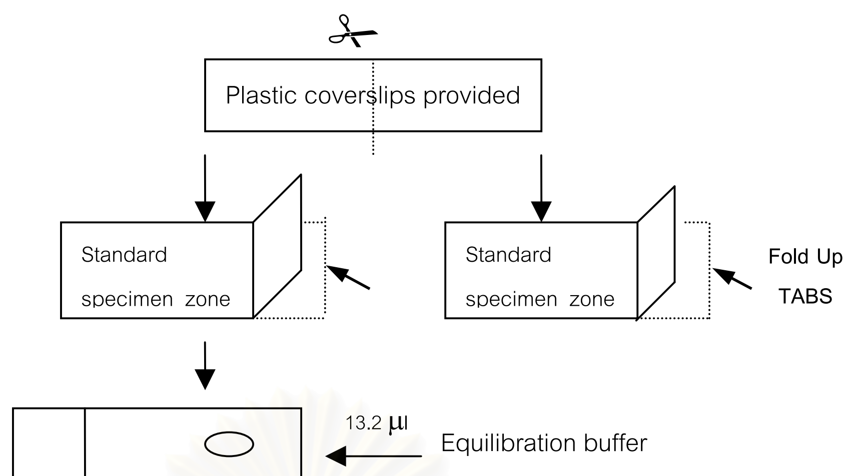


Figure 14. The slide making and the plastic coverslip.[54]

11. After incubation, carefully remove the coverslip and dry around the marker area with absorbent. (Note: The positive control slide was treated with DNase I, and incubated in CO_2 incubator 1 hour before going to step 12.)

12. Add 13.2 μl of TdT mixture prepared freshly or within 6 hours to the specimen area. Cover with a plastic coverslip and incubate in CO_2 incubator at 37°C, 97% humidity, 0.35% CO_2 for 1 hour. (Note: The negative control slide was created by omitting TdT enzyme.)

13. After incubation, carefully remove the coverslip and dry off around the marked area with absorbent.

14. Place the slides into Coplin staining jar containing a warm stop wash buffer; incubate at 37°C for 30 minutes.

15. Wash slides for 5 minutes in PBS at room temperature for 3 times.

16. Allow the slides to air dry in a fume hood or laminar flow cabinet.

17. Add 13.2 μl Fluorescein Isothio-Cyanate-Dextran (FITC) mixture prepared freshly or within 6 hours onto the specimen area. Cover with plastic coverslip; incubate 45 minutes in a humidified chamber place in a dark room at room temperature. (Note : Avoid exposure to light as much as possible)

18. After incubation, carefully remove the coverslip and dry off around the sample area. Wash the slides 3 times for 5 minutes in PBS in the dark room.

19. Rinse the slides 1 minute with Hoechst dry solution, and then rinse the slides in PBS for 1 minute in the dark room.

20. Allow the slides to air dry in a fume hood or laminar flow cabinet. Add 10 μl DAPI-Antifade solution to the specimen areas and cover with a standard glass coverslip and mount. Develop the slides in dark room for 10 minutes before viewing under fluorescence microscope. If storage of the slides is required, put some rubber cement to the edges of the coverslip and store at $-20^{o}C$ in a dark box.

21. The slides were viewed under a florescence microscope for monitor apoptotic cells. The lymphocyte cells were determined by counting the total lymphocytes 1,000 cells per slide, and counted the apoptotic cells in the same field under the blue filter. The apoptotic index of lymphocytes were calculated.

$$\text{Apoptotic index} = \frac{\text{Number of apoptotic cells}}{\text{Total number of cells}} \times 100$$

Apoptotic activity = Apoptotic index test – Apoptotic index control (distilled water)

F. Apoptotic activity of the extract on cervical cancer patient lymphocytes

From the study of apoptotic activity in normal human lymphocytes, the water extract of *S.venosa* presented apoptotic activity against normal lymphocytes, to evaluate whether the water extract of *S.venosa* induces apoptosis in cervical cancer patient lymphocytes; we used ApopTag *In situ* Apoptosis detection kit to confirm the death process as described in **E.**

G. Proliferation assay

The extract exhibited cytotoxic effect on normal human lymphocytes with the IC_{50} of 300 μg/ml; the high IC_{50} dosage was possibly due to the fact that the used lymphocytes were non-dividing mature cells. To simulate the condition mostly found in cancerous stage, phytohemagglutinin A (PHA) was applied to enhance cell division and cell proliferation before adding *S.venosa*. The antiproliferative effect of *S.venosa* was assayed by ^{3}H – thymidine incorporation methods which is described below.

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1. Normal human lymphocytes are suspended in complete RPMI medium to a density of 2.2×10^6 cells/ml.

2. Aliquots of 100 μl of the cell suspension are placed into each well of a 96 – well plate.

3. A 100 μl of 0.01mg/ml of Phytohemagglutinin (PHA) is added into each well and mixed.

4. Subsequently, 10 μl of the *S.venosa* water solution is added to final concentrations of 3, 10, 30, 100, 300 and 1000 μg/ml. An aliquot of 10 μl of medium is added to a control well. Each test is performed in triplicates. The plate is incubated for 48 h in a CO_2 incubator at 37°C, 97% humidity, 0.35 % CO_2.

5. After 42 hours of incubation 1 μCi of [^{3}H] thymidine is added to each well and incubated for 6 hours. Then, the cells are collected onto a glass fiber filter paper, using a multiharvester device, washed 10 times with the distilled water and dried.

6. The radioactivity retained on the glass fiber filter is transferred into the scintillant, and determine the radioactivity on a liquid scintillation counting. The mean of the counts from the triplicates for each sample is determined.

Statistical analysis

All data were presented as means and standard error of means (mean ± SEM). Repeated measurement test was used to compare the significance between the control and the treatment groups. The p- value of less than 0.05 was set for the significant difference.

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CHAPTER III

Results

1. Cytotoxic activity of the water extract of *Stephania venosa* tuber

The cytotoxic effects of *S.venosa* water extract against normal human lymphocytes were determined by incubating 190 μl of $4x10^5$ lymphocytes/ml with 10 μl of various concentrations (0,18.75,37.5, 75, 150, 300 and 600 μg/ml) of *S.venosa* for 48 hours. The percentages of cell death were determined by trypan blue dye exclusion test. The result showed that the water extract of *S.venosa* exhibited its cytotoxic activity in a dose dependent manner as presented in Figure 15. There was no significant difference in the number of cell death found in the solvent control (water) as compared to the blank control. The water extract of *S.venosa* at the concentration of 150 μg/ml gave the same percentage of cell death as the positive control. The positive control was human lymphocytes that were treated with 0.08 μM of Actinomycin D (Table 4).

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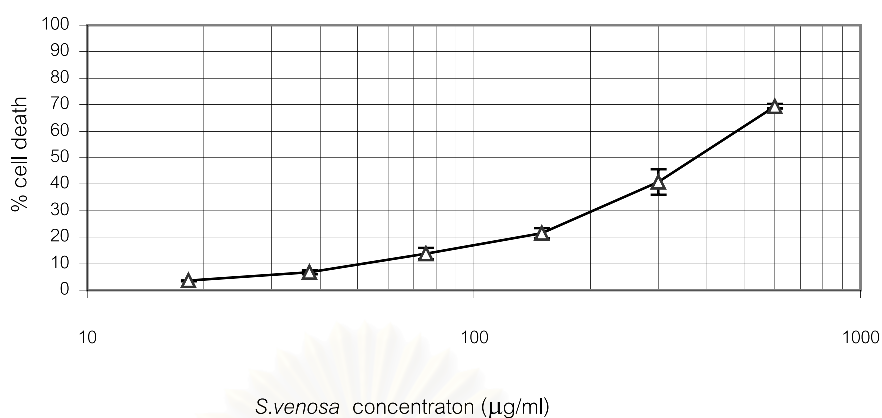

Figure 15. Trypan blue dye exclusion test showed the percentage of cell death (cytotoxicity) at 48 hours after the normal human lymphocytes were exposed to various concentration of *S.venosa.*

Table 4. Cytotoxic effect of *S.venosa* on normal human lymphocytes after exposed to various concentration of the water extract of *S.venosa* for 48 hours. The percentages of cell death were estimated by trypan blue dye exclusion test.

