CANCER CHEMOTHERAPY AND CHEMOPREVENTION OF *PHYLLANTHUS AMARUS, STEPHANIA VENOSA* EXTRACTS AND OVS1 MONOCLONAL ANTIBODY

SUNA JONGSOMBOONKUSOL

A THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF MASTER OF SCIENCE
(BIOPHARMACEUTICAL SCIENCES)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNIVERSITY
2005

ISBN 974-04-5702-9

Thesis
entitled

CANCER CHEMOTHERAPY AND CHEMOPREVENTION OF *PHYLLANTHUS AMARUS, STEPHANIA VENOSA* EXTRACTS AND OVS1 MONOCLONAL ANTIBODY

Suna Jongsomboonkusol
Miss Suna Jongsomboonkusol
Candidate

Primchanien Moongkarndi
Assoc.Prof. Primchanien Moongkarndi,
Dr.rer.nat.
Major-Advisor

Omboon Luanratana
Assoc.Prof. Omboon Luanratana,
Ph.D.
Co-Advisor

Rassmidara Hoonsawat
Assoc.Prof. Rassmidara Hoonsawat,
Ph.D.
Dean
Faculty of Graduate Studies

Primchanien Moongkarndi
Assoc.Prof. Primchanien Moongkarndi,
Dr.rer.nat.
Chair
Master of Science Programme
in Biopharmaceutical Sciences
Faculty of Pharmacy

Thesis
entitled

CANCER CHEMOTHERAPY AND CHEMOPREVENTION OF *PHYLLANTHUS AMARUS, STEPHANIA VENOSA* EXTRACTS AND OVS1 MONOCLONAL ANTIBODY

was submitted to the Faculty of Graduate Studies, Mahidol University
for the degree of Master of Science (Biopharmaceutical Sciences)
on
April 7, 2005

Suna Jongsomboonkusol.
.....
Miss Suna Jongsomboonkusol
Candidate

Primchanien Moongkarndi
.....
Assoc.Prof. Primchanien Moongkarndi, Dr.rer.nat.
Chair

Omboon Luanratana
.....
Assoc.Prof. Omboon Luanratana, Ph.D.
Member

Kovit Pattanapanyasat
.....
Prof. Kovit Pattanapanyasat, Ph.D.
Member

Neelobol Neungton
.....
Prof. Neelobol Neungton, M.D.
Member

Rassmidara Hoonsawat
.....
Assoc.Prof. Rassmidara Hoonsawat, Ph.D.
Dean
Faculty of Graduate Studies
Mahidol University

Ampol Mitrevej
.....
Prof. Ampol Mitrevej, Ph.D.
Dean
Faculty of Pharmacy
Mahidol University

ACKNOWLEDGEMENT

I would like to express my sincere gratitude and deep appreciation to Assoc. Prof. Dr. Primchanien Moongkarndi my Principal Supervisor for her guidance, support, invaluable advice, supervision, encouragement throughout and intensive supervisor throughout the laboratory works and the preparation of this thesis. She was never lacking in kindness.

I am equally grateful to Prof. Kovit Pattanapanyasat, Prof. Neelobol Neungton and Assoc. Prof. Omboon Luanratana for their guidance, constructive comments, assistance, supervision and encouragement. They were always nice and friendly.

I would like to thanks Mr. Sarawut Junnu for his great assistance, solving technical problems and advise about the monoclonal antibody purification. I would like to thank Mr. Narongchai Pongpan for his advice and support on medicinal plant extraction. I also would like to thank Mrs. Songjun Puthong for her assistance, advice and support on cell culture. I wish to thank Miss Surada Lerdwana for her guidance and assistance on flow cytometry.

I am particularly indebted to Mr. Amal Naj, President of Pfizer (Thailand) foundation and staff of Pfizer (Thailand) foundation for the scholarship which enabled me to undertake this study.

Special thanks to all my friends and staff in Faculty of Pharmacy, Mahidol University for their encouragement and wonderful friendship.

Finally, I wish to express my infinite gratitude to my mom and my family for their love, assistance, understanding and encouragement.

Suna Jongsomboonkusol

CANCER CHEMOTHERAPY AND CHEMOPREVENTION OF *PHYLLANTHUS AMARUS, STEPHANIA VENOSA* EXTRACTS AND OVS1 MONOCLONAL ANTIBODY

SUNA JONGSOMBOONKUSOL 4337552 PYBS/M

M.Sc. (BIOPHARMACEUTICAL SCIENCES)

THESIS ADVISORS : PRIMCHANIEN MOONGKARNDI, Dr.rer.nat. (Immunology), OMBOON LUANRATANA, Ph.D. (Phytochemistry)

ABSTRACT

Phyllanthus amarus, Stephania venosa extracts and OVS1 monoclonal antibody (MAb) were studied on antiproliferation, apoptosis, cell cycle analysis and antioxidation against SKOV3 human ovarian cancer cells and SKBR3 human breast cancer cells.