Test	% Cell death* Mean ± SEM (n=5)
Solvent control (distilled water)	0.99 ± 0.276
Actinomycin D 0.08 μg/ml(positive control)	22.51 ± 0.99
S.venosa (μg/ml)	
18.25	3.55 ± 0.181
37.5	6.75 ± 0.757
75	13.72 ± 2.161
150	21.45 ± 1.913
300	40.85 ± 4.745
600	69.39 ± 0.903

% Cell death of each condition after subtracted with the background (no treatment), which % cell death is 12.92 ± 0.365

2. **The stability study of the water extract of *S.venosa***

The stability of the stock solution of 20 mg/ml *S.venosa* water extract was studied with two tests: pH change and the change in cytotoxic activity for 12 weeks. The result showed that there was no significant change in the pH of *S.venosa* stock solution from 1st week through the 12th week as shown in Figure 16A. Furthermore, the various concentrations of *S.venosa* (0,3,10,30,100, 300 and 1,000 μg/ml) prepared weekly from the same stock solution gave similar cytotoxic activity throughout the 12 weeks of experimental period (Figure 16B). The cytotoxic activity detected by trypan blue dye exclusion is dose dependent with IC_{50} of 300 μg/ml, which is similar to a prior experiment with freshly prepared *S.venosa*. (Appendix c)

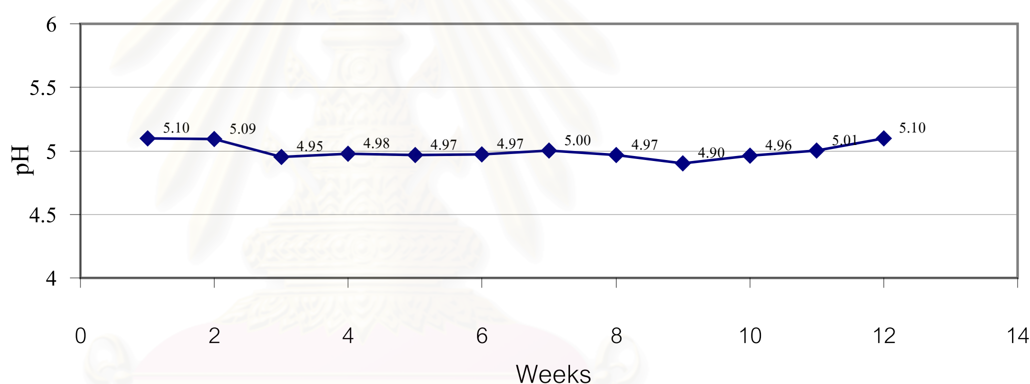

Figure 16A. The mean ± SE of pH change of *S.venosa* stock solution at the concentration of 20 mg/ml for 12 weeks.

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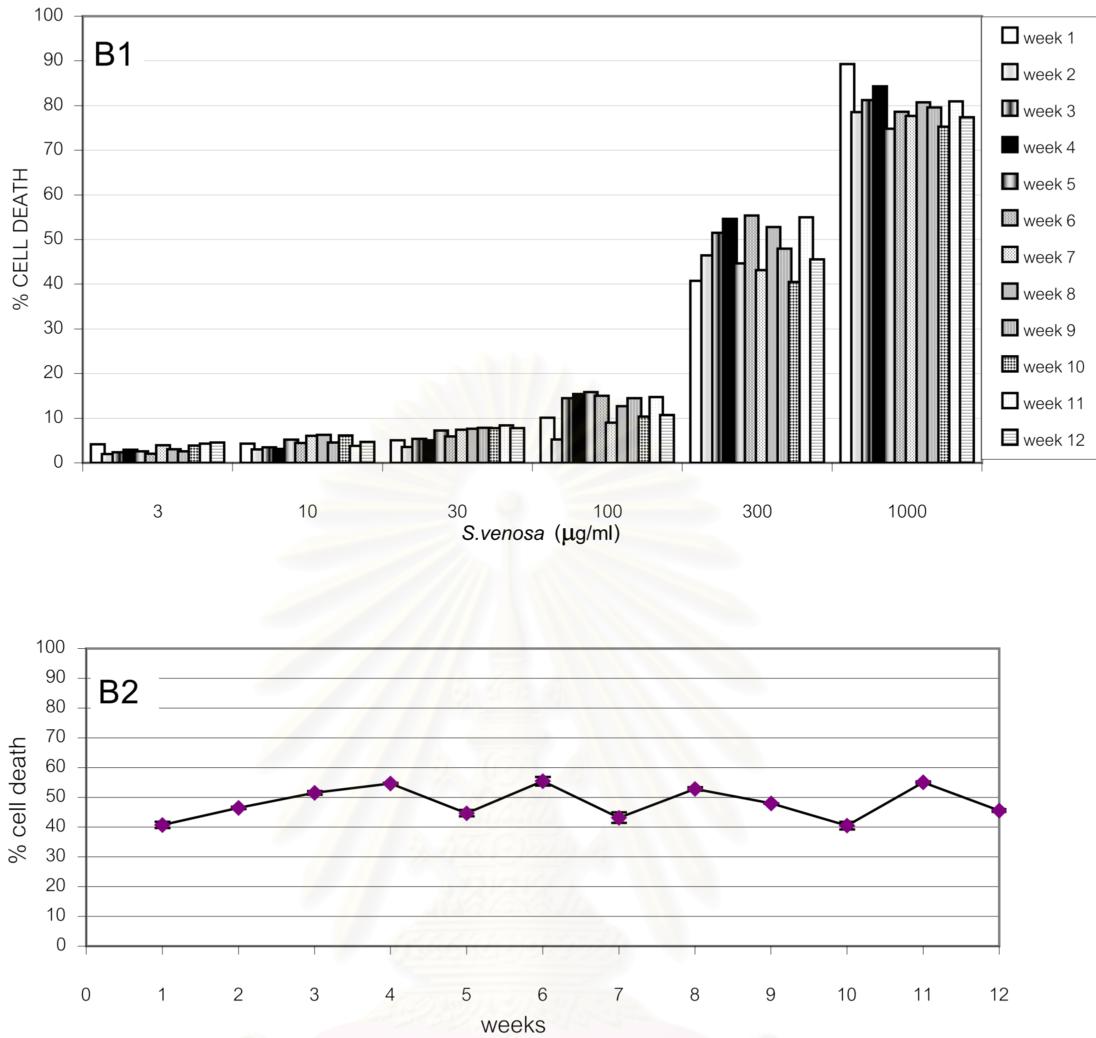

Figure 16B. Stability of the water extract of *S.venosa* stock solution at the concentration of 20 mg/ml for 12 weeks.

B1. The cytotoxic effects by trypan blue dye exclusion technique of various concentration of *S.venosa* water extract. (mean of triplicates)

B2. The means of the % cell death of lymphocyte after exposing to *S.venosa* stock solution of 300 µg/ml for 12 weeks.

3. Apoptotic activity of *S.venosa* on normal human lymphocytes

The extract was investigated for its apoptotic activity against human lymphocyte. The concentrations of the extract at 100 and 300 μg/ml were used in this study. Apoptotic cell were detected by ApopTag *In situ* Apoptosis detection kit (TdT assay) and viewed under a fluorescence microscope with 200-1000X of magnification. Without any filter, all the cells in the microscopic field gave blue fluorescent appearance due to Hoechst fluorescent DNA stain (Figure 17A). Then the same field was re-examined under blue filter, which served as a barrier filter to block the residual excitation light (approximately 400-490 nm). Only the green fluorescence light of FITC (emission light 520 nm), which stained DNA fragments of apoptotic cell was visualized (Figure 17B). A positive control slide (Figure 18A & B) was prepared by treating the lymphocytes with DNase I to yield DNA fragmentation. A negative control slide shown in Figure 19A & B was simultaneously prepared from the lymphocytes by omitting TdT enzyme from ApopTag slide-making protocol. There were no apoptotic light green cells visualized under blue filter (Figure 19B). Both the positive control and the negative control were included in each test. Figure 20 & 21 presented the apoptosis of normal human lymphocytes for 48 hours after being exposed to 100 μg/ml and 300 μg/ml of *S.venosa*, respectively. The results showed that *S.venosa* induced apoptosis in normal human lymphocytes in dose dependent manner. The apoptotic index of the solvent control (distilled water), 100 μg/ml and 300 μg/ml of *S.venosa* were 10.65 ± 0.714, 15.77 ± 0.996, and 31.61 ± 1.467 %, respectively. The results indicated that the percentage of apoptotic lymphocytes treated with 100 μg/ml *S.venosa* was lower than 300 μg/ml *S.venosa* group with statistical significance ($p < 0.05$). The apoptotic activity of *S.venosa* water extract at 300 μg/ml on cultured normal human lymphocytes was equivalent to that of low dose radiation of 0.5 Gy ^{60}Co gamma ray, apoptotic index of 29.33 ± 1.143 (Figure 21, 22 & 23). When the apoptotic index of normal human lymphocytes exposed to 300 μg/ml *S.venosa* was compared with those exposed to ^{60}Co irradiation, the data showed no significant difference ($p = 0.061$) as in Figure 23.

In Figure 24, the apoptotic effects of the combination of *S.venosa* and 0.5 Gy. ^{60}Co radiation on normal human lymphocytes were evaluated. There was no significant difference in either combination 1 (*S.venosa* prior to irradiation) or combination 2 (irradiation prior to *S.venosa*); Table 5. The comparisons between both combination groups and the 300 μg/ml *S.venosa* group showed significant increase in the percentage of apoptotic index as well as the comparison between both combination groups and the ^{60}Co irradiation groups (Figure 24; Table 5).