Cancer-chemotherapeutic activities of *P. amarus, S. venosa* extracts and OVS1 MAb were demonstrated to have a significant effect on antiproliferation against both cancer cells in a dose-dependent manner by MTT assay. The Ethanol extract from *P. amarus* against SKOV3 and SKBR3 expressed ED_{50} at 13.62 and 10.44 μg/ml respectively. The ED_{50} of Ethanol, Acetone, Ether, Petroleum ether, Ethyl acetate, Silica gel Fractions: 12-17, 18-19 and 20-23 from *S. venosa* on: SKOV3 cells were 35.11, 3.91, 6.67, 7.03, 4.38, 3.01, 1.95 and 3.13 μg/ml respectively; and on SKBR3 cells were 39.67, 5.24, 4.07, 6.42, 4.90, 4.19, 2.80 and 7.03 μg/ml respectively. The ED_{50} of OVS1 MAb against SKOV3 and SKBR3 were 6.71, 7.91 μg/ml respectively. All extracts and OVS1 MAb could clearly expressed apoptosis in cancer cells by determination on morphological changes with Ho33342 and propidium iodide staining, DNA fragmentation, DNA damage by comet assay and phosphatidylserine translocation by Annexin V-FITC assay. The effect of cell cycle analysis by flow cytometry from our study suggested that the extracts from *P. amarus* and *S. venosa* may arrest cancer cells at G2/M phase in a time- and dose-dependent manner. We proposed that this phenomenon could be clearly demonstrated by using a higher concentration of extracts.

Roles of extracts on chemoprevention as antioxidation which would prevent oxidative damage considered to be an important factor on carcinogenesis. All extracts studies, exhibited significant reduction in reactive oxygen species (ROS) and partly in reactive nitrogen species (RNS) with the system tested on both cancer cells.

This study provided the information on *P. amarus* and *S. venosa* which showed significant potential in cancer chemotherapy and chemoprevention. Identification of certain mechanisms of action of these extracts and the purified constituents were proposed for future study. OVS1 MAb showing high sensitivity and specificity on ovarian and breast cancer cells in this study could be considered as a transporter delivering active substances from plants to the target cells. This would be an alternative aspect for scientists to combat cancers.

KEY WORDS: CHEMOTHERAPY / CHEMOPREVENTION / MONOCLONAL ANTIBODY / *PHYLLANTHUS AMARUS* / *STEPHANIA VENOSA* / ANTIPROLIFERATION / APOPTOSIS / ANTIOXIDATION/ CELL CYCLE ANALYSIS

140 P. ISBN 974-04-5702-9

ฤทธิ์ในการต้านและป้องกันมะเร็งของสารสกัดสมุนไพรลูกใต้ใบ, สบู่เลือด และ OVS1 โมโนโคลนอล แอนติบอดี (CANCER CHEMOTHERAPY AND CHEMOPREVENTION OF *PHYLLANTHUS AMARUS*, *STEPHANIA VENOSA* EXTRACTS AND OVS1 MONOCLONAL ANTIBODY)

สุนา จงสมบูรณ์กุศล 4337552 PYBS/M

วท.ม. (เภสัชศาสตร์ชีวภาพ)

คณะกรรมการควบคุมวิทยานิพนธ์ : ปริ่มเฉนียน มุ่งการดี, Dr.rer.nat. (Immunology),
อ้อมบุญ ล้วนรัตน์, Ph.D. (Phytochemistry)

บทคัดย่อ

การศึกษาสารสกัดจากลูกใต้ใบ, สบู่เลือด และ OVS1 โมโนโคลนอล แอนติบอดี เพื่อหาฤทธิ์ต้านการเพิ่มจำนวน, การทำให้เกิด apoptosis, ผลกระทบต่อวงจรชีวิตของเซลล์มะเร็ง และการต้านอนุมูลอิสระที่มีผลต่อเซลล์มะเร็งรังไข่ SKOV3 และเซลล์มะเร็งเต้านม SKBR3