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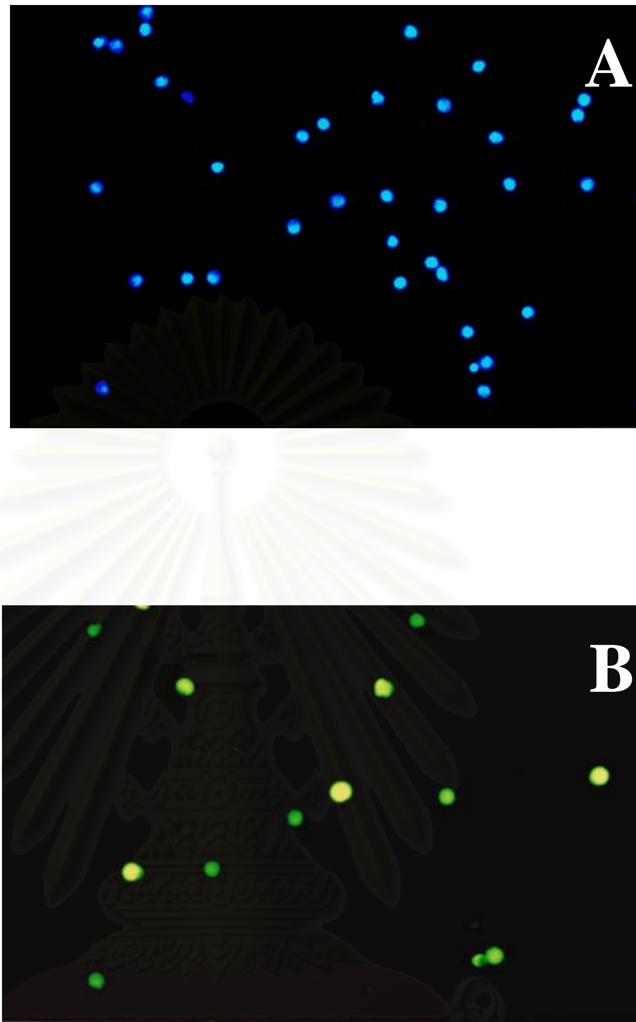

Figure 17. Pictures (A, B) present TdT assay of normal human lymphocytes to detect apoptosis cells in the control group.

A. Without blue filter, every cell in the field stained blue from Hoechst dye.

B. With blue filter, in the same microscopic field, only apoptotic stained light green with FITC appeared.

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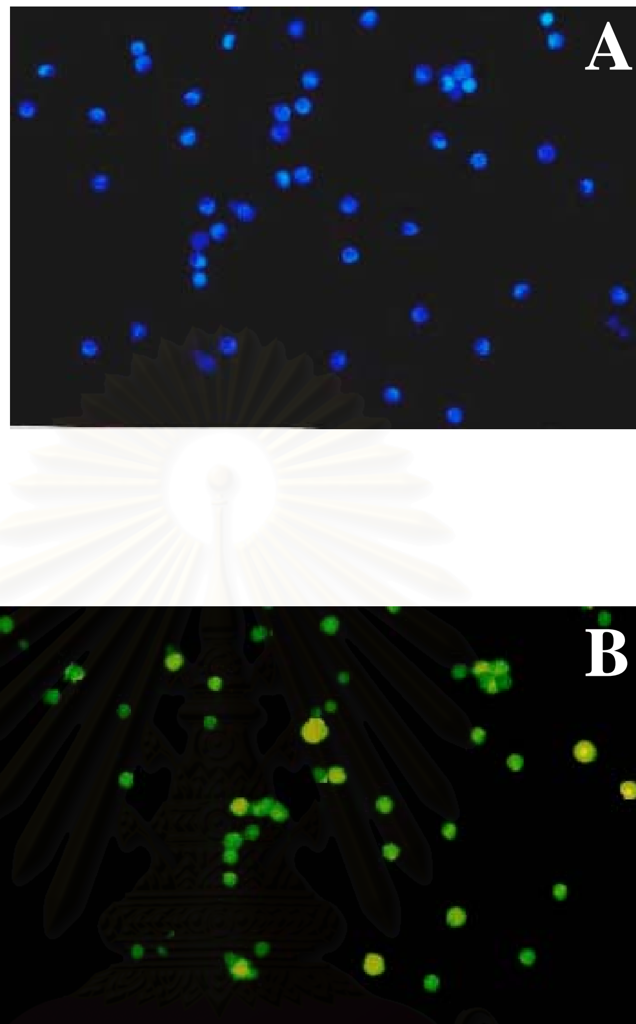

Figure 18. TdT assay of positive control (A, B), the positive control slide was prepared by treating normal human lymphocytes with DNase I to produce DNA fragmentation.

A. Without blue filter, the entire cells are seen as the blue cells from Hoechst staining.

B. With blue filter, in the same microscopic field, all lymphocytes having undergone apoptosis and stained light green with FITC.

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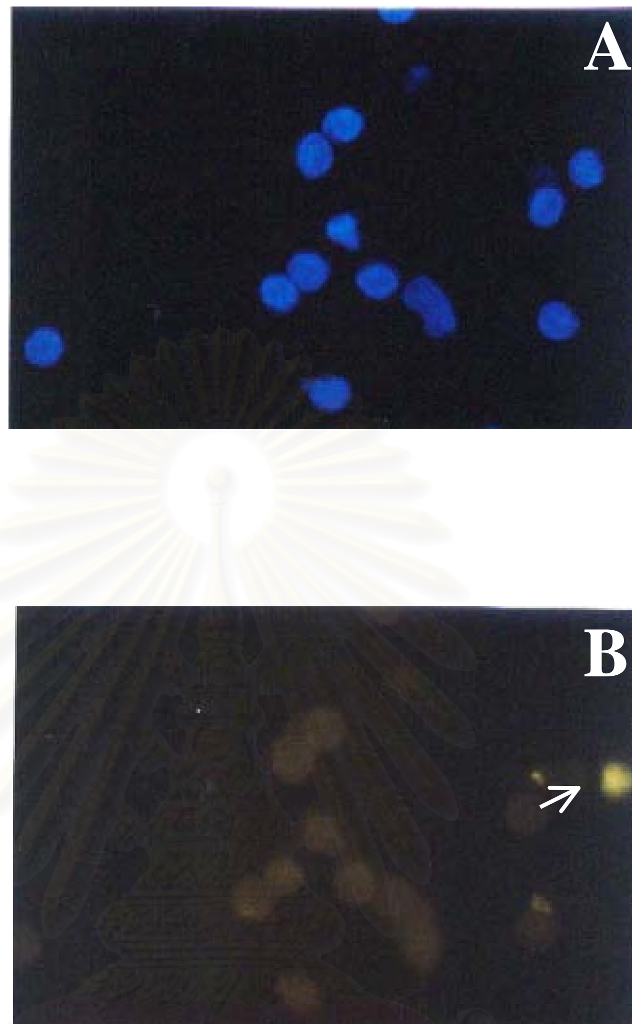

Figure 19. TdT assay of negative control (A, B). The negative control was obtained by omitting the TdT enzyme from the labeling mix in the ApopTag slidemarking protocol.

A. Without blue filter, the entire cells are seen as the blue cells from Hoechst staining.

B. With blue filter, in the same microscopic field, no apoptotic cell can be visualized. The arrow head indicates the residual of fluorescent dye.

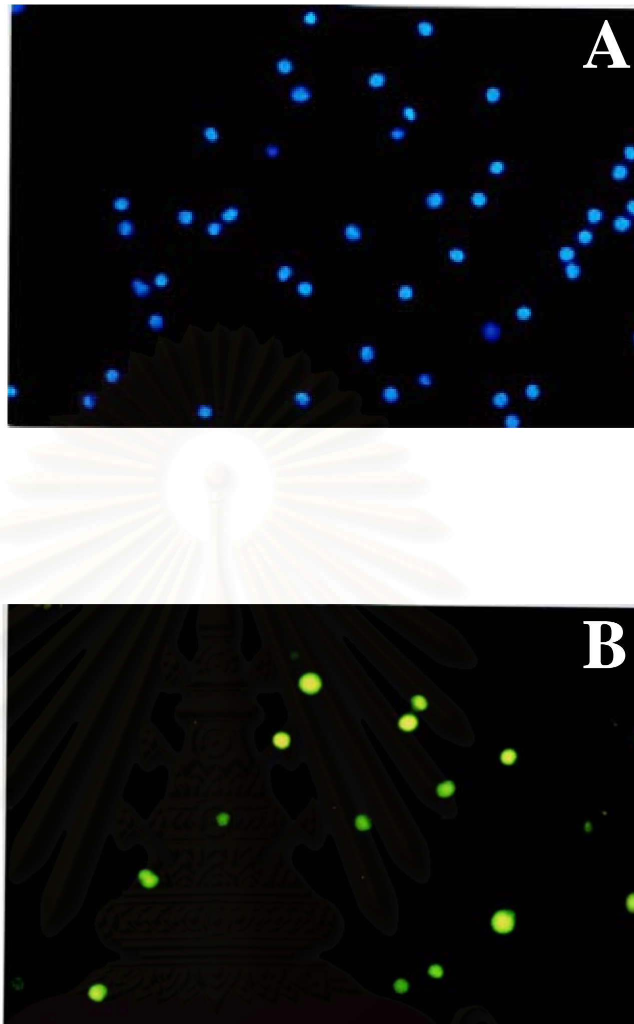

Figure 20. TdT assay of normal human lymphocyte (A, B) cultures at 48 hours after exposed to 100 μg/ml water extract of *S.venosa.*

A. Without blue filter, every cells in this field are seen as the blue cells from Hoechst staining.

B. With blue filter, in the same microscopic field, only those lymphocytes undergone apoptosis stained with light green fluorescent of FITC are visualized.

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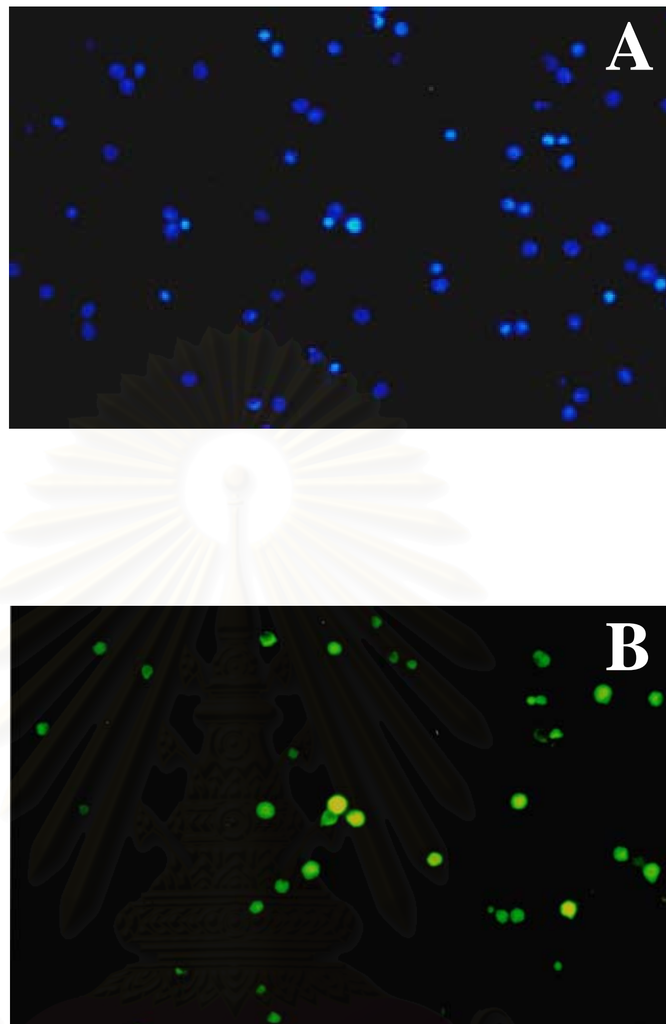

Figure 21. TdT assay of normal human lymphocyte (A, B) cultures at 48 hours after exposed to 300 μg/ml water extract of *S.venosa*.

A. Without blue filter, the entire cells in this field are seen as the blue cells from Hoechst staining.

B. With blue filter, in the same microscopic field, only those lymphocytes that have undergone -apoptosis, stained with light green fluorescent of FITC, are visualized.

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Figure 22. TdT assay of normal human lymphocyte (A, B) cultures at 48 hours after exposed to 0.5 Gy ^{60}Co radiation.

A. Without the blue filter, the entire cells in this field are seen as the blue cells from Hoechst staining.

B. With blue filter, in the same microscopic field, only those lymphocytes that having undergone –apoptosis, stained with light green fluorescent of FITC, are visualized.

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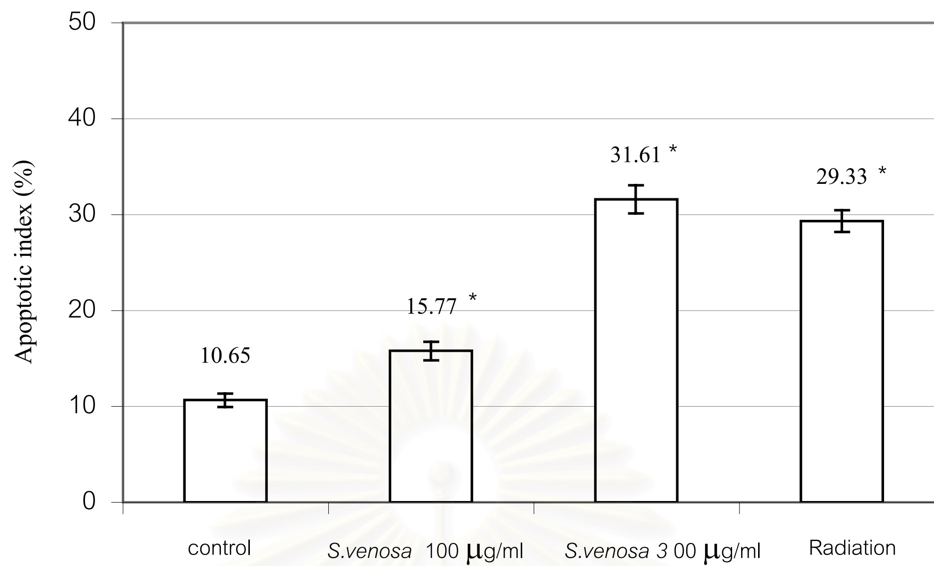

Figure 23. Comparison of the apoptotic index (mean ± SE) of normal human lymphocyte cultures for 48 hours among the control group (treated with distilled water) and the groups exposed to 100, 300 μg/ml of *S.venosa* water extract and 0.5 Gy ^{60}Co radiation. (n=10)

* Significance when compared to the control group at $p < 0.05$

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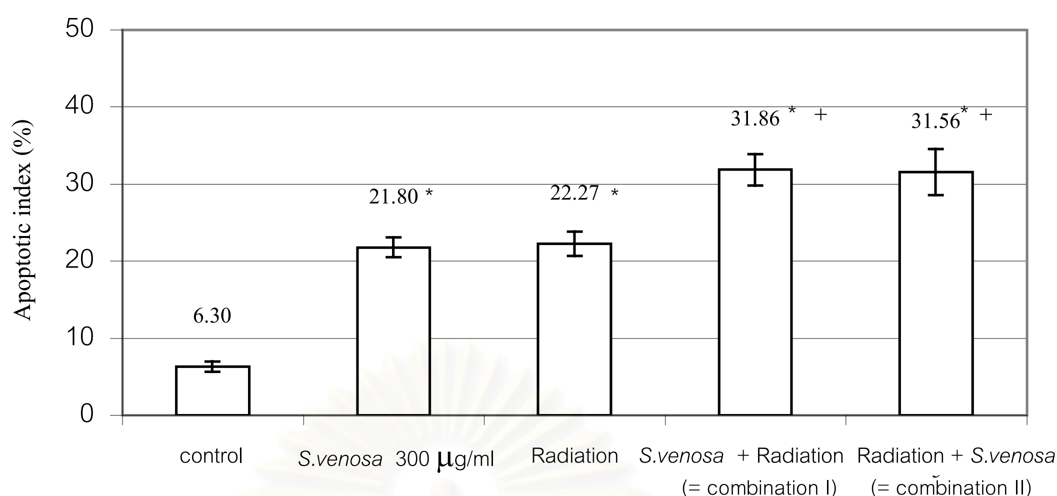

Figure 24. The apoptotic index (mean ± SE) of normal human lymphocyte cultures at 48 hours of: the control group (distilled water), the groups exposed to 300 μg/ml of *S.venosa* water extract and the group exposed to 0.5 Gy ^{60}Co iradiation, the Combination I group (300 μg/ml *S.venosa* prior to 0.5 Gy ^{60}Co irradiation), and the Combination II group (0.5 Gy ^{60}Co irradiation prior to 300 μg/ml *S.venosa*)

* Significance when compared to the control group at p< 0.05

+ Significance when compared to the *S.venosa* group or radiation groups alone at p< 0.05

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Table 5. Comparison of various treatments on the apoptotic index of normal human lymphocytes.

Comparison conditions	Apoptotic index (%) (Mean + SE)
300 µg/ml *S.venosa* : Control	21.80 + 1.292 : 6.30 + 0.373*
0.5 Gy ^{60}Co irradiation : Control	22.27 + 1.610 : 6.30 +0.373*
Combination I : Control	31.86 + 2.062 : 6.30 + 0.373*
Combination II : Control	31.56 + 3.004 : 6.30 + 0.373*
0.5 Gy ^{60}Co irradiation : 300 µg/ml *S.venosa*	22.27 + 1.610 : 21.80 + 1.292
Combination I : 300 µg/ml *S.venosa*	31.86 + 2.062 : 21.80 + 1.292*
Combination II : 300 µg/ml *S.venosa*	31.56 + 3.004 : 21.80 + 1.292*
Combination I : 0.5 Gy ^{60}Co irradiation	31.86 + 2.062 : 22.27 + 1.610*
Combination II : 0.5 Gy ^{60}Co irradiation	31.56 + 3.004 : 22.27 + 1.610*
Combination I : Combination II	31.86 + 2.062 : 31.56 + 3.004

* The mean difference is significant at p<0.05

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4. Apoptotic activity of the extract on lymphocytes from cervical cancer patients

The lymphocytes obtained from cervical cancer patients stage IIB & IIIB were evaluated by ApopTag *In situ* Apoptosis detection kit (TdT assay) after exposure to water extract of *S.venosa.* The apoptotic index shown as mean $\pm$ SE of the control (treated with distilled water), 300 μg/ml of *S.venosa* treated and the ^{60}Co irradiation group were 10.03$\pm$1.748, 28.61$\pm$3.922 and 29.18$\pm$2.998, respectively. Figure 25 presented the percentage of apoptotic index of lymphocytes obtained from cervical cancer patients after exposure to various treatments. The results showed that percentage of apoptotic index of the combination group (radiation + *S.venosa*) was 33.04$\pm$2.950 which was higher than the *S.venosa* treated or the ^{60}Co irradiation group without any statistical significances (p = 0.088 and 0.204, respectively). Also, there was no significant difference detected between the *S.venosa* group and the radiation group (p = 0.692) (Figure 11).