สารสกัดทั้งหมดของลูกใต้ใบ, สบู่เลือด และ OVS1 MAb มีฤทธิ์ต้านการเพิ่มจำนวนของเซลล์มะเร็งทั้งสองชนิดอย่างมีนัยสำคัญ ซึ่งความแรงขึ้นกับความเข้มข้นของสารทดสอบด้วยวิธี MTT assay ฤทธิ์ของสารสกัดเอทานอลจากลูกใต้ใบต่อ SKOV3 และ SKBR3 มีค่า ED_{50} 13.62 และ 10.44 มคก/มล ตามลำดับ ส่วน ED_{50} ของสารสกัดเอทานอล, อะซิโตน, อีเทอร์, ปิโตรเลียมอีเทอร์, เอทิลอะซีเตต, สารสกัดจากซิลิกาเจลคอลัมน์ส่วนที่ 12-17, 18-19 และ 20-23 จากสบู่เลือดมีฤทธิ์ต่อ SKOV3 คือ 35.11, 3.91, 6.67, 7.03, 4.38, 3.01, 1.95 และ 3.13 มคก/มล ตามลำดับ และต่อ SKBR3 คือ 39.67, 5.24, 4.07, 6.42, 4.90, 4.19, 2.80 และ 7.03 มคก/มล ตามลำดับ และ ED_{50} ของ OVS1 MAb ต่อ SKOV3 และ SKBR3 คือ 6.71 และ 7.91 มคก/มล ตามลำดับ นอกจากนี้สารสกัดทั้งหมด และ OVS1 MAb สามารถกระตุ้นเซลล์ให้เกิดการตายแบบ apoptosis อย่างชัดเจน โดยพบการเปลี่ยนแปลงทางสัณฐานวิทยาของเซลล์เมื่อย้อมด้วย Ho33342 และ propidium iodide, พบสาย DNA เกิดการแตกหัก, มีการทำลาย DNA ตรวจโดยวิธี comet assay และพบการเปลี่ยนแปลง phosphatidylserine ของเซลล์เมมเบรนด้วย Annexin V-FITC ผลต่อวงจรชีวิตของเซลล์ตรวจด้วย flow cytometry พบว่า สารสกัดจากลูกใต้ใบ และสบู่เลือดอาจมีฤทธิ์ยับยั้งเซลล์มะเร็งที่ช่วง G2/M ซึ่งขึ้นกับเวลาและความเข้มข้นของสาร เพื่อเป็นการยืนยันผลการทดลอง ควรทำการทดลองซ้ำโดยการใช้สารสกัดที่มีความเข้มข้นที่สูงในการทดลอง

บทบาทของสารสกัดในการเป็นสารป้องกันมะเร็ง คือการต้านอนุมูลอิสระของออกซิเจนซึ่งเป็นปัจจัยสำคัญในการก่อมะเร็ง สารสกัดที่ทำการทดลองทั้งหมดแสดงฤทธิ์ต้านอนุมูลอิสระของออกซิเจน และ อนุมูลอิสระของไนโตรเจนได้ในการทดลองกับเซลล์มะเร็งทั้งสองชนิด

การศึกษานี้ได้แสดงผลการทดลองของสารสกัดลูกใต้ใบ และสบู่เลือด ซึ่งมีฤทธิ์ที่ดีในการทำลายและป้องกันมะเร็งอย่างมีนัยสำคัญ ซึ่งควรศึกษาเพิ่มเติมในด้านกลไกการออกฤทธิ์ และควรมีการแยกสารบริสุทธิ์จากสารสกัดเหล่านี้โดยใช้ร่วมกับ OVS1 โมโนโคลนอล แอนติบอดี ซึ่งเป็นสารที่มีไว และความจำเพาะสูงต่อมะเร็งรังไข่ และมะเร็งเต้านม โดยการใช้ OVS1 โมโนโคลนอลเป็นตัวพาสารบริสุทธิ์จากสมุนไพรที่สกัดได้ไปออกฤทธิ์ยังเซลล์เป้าหมาย ซึ่งอาจเป็นทางเลือกของนักวิทยาศาสตร์ในการต่อต้านมะเร็งในอนาคต.

140 หน้า ISBN 974-04-5702-9

LIST OF CONTENTS

LIST OF CONTENTS (continued)

Page

LIST OF CONTENTS (continued)

Page

LIST OF CONTENTS (continued)

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
RPMI	Rosewell Park Memorial Institute
FCS	fetal calf serum
MAb	monoclonal antibody
IgG	gamma immunoglobulin
PBS	phosphate-buffered saline
DMSO	dimethylsulfoxide
ELISA	Enzyme-linked immunosorbent assay
BSA	bovine serum albumin
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TEMED	*N,N′,N″,N‴*-tetramethylethylenediamine
Tris	Tris (hydroxylmethyl) aminomethane
MTT	[3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]
HBSS	Hanks' balanced salt solution
ED_{50}	the 50% reduction of absorbency in MTT assay
Ho33342	bisbenzimide dye Hoechst No. 33342
PI	propidium iodide
DNA	deoxyribonucleic acid
EDTA	ethylene diamine tetra acetic acid
bp	base pair
RNase A	ribonuclease A
TAE	Tris-acetate-EDTA buffer
FITC	fluorescein isothiocyanate
FACS	Fluorescence Activated Cell Sorter
ROS	reative oxygen species
DCFH-DA	(2′,7′-dichlorodihydro fluorescein diacetate)

LIST OF ABBREVIATIONS (continued)

NO	nitric oxide
H_2O_2	hydrogen peroxide
CO_2	carbon dioxide
^oC	degree Celsius
O.D.	optical density
%	percentage
rpm	revolution per minute
h	hour
min	minute
l	liter
ml	milliliter
µl	microliter
g	gram
mg	milligram
µg	microgram
µm	micrometer
nm	nanometer
et al.	et alli (Latin), and other people
e.g.	for example
i.e.	id est (Latin), which is to say, in other words
etc.	et ectera (Latin), other things

CHAPTER I
INTRODUCTION

Cancer is the third common leading cause of death in Thailand and death from cancer still increasing each year (1,2,3). The present drugs to use in cancer therapy are directly against all rapid proliferative cells which affect not only cancer cells, but also to normal cells. Therefore, the medicinal plants for treatment cancer patients have been searching for the alternative drugs or substances that will participate with standard methods, i.e., surgery, chemotherapy, radiation, biological products. The administrations of monoclonal antibodies (MAb) are a specific immunotherapy and become one of advance technology in cancer therapy (4,5,6). MAb is used to eliminate tumor cells and also are served as a vehicle to transport the cytotoxic agents specifically to target tumor cells expressing the tumor associated antigen (7).