The apoptotic activity of the extract on lymphocytes from the cervical cancer patients was compared to its activity on the lymphocytes taken from matched female blood donors after various treatments for 48 hours as shown in Figure 26. The results demonstrated that the apoptotic activity of the extract on the lymphocytes from the cervical cancer patients in every treatment was not significantly different from that of normal human lymphocytes subjected to similar treatments (Figure 26).

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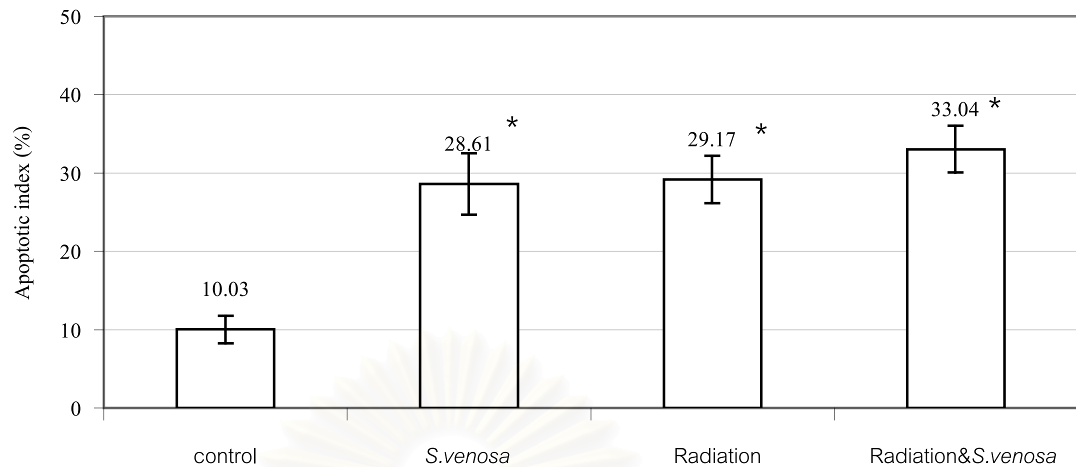

Figure 25. Apoptotic index (mean ± SE) of lymphocytes from cervical cancer patients stage IIB & IIIB (n=9) at 48 hours after exposed to control (distilled water), 300 μg/ml of *S.venosa*, 0.5 Gy ^{60}Co radiaton and the combination between radaition and *S.venosa*.

* Significance when compared to the control at $p < 0.05$

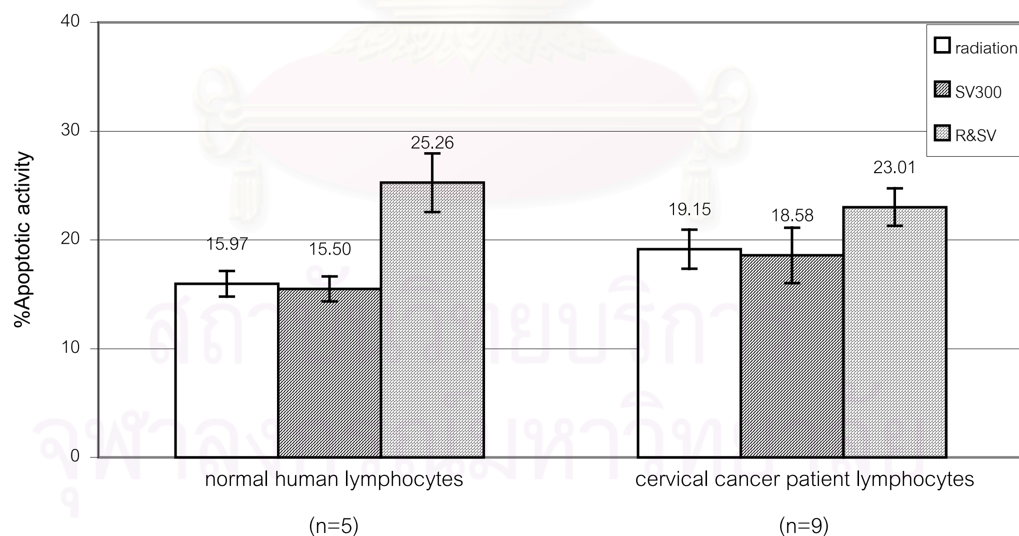

Figure 26. Apoptotic activity on the lymphocytes from normal healthy donors and cervical cancer patients (stage IIB & IIIB) at 48 hours after being exposed to low dose radiation (0.5 Gy ^{60}Co gamma ray), *S.venosa* 300 μg/ml and the combination between radiation and *S.venosa*.

5. Antiproliferative effect of the water extract of *S.venosa*

Normal human lymphocytes were stimulated with PHA before being treated with various concentrations of *S.venosa*. After 48 hours, the inhibition of cell proliferation was measured by [^{3}H] thymidine incorporation. The result in Figure 27 showed that [^{3}H] thymidine incorporation (c.p.m.) decreased as the concentrations of *S.venosa* increased. This indicated that water extract of *S.venosa* exhibited an inhibitory effect on cellular proliferation with IC_{50} at 40 μg/ml as shown in Figure 28.

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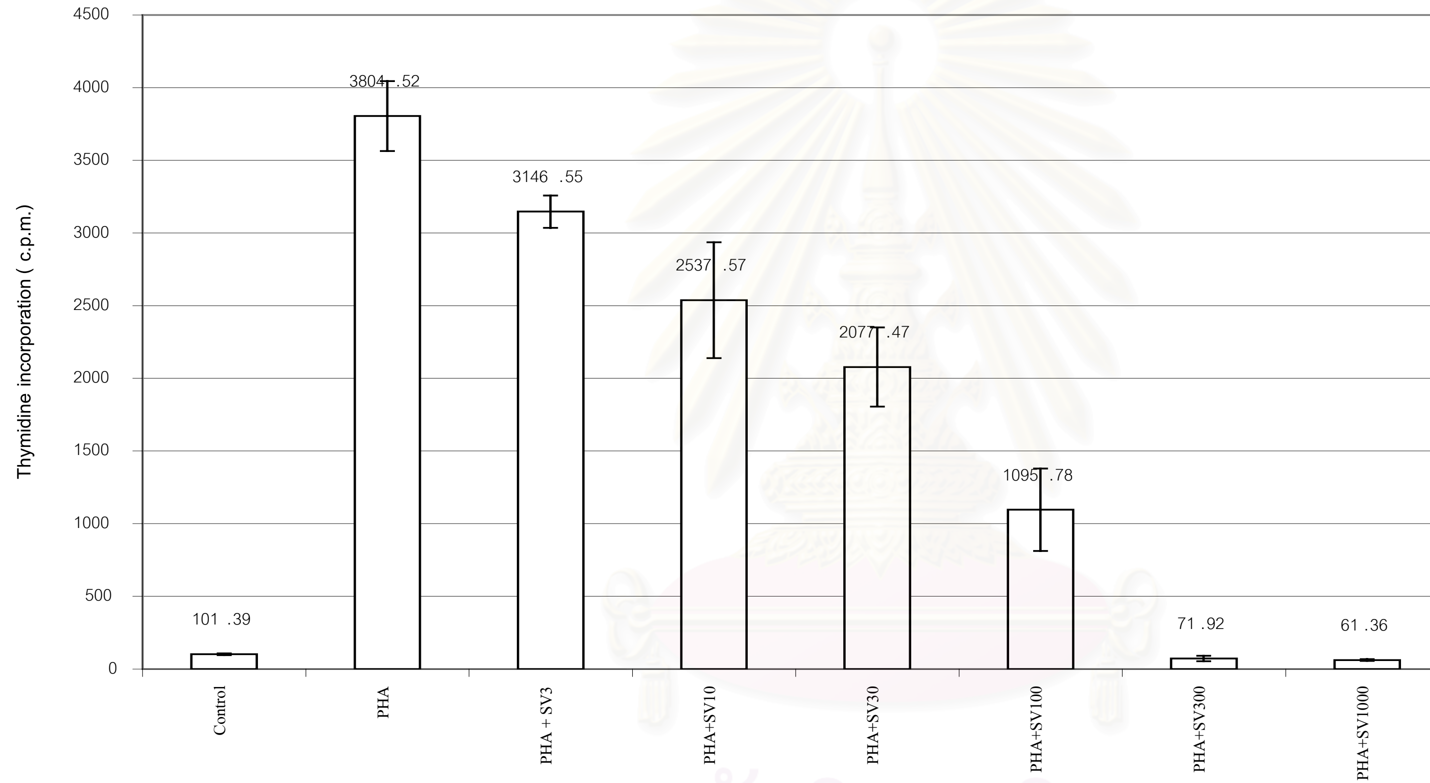

Figure 27. [^{3}H]thymidine incorporation (c.p.m.) in normal human lymphocytes after being stimulated with PHA and treated with various concentration of *S.venosa* (SV.; n=6). Control group was non -PHA stimulated lymphocytes and no SV exposure. The PHA group is PHA stimulated lymphocytes.

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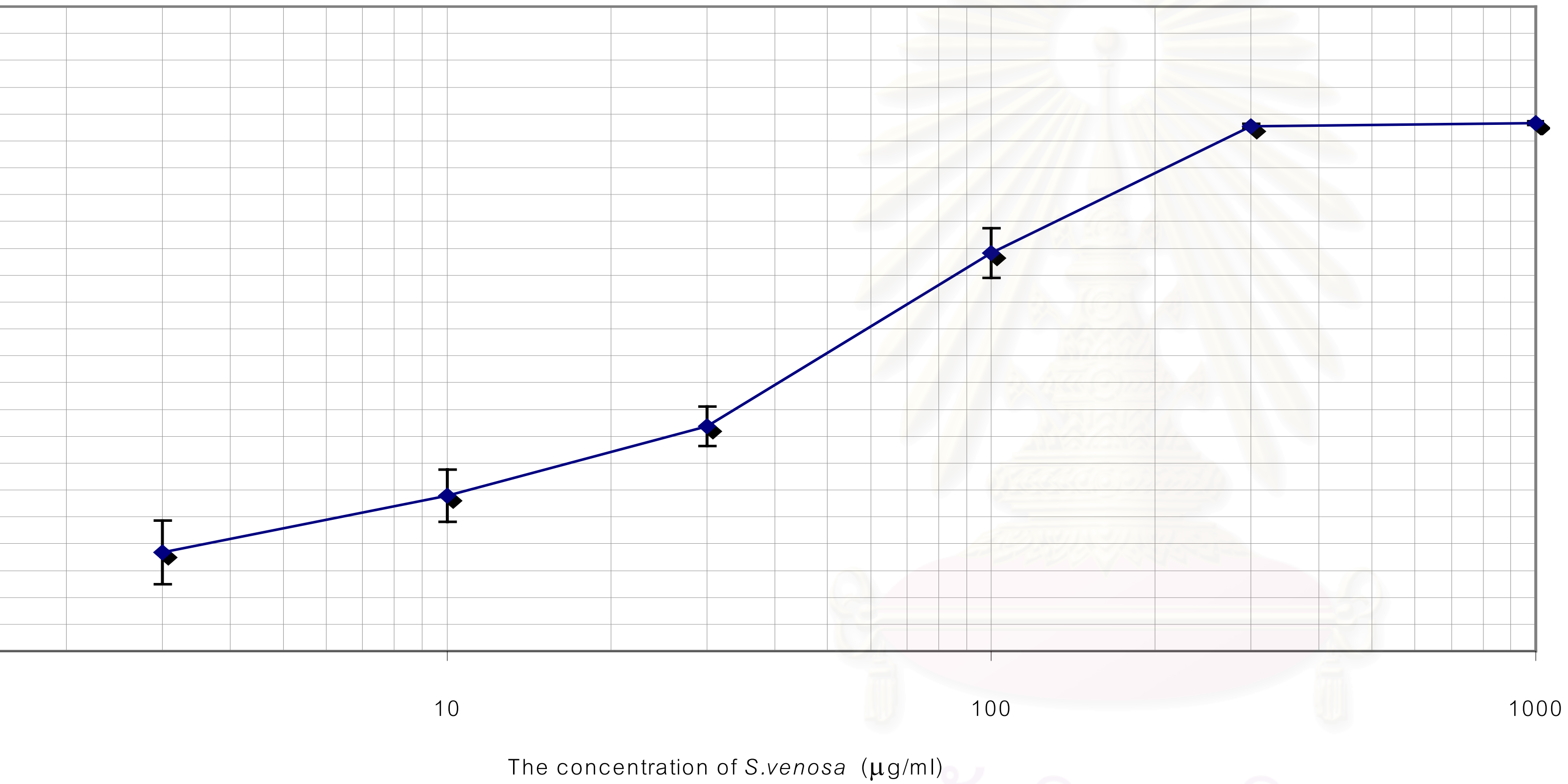

Figure 28. Proliferative inhibitory effect of *S.venosa* on normal human lymphocytes after being stimulated by PHA at 48 hours.

CHAPTER IV

DISCUSSION AND CONCLUSION

Herbal medicines have been used for thousands of years as a valuable and readily available resource for primary health care. Recently, herbal medicines are becoming a popular alternative medication for the remedy of various diseases including cancer. But only a few herbal medicines have withstood scientific testing. Most herbal medicines still need to be studied scientifically, although the experience obtained from their traditional use over the years should not be ignored. *Stephania venosa (S.venosa)* is a Thai herbal medicine which the decoction of its tuber has been used in the remedy of various disease including cancer. In this study, the anticancer effect of the water extract compound from *S.venosa* tuber was investigated using the model of human lymphocytes primary culture. The cytotoxic effect of the water extract of *S.venosa* against human lymphocytes was evaluated through apoptotic activity, an evolutionary conserved intracellular pathway distinct from necrosis. Apoptosis is essential in the homeostasis of normal tissue and the therapeutic target of anticancer research.

In the cytotoxic study, the normal human lymphocyte had shown a dose dependent response in which 300 μg/ml of water extract of *S.venosa* caused 50% cell death. It seems that the IC_{50} of the water extract of *S.venosa* is rather high in this study which may possibly due to both the cell type used and the nature of compounds found in the tuber of this plant. It is known that the tuber of *S.venosa* contains mostly the alkaloids, which are rather scantly water soluble. Whereas the *S.venosa* tuber contains various isoquinoline alkaloids namely: stephanine, crebanine, sukhodianine, dehydrocrebanine, tetrahydropalmatine, jatrorhizine and stephatine. These alkaloids were shown to possess certain degree of growth inhibition to *Plasmodium falciparum.*[69] Both the water and ethanol extracts from the tuber of *S.venosa* exhibited cytotoxic activity against the brine shrimps. The ethanol contained palmatine and crebanine which are the most active fraction from the tuber of *S.venosa* demonstrate variable degree of cytotoxic activity against MCF-7 (human breast adenocarcinoma cell line) and many

other cell lines with lesses toxic to kidney and hepatic cells.[78,83] The fraction also shows antituberculosis activity with a minimum inhibitory concentration of 200 μg/ml.[83]

Apoptosis is a discrete way of cell death different from necrotic cell death and is regarded as an ideal way to eliminate unwanted cells. A variety of anticancer drugs have been demonstrated to inhibit the growth of carcinoma cell by the induction of apoptotic cell death. Recently, many herbal medicines were shown to possess anticancer activity via their induction of apoptosis, namely: Green tea, *Viscum album* L. and *Lithospermum erythrohizon*.[101-103] The main purpose of this study is to elucidate the cytotoxic effect of the water extract of *S.venosa* via the apoptosis phenomenon.

This study has demonstrated for the first time that, the water extract of *S.venosa* can efficiently induce apoptosis in human lymphocytes, by demonstrating the presence of DNA fragmentation with ApopTag *In situ* Apoptosis detection kit (TdT assay). It also presents that the water extract of *S.venosa* causes cell death by either apoptosis or other means in a dose dependent manner as demonstrated by trypan blue dye exclusion test and the TdT assay. It has been shown that low dose radiation, 0.5 Gy ^{60}Co irradiation, induced a maximum apoptotic activity in normal human lymphocytes at 48 hours post exposure.[13,81-82] The 300 μg/ml of water extract of *S.venosa* (IC_{50} dose from the cytotoxicity test) is equivalent to the low dose radiation (0.5 Gy ^{60}Co gamma ray) for the induction of apoptosis activity in normal human lymphocytes as well as the lymphocytes from cervical cancer patients.

In the medical practice on cancer therapy, local tumor recurrence after radiation occurs if the radiation is not effective or the tumor cells are resistant to radiation. Using chemotherapy combined with radiation in this situation may be expected to improve local tumor outcome and therefore minimize cancer recurrence rates.[84] The data from this study by using the combination of 0.5 Gy ^{60}Co gamma ray either prior or after to the exposure of 300 μg/ml water extract of *S.venosa* has clearly suggested an additive effect on the induction of apoptotic activity in normal human lymphocytes. This may have significant implication on the cancer therapy in the further.