Neungton et al. (8) reported the production of OVS1 MAb by fusing murine myeloma cell line NS1/1-Ag4-1 with spleen cells from mice immunized with fresh mucinous cystadenocarcinoma which was a prevalent type of human ovarian cancer in Thailand (9,10,11). Furthermore, Kaslungla (12) found that the combination of a chemotherapeutic drug, paclitaxel, and OVS1 MAb can increase the occurring of apoptosis and the antiproliferative effect on ovarian and breast cancer cells comparing with either OVS1 MAb or paclitaxel was given alone. Kosem (13) showed that OVS1 MAb can enhance antiproliferative and apoptotic activities when combined with extracts from *Garcinia mangostana* or *Curcuma longa* on ovarian and breast cancer cells comparing with either OVS1 MAb or plant extracts were given alone.

The therapeutic utilization of plants is part of universal human culture. Nowadays, phytotherapy is considered as an alternative to reduce the side effect on the indiscriminate use of synthetic drug. Moreover, the advancement in biochemical and pharmacological studies of plant-derived drugs have opened a chance for new drug development. These include extracts of plants as well as plant-derived products (14).

In our preliminary screening on antiproliferative activities of many Thai medicinal plant extracts against SKOV3 and SKBR3 cancer cells, i.e., Ethanol extract from *Heliotropium indicum* (ED_{50} 138.53 and >200 μg/ml, respectively), Ethanol extract from *Angle marmelos* (>200 and >200 μg/ml), Ethanol extract from *Morinda citrifolia* (>200 and >200 μg/ml), Ethanol extract from *Thunbergia laurifolia* (112.42 and >200 μg/ml), Ethanol extract from *Phyllanthus amarus* (13.62 and 10.44 μg/ml), and Ethanol extract from *Stephania venosa* (35.11 and 39.67 μg/ml). We determined the ED_{50} of cytotoxicity method and we found that *P. amarus* and *S. venosa* expressed strong antiproliferative property (<30 μg/ml) (13). Therefore, this study aimed to evaluate the biological and pharmacological activities of *P. amarus* and *S. venosa* for chemotherapeutic and chemopreventive properties on ovarian and breast cancer cells. However, we emphasized to investigate on *S. venosa* due to only a little number of authentic scientific studies is available.

Phyllanthus amarus Schum. and Thonn. was classified in Euphorbiaceae. *Phyllanthus* sp. has long been used as traditional medicine for the treatment of various diseases, i.e., diuretics, jaundice and liver diseases (15). In recent studies, *P. amarus* was reported that possessed an anti-viral activity (16), inhibited DNA polymerase of hepatitis B virus and other hepatitis viruses (16). Aqueous extract of *P. amarus* exhibited potent anticarcinogenic property, and inhibited cell cycle enzyme *cdc 25* tyrosine phosphatase (16,17).

Stephania venosa Spreng. in Menispermaceae (18) is a vine tropical plants that rhizomes have been traditionally used as health tonic and relief fever (19). It has known in Thailand under the name of sabu-le-ad or blood soap due to its red latex has a lot of isoquinoline alkaloids (19). In recent study, *S. venosa* was found that showed antimalarial activity (20) and antiproliferation on MCF-7 breast adenocarcinoma cell line (19).

The antiproliferative activity was investigated with cytotoxic MTT assay which measured the level of MTT reduction in mitochondria. Determination of MTT method is very useful for assaying cell survival and proliferation, which only a living cell can reduce MTT and produce blue formazan crystal that is detected by spectrophotometer. This method offers major advantages in speed, simplicity, cost, safety and conventional assay (21,22,23).

Apoptosis is essential for the appropriate development and function of multicellular organisms. Apoptosis can activate intracellular through a genetically defined developmental program or extracellular by endogenous protein such as cytokines, hormones, radiation, oxidative stress, and toxic insult especially by agents that damage DNA (2,24). The most of cancer chemotherapeutic drugs induce cancer cell to death by apoptotic pathway. Thus, this pathway can indicate the influence of chemotherapeutic activities of cancer drug or plant extracts (25,26,27,28). Various techniques had proposed to identify apoptosis by medicinal plants from *P. amrus* and *S. venosa* against human ovarian cancer SKOV3 and human breast cancer SKBR3:

1. Morphological changes of cells were observed by Ho33342 and PI staining which depended on the different property of dye against cancer cells (29,30).