There has been report that, approximately 0.3$\pm$0.2% of the lymphocytes exhibit apoptotic change immediately after the isolation process.[13] Other reports have demonstrated that 8-10% of lymphocytes in the cell culture will undergo spontaneous

apoptosis.[81,85] These results agree with the present study, which evidences can be found in the control group. These spontaneous or background cell death from the control tube had been subtracted from total apoptotic index to yield actual apoptotic activity. The apoptotic activity of the extract on lymphocytes taken from cervical cancer patients (stage IIB & IIIB) was compared to its activity on female blood donors with closet age matched to the patients. The results demonstrated that the apoptotic activity of lymphocytes in the cervical cancer group (stage IIB & IIIB) was slightly higher than the apoptotic activity from the blood donor control group but without statistical significance. However, there is a high variability in apoptotic activity between persons for the radiation induced apoptosis of lymphocytes and that individual responses can change over a long time period or be modified by factor such as health status, environmental stress, and life style.[13] There still are many controversies on the changes of apoptotic activity occurred in the cancerous tissues and the lymphocytes or other peripheral blood hematocytes taken from the same cancer patient. Recently, it has been observed that peripheral blood mononuclear cells (PBMCs) obtained from patients with cancer, showed a variety of functional abnormalities, which may vary in magnitude from patient to patient and may be related to the extent of disease. The tumor microenvironment may influence the function potential of immune cells that accumulate at the site of tumor growth or metastasis. More recent data indicate that lymphocytes undergo apoptosis at the tumor site.[47,97] PBMCs obtained from the cancer patients has significantly higher proportion of apoptotic cells than PBMCs of normal control before incubation. Recent studies from several laboratories have demonstrated expression of Fas ligand (FasL) on human tumors, including colon, hepatocellular carcinoma, melanoma and lung carcinoma may be involved in induction of apoptosis in Fas-sensitive T cells. T cells coincubated with ovarian carcinoma, which also express function FasL are induced to apoptosis through Fas-medidiated and involves activation of caspase.[98] There is an evidence showed that, Hela cells (a human cervical carcinoma cell line) when co-cultured with peripheral blood lymphocytes (PBLs) acquired the capacity to inhibit PBLs proliferation in response to interleukin –2 (IL-2).[99]

The growth activity of most cancer cells, but not all are known to be much higher than normal (noncancerous) cells. Cancer cells are out of contact inhibition control, and

the antitumor agents generally show greater cytotoxic effect against actively divided cell. As a result, the hematopoietic cells, intestinal epithelial cell and the hair matrix keratinocytes which are the highly proliferative cell types are more susceptible to cytotoxic drugs than the less proliferated cells such as the muscle cells.[11] In the proliferation assay, the normal human lymphocytes were stimulated with phytohemagglutinin A (PHA) to imitate the cancerous condition. The result showed that the water extract of *S.venosa* inhibited the PHA- activated lymphocytes with an IC_{50} of 40 μg/ml, which was 7.5 times lower than the IC_{50} of 300 μg/ml of non-stimulated lymphocytes. However the difference of the technique used to quantitate the IC_{50} of the water extract of *S.venosa* also attributes to the variation in IC_{50} concentration. Alternatively, the anti-proliferative activity of the water extract of *S.venosa* may possibly due to its direct inhibitory effect on certain step in the apoptotic pathway. There are reports that, mitogens control the rate of cell division by acting to the cyclins/cyclin-dependent kinase (cdks) processes in the G_1 phase of the cell cycle and also binding to cell surface receptors to initiate a complex array of intracellular signals. The action of the cyclin/cdks is modulated by various negative regulatory forces-proteins that bind to the cdks and inhibit their action. These proteins are induced by various genes, e.g. *p53* and the retinoblastoma (*Rb*) gene. If there is DNA damage during the cell cycle process, these inhibitors normally halt the cycle at checkpoint, allowing for repair. If repair fails, apoptosis is initiated. However, many different promoters of cell proliferation have been found to possess proapoptotic activity, the tendency of cells to undergo apoptosis is a normal consequence of engaging the cell proliferative machinery – cell proliferative and apoptotic pathways are coupled.[38,99-100] There are studies demonstrated that, although the freshly isolated T cells are largely resistant to apoptosis, T cells that are stimulated via the T-cell receptors (TCRs) not only can proliferate but also undergo subsequent apoptosis by an active process termed activation induced cell death which play a role in the clonal deletion or in the induction of transplantation tolerance.[94] Studies on the diverse effects of IL-2 on T- cell apoptosis indicate that, IL-2 is generally causes activated T cell to become susceptible to apoptosis. But IL-2 can also rescue antigen specific T cells from radiation or dexamethasone- induced apoptosis via *bcl-2* induction. These diversified phenomena of the cited T cell can possibly use to elaborate the

activities found in the water extract of *S.venosa* as both pro- apoptotic and antiproliferative agent. Therefore, the data from this study which exhibited that the water extract of *S.venosa* possessed both pro-apoptotic and anti-proliferative activities is complied with the previously proposed efficacy of *S.venosa* which has been told among Thai folk medicine practitioners. However, further studies on the mechanisms of both activities of *S.venosa* need to be explored in more details.

CONCLUSION

The medicinal effects of almost all Thai folk medicinal plants are told from generations to generations like a legend with a concrete scientific basis. This study is designed to set a scientific *in vitro* cell culture model to test the anticancer effect of Thai folk medicinal plants. The water extract of *S.venosa* was first test for its stability via two measures namely: pH change of the $-20^{o}C$ storage aliquots and its cytotoxic effects on normal human lymphocytes viability for a period of 12 weeks. The IC_{50} at 300 μg/ml of *S.venosa* calculated from the quantitation of lymphocyte viability using the trypan blue dye exclusion was then used in the apoptosis studies.

Apoptosis can be considered as both a preventive and therapeutic measures in cancer therapy. The present study has applied the TdT assay using ApopTag® *in situ* hybridization kit to detect apoptosis in lymphocytes taken from female blood donors and cervical cancer patients. Comparison of apoptosis index or apoptosis activity between low dose radiation (0.5 Gy ^{60}Co gamma ray) induced apoptosis to *S.venosa* induced apoptosis was carried out. Combination of low dose radiation with *S.venosa* suggested that, an additive effect of apoptosis induction in normal human lymphocytes had occurred.

Anti- proliferation of the actively divided cancerous tissue can be another target for anticancer drug action. This study presented an inhibitory effect of the water extract of *S.venosa* in the PHA-activated blood lymphocytes with the IC_{50} at 40 μg/ml.

The overall results presented in this study have shown some scientific clues to confirm the legend of *S.venosa* as a potential anticancer Thai folk medicinal plant. Further in depth studies in cancer cell lines and animal models are strongly recommended.

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APPENDICES

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APPENDIX A

Cell counting by Hemocytometer

The concentration of a cell suspension can be determined by using a hemocytometer slide

Protocol

1. Clean the surface of the slide with 70% alcohol, taking care not to scratch the semisilvered surface. Clean the coverslip, and, wetting the edges very slightly, press it down over the grooves and semisilvered counting area (Figure 29).

2. Mix the cell suspension thoroughly pipetting to disperse any clumps.

3. Transfer the cell suspension immediately to the edge of the hemocytometer chamber, and let the suspension run out of the pipette and be drawn under the coverslip by capillarity. Do not overfill or underfill the chamber, or else its dimensions may change, due to alterations in the surface tension; the fluid should run only to the edges of the grooves.

4. Transfer the slide to the microscope stage.

5. Select a 10 x objective, and focus on the grid lines in the chamber (Figure 29). Move the slide so that the field is the central area of the grid and is the largest area that bounded by three parallel lines. This area is 1 mm^2.

6. Count the cells lying within this 1 mm^2 area, using the subdivisions (also bounded by three parallel lines) and single grid lines as an aid for counting. Count cells that lie on the top and left-hand lines of each square, but not those on the bottom or right-hand lines, in order to avoid counting the same cell twice.

7. Move to the second chamber and do a second count.

Analysis

Calculate the average of the two counts, and derive the concentration of sample using the formula

$$c = n/v$$

c = cell concentration (cells/ml)

n = number of cells counted

v = volume counted (ml)

For the Improved Neubauer slide, the depth of the chamber is 0.1 mm (Figure 29 a), and, assuming that only the central 1 mm^2 (Figure 29 c) is used, v is 0.1 mm^3, or 1×10^{-4} ml. The formula then becomes

$$c = n \times 10^4$$

Only the 5 diagonal squares within the central 1 mm^2 (Figure 29 d) were counted this equation becomes

$$c = n \times 5 \times 10^4$$

Reference

- Freshney RI. Culture of animal cells : a manual of basic technique. p309 4 th ed. New York: Wiley- liss; 2000.

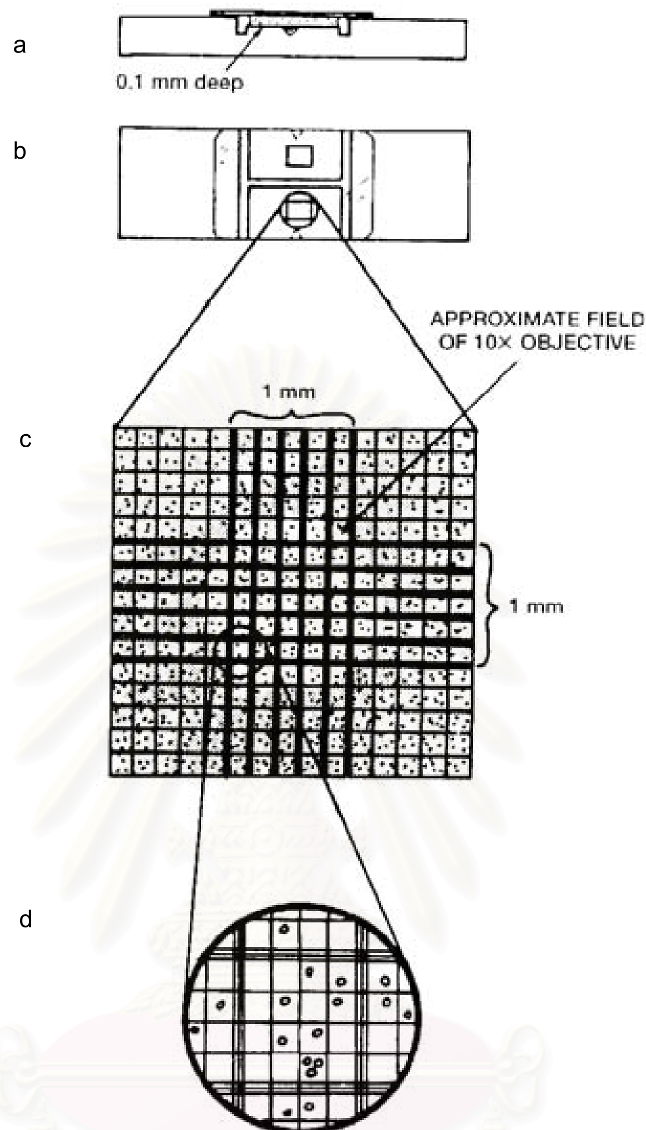

Figure 29. Hemocytometer slide. a) Longitudinal section of the slide, showing the position of the cell sample in a 0.1-mm-deep chamber. b) Top view of the slide. c) Magnified view of the total area of the grid. The light central area is that area which would be convered by the average 10 x objective. This area covers approximately the central 1 mm^2 of the grid. d) Magnified view of one of the 25 smaller squares, bounded by the triple parallel lines that make up the 1- mm^2 central area. This view is subdivided by single grid lines into the 16 smallest squares to aid counting.

APPENDIX B

Buffers and Reagents

1. RPMI 1640 stock solution 1 liter

RPMI powder	10.4 g
Na HCO_3	2 g
ddH_2O	900 ml

Adjust pH to 7.2 with 1 M HCl.

Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μ membrane filter.

2. HBSS stock solution 1 liter

HBSS powder	9.52 g
$NaHCO_3$	0.35 g
ddH_2O	900 ml

Adjust pH to 7.2 with 1 M HCl.

Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μ membrane filter.

3. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80 g
KCl	2 g
Na_2HPO_4	9.136 g
KH_2PO_4	2 g
ddH_2O	900 ml

Adjust pH to 7.4 with 1 M HCl.

Add ddH_2O to 1 liter and sterilized by autoclaving.

4. 1x Phosphate Buffer Saline (PBS) 1 liter

10x PBS	100 ml
ddH_2O	900 ml

Sterilized by autoclaving.

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5. 10% Buffered Formalin Acetate solution 1 liter

Paraformaldehyde powder	10 g
1 M NaOH	1 ml
Formaldehyde	40 ml
10Xpbs	100 ml
warm ddH_2O	800 ml

Adjust pH to 7.0 with 1 M HCl.

Add ddH_2O to 1 liter and sterilized by filtering through a 0.45 μm membrane filter.

6. PBS/ 1 % Paraformaldehyde fixative 1 liter

1XPBS	600 ml
10% Buffered Formalin Acetate	400 ml

Adjust pH to 7.1 and sterilized by filtering through a 0.45 μm membrane filter.

7. 200 mM L-Glutamine

L- Glutamine	29.22 g
ddH_2O	1000 ml

Sterilize by filtering through a 0.45 μm membrane filter.

Store at –20°C. (Do not heat; Since heat will destroy glutamine.)

8. Hoeschts 33258 Dye stock solution

Hoeschts dye	0.005 g
ddH_2O	50 ml

Store in polypropylene centrifuge tube at –20°C.

9. Hoeschts Dye solution for 8 slides

Hoeschts dye 33258 stock solution	180 μl
1Xpbs	45 ml

** freshly prepare before use.

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10. 2μl / ml Heparin in HBSS

HBSS stock	22.5 ml
Heparin	45 μl

11. Complete RPMI 1640 medium 69 ml.

RPMI stock	65 ml
L-Glutamine	338 μl
Gentamycin	16.25 μl
Fetal Bovine Serum	3.58 ml

12. HBSS/ 2 μL/ml Heparin / 1% Fetal Bovine Serum

HBSS	49.5 ml
Heparin (5000i.u./u.i./ml)	100 μl
Fetal Bovine Serum	3.58 ml

13. Stop Wash Buffer (Apoptag kit) 400 ml

Stop Wash Buffer	11.43 ml
ddH_2O	388.57 ml

Stored at 4°C

14. TdT mixture (for 8 slides)

Reaction Buffer	152 μl
TdT enzyme	64 μl

** prepare freshly before use

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15. FITC mixture (for 8 slides)

Blocking solution	112 μl
Anti-Digoxygenin	98.67 μl

** prepare freshly before use

APPENDIX C

The stability study of the water extract of *S.venosa*

Table 6. The mean of % cell death of normal human lymphocytes after exposed to various concentrations of *S.venosa* water extract for 12 weeks.

	control		positive control		*S.venosa* (μg/ml)											
	(distilled water)		(Actinomycin D)		3		10		30		100		300		1000	
weeks	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE	MEAN	SE
1	1.313	0.399	21.45	0.597	4.18	0.289	4.3	0.754	5.06	0.699	10.14	0.244	40.75	1.103	89.28	0.353
2	5.543	0.316	14.04	2.454	1.96	0.997	3.02	0.153	3.52	2.399	5.237	1.977	46.47	0.452	78.58	1.062
3	2.367	0.457	12.93	0.356	2.41	1.245	3.47	0.702	5.397	1.188	14.45	1.219	51.52	0.638	81.24	1.257
4	-0.12	0.415	15.93	1.063	2.943	0.188	3.163	0.615	5.057	0.43	15.46	3.139	54.66	0.234	84.3	0.367
5	2.71	0.372	10.56	0.689	2.627	1.018	5.19	0.639	7.227	1.724	15.83	2.068	44.68	1.075	74.83	0.734
6	3.533	0.989	28.61	0.692	2.08	0.323	4.42	0.106	5.917	0.704	15	0.677	55.41	1.457	78.61	0.939
7	4.827	1.235	17.88	0.817	3.96	1.977	6.077	0.771	7.457	0.704	9.01	0.188	43.15	1.763	77.67	0.119
8	3.477	1.985	15.42	0.328	3.06	1.527	6.263	0.771	7.67	0.255	12.72	1.028	52.8	0.609	80.76	0.537
9	1.897	0.807	11.72	0.375	2.623	0.469	4.573	1.294	7.84	0.714	14.48	0.862	47.95	0.072	79.59	0.246
10	2.583	0.102	13.39	1.702	3.913	0.785	6.157	0.3	7.8	0.741	10.39	0.797	40.49	1.347	75.28	1.135
11	0.533	0.632	14.71	1.362	4.317	0.869	3.85	0.826	8.38	0.53	14.76	0.224	55.01	0.337	80.94	0.26
12	3.717	0.375	14.57	0.426	4.57	0.362	4.723	0.711	7.77	1.257	10.71	1.998	45.59	0.428	77.4	1.284

% cell death of lymphocyte from each condition was substracted with its background (no treatment)

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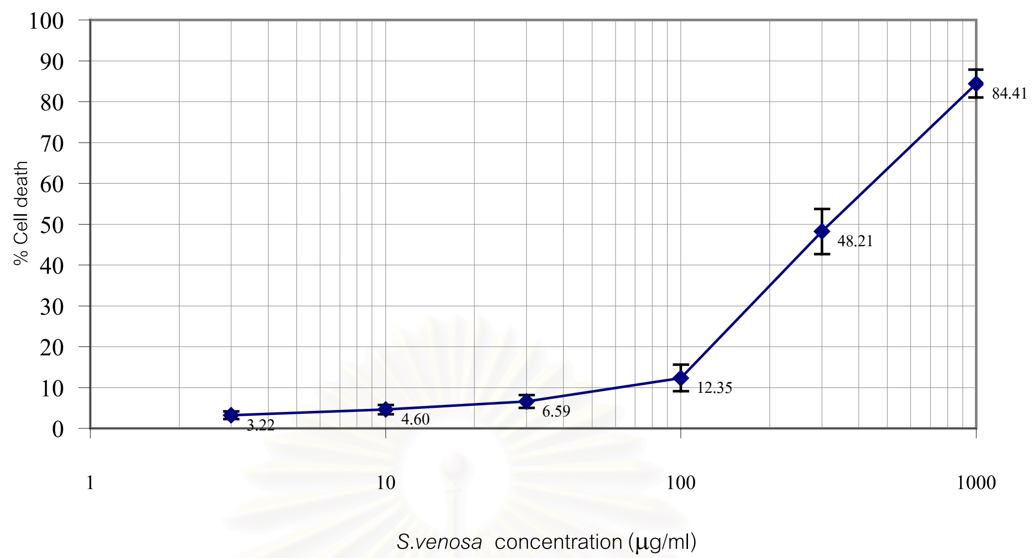

Figure 30. The cytotoxic activity on normal human lymphocytes after exposed to various concentrations of *S.venosa* water extract detected by trypan blue dye exclusion technique, a study period of 12 weeks.

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BIOGRAPHY

Miss Tipsuda Plumchai was born on September 14, 1979 in Trang provice, Thailand. She received her Bachelor degree of Science in Medical Technology in the year 2000 from the Faculty of Allied Health Science, Chulalongkorn University, Bangkok, Thailand. She has enrolled in a graduate program at Chulalongkorn University for the degree of Master of Science in Pharmacology and graduated in the year 2003.

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