2. DNA laddering was one of the late character events of apoptosis. The ladder products could visualize after ran on agarose gel electrophoresis as discontined DNA bands (laddering) and were a hallmark of apoptosis.

3. The DNA appearance as comet was measured to evaluate the dose effect of medicinal plant compounds. The staining intensity and length of the comet tails reflected the level of DNA damage (31,32).

4. Phosphatidylserine externalization was used to investigate the early apoptotic cells. Phosphatidylserine had a high affinity with Annexin V-FITC and can measure by flow cytometry and used to identify early apoptotic cells (33).

The study of DNA content of cell cycle was detected by DNA staining for to search chemotherapeutic activity. The DNA degradation depended on the stage of apoptosis, cell types and the nature of the apoptosis-inducing agent. DNA content was recorded by flow cytometry to express cell cycle phases which presented the life time of cell and also showed the effects of cancer drugs in each phase of cancer cells. Therefore, we can determine and understand the kinetic of drugs or plant extracts on cancer cells (34,35).

Recently, chemoprevention is introduced and rapidly interested in field of oncology. Chemoprevention is the prevention of cancer by using pharmacological agents to inhibit or reverse the process of carcinogenesis (36). Natural or synthetic compounds, such as vitamin A and derivatives of vitamin A, vitamin C, vitamin E, calcium and selenium were used as cancer chemopreventive agents (37,38). These

compounds can prevent, block, inhibit or reverse the process of invasive cancer. Chemoprevention focuses on intervening in the processes in the cascade of carcinogenic events to prevent the final progression to neoplastic diseases, unlike chemotherapy which concentrates on containing or eradicating cells that have already undergone malignant transformation. While chemotherapy is targeted at people with manifest disease, chemoprevention is directed individuals who are apparently well, although those in high-risk groups have existing premalignant conditions (39,40).

Antioxidation of cells after treating with medicinal plant extracts would be evaluated by reactive oxygen species (ROS) assay and nitric oxide ($^{\bullet}$NO) assay. Chemopreventive activity of ROS are measured by detection with DCFH-DA, 2',7'-dichlorodihydro fluorescein diacetate, a fluorescent probe was used to estimate intracellular ROS level by luminescence spectrophotometer (41,42).

Nitric oxide is an intra- and intercellular important bioregulatory mediator that involved in many processes in cardiovascular, nervous and immune system. The Griess reaction provides simple method to determine the cellular production of $^{\bullet}$NO by measuring its stable product, nitrite, in the cell culture supernatant which uses sulfanilamide and N-1-napthylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions (43,44,45).

The crude extract of 80% Ethanol from *P. amarus* and eight extracts from *S. venosa*, i.e., 80% Ethanol extract, the extracts from solubility property method: Acetone, Ether, Petroleum ether, Ethyl acetate, and the extract fractions from silica gel column: Fraction 12-17, 18-19 and 20-23. All extracts and OVS1 MAb would be tested and evaluated on the chemotherapeutic and chemopreventive activities against SKOV3 and SKBR3 cancer cells. The study was divided into three parts. The first part related chemotherapeutic activities of these extracts and OVS1 MAb on both cancer cells. Cytotoxicity and apoptosis assay would be investigated by various methods. The second part contained cell cycle analysis or DNA content assay by flow cytometry. The third part concerned chemopreventive activities of these extracts on both cancer cells by measure the inhibition of intracellular reactive oxygen species (ROS) and reactive nitrogen species (RNS). The results of all studies would be summarized and evaluated the plant extracts for their potentials in application and future aspects of study to bring these plants as alternative treatment or new drugs for cancer patients.

CHAPTER II
LITERATURE REVIEW

1. The knowledge of cancer

The transition of a normal somatic cell to a cancer cell is generally the results of many genetic changes, involving activation of oncogenes or inactivation of tumor-suppressor genes. These changes allow the cell to escape normal control mechanisms in cell proliferation, differentiation, migration and death which collectively maintain the normal cellular architecture and functions in an organized tissue. The frequency of somatic mutations leading to cancer in human beings is dictated largely by chemicals in the cellular microenvironment and to a small extent by heritable genetic predisposition.

Cancer is the uncontrolled growth and spread of cells that may affect almost any tissue of the body. Lung, colorectal and stomach cancer are among the five most common cancers in the world for both men and women. Among men, lung and stomach cancer are the most common cancers worldwide. For women, the most common cancers are breast and cervical cancer (46). More than 10 million people are diagnosed with cancer every year. It is estimated that there will be 15 million new cases every year by 2020. Cancer causes 6 million deaths every year or 12% of deaths worldwide (46,47).

Although there are many kinds of cancer, the prevalent Thai female cancer is ovary (the seventh rank) and breast (the second rank) cancers from Siriraj Cancer Center Report, 2003 (Table 1) (1). In the same year, Ramathibodi Cancer Registry reported ovary is the sixth rank and breast cancer is the first rank (Table 2) (48). Ovray cancer occurs during age 45-60 and the most patients come to diagnoses in the late stage of disease. Breast cancer also the most case incidences during age 40-55 (1,48,49). Cancer can occur in all ages and many studies found the relation between age and cancer, i.e., leukemias and lymphoma in children or cancer and gender, i.e., the most case in male is lung cancer whereas the case in female is cervix cancer (49).

Table 1 The common malignancy in Thai female from Siriraj Cancer Center (1).

Sites	Population	Percentage
All sites	2727	100.00
Cervix	618	22.66
Breast	545	19.99
Colon and rectum	164	6.01
Lymphoma	151	5.54
Leukaemia	148	5.43
Ovary	111	4.07
Corpus	92	3.37
Lung	91	3.34
Thyroid	83	3.04
Skin	74	2.71

Table 2 The leading sites of cancer in female from Ramathibodi Cancer Registry (2).

Sites	Population	Percentage
Breast	375	24.69
Cervix uteri	208	13.69
Leukaemia	83	5.46
Bronchus&lung	83	5.46
Colon	72	4.74
Brain	66	4.35
Ovary	66	4.35
Lymph nodes	62	4.08
Corpus uteri	54	3.55
Thyroid gland	49	3.23
Skin	46	3.03
Liver	37	2.44

1.1 Ovarian cancer

Ovarian cancer occurs during age 45 to 60. The 90% of ovarian cancers are adenocarcinomas arising from the epithelial cells covering the ovary. Ovarian epithelial carcinomas may be subclassified by cell type to include serous, mucinous, endometroid, mesonephroid and clear cell. Epithelial ovarian carcinoma is arising from the germinal epithelium of the ovary. Other malignancies of the ovary include those of germ cell origin, i.e., dysgerminomas, yolk sac carcinomas, teratomas and choriocarcinomas, stromal and sex cord tumor, mesenchymal tumors of the ovary and cancer metastatic to the ovary, in particular from the gastrointestinal tract and breast or from other ovarian cell types, such as granulosa, theca and Sertoli-Leydig cells (50). There are non-specific symptoms of ovarian cancer. Some of the symptom of ovarian cancer such as: stomach swelling or bloating or stomach discomfort, anxiety, loss of appetite and not feeling well, going to the bathroom a lot and losing or gaining weight without trying (51).

1.1.1 Epidemiology of ovarian cancer

The incidence of epithelial ovarian cancer begins to highly in the fifth decade of life and increases until the eighth decade. The average age to diagnosis is approximately 53 years. The factor of ovarian cancer appears that is hormonal factors which are important in cancer development. The associated with a higher incidence of ovarian cancer is the women who were never pregnant have about twofold increased chance and women who have had either breast or colon cancer are the higher chance of developing ovarian cancer. The chance also decreases when women who were pregnant or take birth control pill which like pregnancy and prevent ovulation. Ovarian cancer is involved inactivation of the *p53* tumor suppressor gene. Amplification of the c-*erbB-2* oncogene is also a frequent event which appears relative with more aggressive tumor growth. The *gip* oncogene is often involved in the relatively rare ovarian tumors arising from granulose and theca cells, which are hormone producer cells that surround the egg in ovarian follicles (52). Thus, the etiology of ovarian cancer has not known but it associated with environmentsl factors, dietary, personal customs contribution to the incidence of ovarian adenocarcinoma.

Table 3 International Federation of Gynecology and Oncology stage grouping for primary carcinoma of the ovary (51).

Stage I	Growth limited to the ovaries.	
	IA	Growth limited to one ovary; no ascites. No tumor on the external surface; capsule intact.
	IB	Growth limited to both ovaries; no ascites. No tumor on the external surface; capsule intact.
	IC	Tumor either stage IA or IB but with tumor on the surface of one or both ovaries, or with capsule ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension.	
	IIA	Growth involving one or both ovaries with pelvic extension.
	IIB	Extension and/or metastases to the uterus and/or tubes.
	IIC	Tumor either stage IIA or IIB, but with tumor on the surface of one or both ovaries, or with capsule (s) ruptured, or with ascites present containing malignant cells, or with positive peritoneal washings.
Stage III	Tumor involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastases equal stage III. Tumor is limited to the true pelvis, but with histologically verified malignant extension to small bowel or omentum.	
	IIIA	Tumor grossly limited to the true pelvis with negative nodes, but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces.
	IIIB	Tumor of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative.
	IIIC	Abdominal implants>2 cm in diameter and/or positive retroperitoneal or inguinal nodes.
Stage IV	Growth involving one or both ovaries with distant metastasis. If pleural effusion is present, there must by positive cytologic test results to allot a case to stage IV. Parenchymal liver metastasis equals stage IV.	

1.1.2 Diagnosis of ovarian cancer

The Early diagnosis is the best usually made by pathologic evaluation of tissue received at exploratory laparotomy or periodic pelvic examination. Ovaries are in the intraperitoneal, epithelial ovarian cancers frequently spread extensively throughout the peritoneal cavity so causing few alarming symptoms. The diagnoses for ovarian cancer are including:

1. Ultrasound or sonography, a screening method, is using of high-frequency sound waves through the body. The pattern of the echoes can creates a picture called a sonogram by special instrument that subsequently creates an image of anatomic parts. For women, pelvic ultrasound is often to use for examine the uterus and ovaries and, during pregnancy. The health tissues, fluid-filled cysts and tumors can produce different echo.

2. Blood test, a screening method, detects the ovarian tumor markers in blood such as CA-125, CA 72-4, CA 19-9, CA 15-3, CASA, OVX1, and M-CSF (53). The most detection is using CA-125 which is an advantage to monitor the status ovarian cancer.

CA-125 or called cancer antigen 125 is a protein that produced on the surface of ovarian cancer cells and released in the blood stream. An excess of these protein molecules is elevated compared to normal cases. CA-125 is sensitive for some epithelial ovarian carcinomas (e.g., serous, endometrioid and clear cell), but this marker is less sensitive for granulosa cell tumor and mucinous carcinoma (54). The CA-125 test has about 50% chance of returning true positive results from stage I and 80% chance from stage II, III, IV ovarian cancer patients, respectively. However in early stage ovarian cancer, this molecule may not necessary to release. Thus, the test is not an effective screening test. It may be used diagnostically in combination with other test such as ultrasound.

3. Computerized axial tomography (CAT) scan is a series of X-rays

4. An intravenous pyelogram is an X-ray of the kidneys and ureters, taken after the injection of a dye.

5. Biopsy: taken tissue from the cancer tissue. The pathologists examine tissue samples under the microscope to identify the presence of cancer (55).

1.1.3 Treatment of ovaian cancer

Ovarian cancer treatment can use only one method or participation method.

1. Surgery is the main treatment for almost woman who had advanced cancer and require adjuvant therapy following surgery. Surgery for ovarian cancer is a major operation. It causes short-term pain and tenderness in the area of the operation. Any kind of surgery also carries a risk of infection, poor wound healing, bleeding or a reaction to the anesthesia used in surgery.

2. Chemotherapy has been used chemotherapeutic agents as an adjuvant therapy. Participation of chemotherapy was developed according to the rationale that participation agents with different mechanism of action and difference of activities, would increase advantage treatment. This method may be used following surgery as adjuvant therapy to kill any cancer cells that can remain in the body. It may also be used at later time if there are some signs to indicate the cancer has recurred (55).

3. Radiation therapy has been used as an adjuvant and for palliation of ovarian cancer. This method may be used in a small number of patients to kill cancer cells that may remain in the pelvic area after surgery.

4. Biological therapy is using the body's immune system (e.g., antibody, interleukin, interferon, cytokine and hormone) that has specific and sensitive property for cancer antigen for combat cancer (56).

1.1.4 Side effects of ovarian cancer treatments

The most of method is advantage to fight cancer but it is difficult to control the side effect of treatment that kills only cancer cells. Because treatment often damages healthy cells and tissue, it can cause unpleasant side effects. The side effects of cancer treatment are depending on the type of treatment and in individual immune response of each woman reacts differently. Physician attempts to control side effects to a minimum that is not interfering to the patient.

1. Side effects of ovarian cancer surgery method. The patient may have difficulty emptying her bladder and having normal bowel movements. Drugs may be given to relieve pain and to prevent or treat infection. In younger women, when the ovaries are removed, the body's natural source of estrogen is lost and menopause starts. Symptoms of menopause are likely to appear soon after the surgery. Hormone

replacement therapy is commonly used to ease such symptoms as hot flashes and vaginal dryness in menopausal women (51,57).

2. Side effects of ovarian cancer chemotherapy method. The drugs used in the treatment of ovarian cancer can cause kidney damage. The protection of the kidneys while taking these drugs, patients are given large amounts of fluid. These drugs also may cause tingling in the fingers or toes, ringing in the ears, or difficulty hearing. These problems may continue after treatment stops (58).

3. Side effects of ovarian cancer radiotherapy method. Radiation treatment to the lower abdomen may cause nausea, vomiting, diarrhea or urinary discomfort. Radiation therapy for ovarian cancer also can cause vaginal dryness and interfere with intercourse. Women may be advised not to have intercourse during treatment. However, most women are able to resume sexual activity a few weeks after radiation treatment ends (59).

4. Side effects of biological therapy method. Hormonal therapy can cause a number of side effects. They depend largely on the specific drug or type of treatment and they vary from patient to patient. Tamoxifen is the most common hormonal treatment. This drug blocks the body's use of estrogen but does not stop estrogen production. Tamoxifen may cause hot flashes, vaginal discharge or irritation and irregular periods. Any unusual bleeding should be reported to the doctor. Younger women taking Tamoxifen may become pregnant more easily and should discuss birth control methods with their doctor. Serious side effects of Tamoxifen are rare, but this drug can cause blood clots in the veins, especially in the legs. In a very small number of women, Tamoxifen has caused cancer of the lining of the uterus. The doctor may do a pelvic exam, as well as biopsies or other tests of the lining of the uterus, to monitor for this condition. Young women whose ovaries are removed to deprive the cancer cells of estrogen experience menopause immediately. The side effects from Tamoxifen are likely to be more severe than the effects of natural menopause (60).

1.2 Breast cancer

Breast cancer continued to be the leading cause of death as the second leading cause of Thai female cancer (Table 1 and 2) (1,48). It means that nearly one in every ten women will develop breast cancer at some point in life and also occurs in men but is more than 100-fold less frequent than in women. Most cases occur during

age 45 to 55 (52). Approximately 90% arise in the ducts and are called “ductal carcinomas”. They are further distinguished according to cell types. The most common is called “invasive ductal”, NOS (for “not otherwise specified”). Other types of ductal carcinomas are medullary, tubular and mucinous. About 5% of breast cancer occurs in the lobules (lobular carcinomas). The remainder is classified as Paget’s disease that involves the nipple, and inflammatory carcinomas that are associated with apparent inflammation of the breast (52,61). Breast cancer is one of the more common type of cancer in women and a leading cause of cancer deaths in women (67). Despite its high prevalence and enormous health impact, the causes of breast cancer are not well understood, but non-genetic factor appear to dominate the etiology of breast cancer. Environmental influences, such as diet, have been proposed to play a major role in mammary gland carcinogenesis (68,69,70), and most individuals are exposed to carcinogens in their daily diet through the eating of cooked meats and fish (71). Cooking protein-rich foods at high temperatures forms heterocyclic amines. Several heterocyclic amines induce mammary gland carcinomas in rodent models (72), and evidence suggests that human breast cancer risk may be to diets high in cooked meats (73,74).

1.2.1 Epidemiology of breast cancer

The epidemiology of breast cancer has been studies extensively to resolved, an important factor to induce breast cancer are widely agreed upon. Some rare forms of breast cancer are directly inherited. Moreover, the overall risk of developing breast cancer increased two-or-three fold of women whose mother or sisters have breast cancer, presumably reflecting inherited diseased susceptibility. Further, women who have had one breast cancer are at higher than average risk of developing a second. Other risk factors for breast cancer relate principally to the effect of hormones on the breast tissue. Breast cancer risk is increased about three fold of women who never had children or who had their first child after age 35. The women who use the birth control pill for several years prior to first pregnancy may result in modest increase in breast cancer risk. Long-term post menopause estrogen replacement therapy may also be associated with a modest increase in breast cancer risk (62,63,64).

Table 4 Staging of breast cancer by Columbia Clinical Classification (CCC) (65).

Stage A	No skin edema, ulceration, or solid fixation of tumor to chest wall. Axillary nodes not clinically involved.
Stage B	No skin edema, ulceration, or solid fixation of tumor to chest wall. Clinically involved nodes, but <2.5 cm in transverse diameter and not fixed to over lying skin or deeper structures of axilla.
Stage C	Any of five grave signs of advanced breast cancer: - Edema of skin of limited extent (involving less than one third of the skin over the breast) - Skin ulceration - Solid fixation of umor to chest wall - Massive involvement of axillary lumph nodes (measuring >= 2.5 cm in transverse diameter) - Fixation of the axillry nodes overlying skin or deeper structures of the axilla
Stage D	All other patients with advanced breast cancer, including: - A participation of any two or more the five grave signs listed under stage C - Extension edema of the skin (involving more than one third of the skin over the breast) - Satellite skin nodules - The inflammatory type of carcinoma - Clinically involved supraclavicular lymph nodes - International mammary metastases, as evidenced by a parasternal tumor - Edema of the arm - Distant metastases

1.2.2 Diagnosis of breast cancer

Early detection is of major importance in reducing breast cancer mortality and screening for breast cancer by breast self-examination.

1. Palpation. The doctor can tell a lot about a lump (its size, its texture and whether it moves easily) by palpation, carefully feeling the lump and the tissue around it. Benign lumps often feel different from cancerous ones.

2. Mammography. The image is examed by a radioligist who looks for changes in the breast tissue.

3. Ultrasonography. This method is using high-frequency sound waves, ultrasonography can often show whether a lump is solid or filled with fluid. This exam may be used along with mammography.

4. Fine needle aspiration. A thin needle is used to remove fluid from the breast lump. This procedure may show whether a lump is a fluid-filled cyst (not cancer) or a solid mass (which may not be cancer).

5. Needle biopsy. Using special techniques, tissue is removed with a needlefrom an area that is suspicious on a mammogram but cannot be felt. Tissue removed in a needle biopsy goes to a lab to be checked by a pathologist for cancer cells.

6. Surgical biopsy. The surgeon cuts out part or all of a lump or suspicious area. A pathologist examines the tissue under a microscope to check for cancer cells.

Recently, through studying blood samples of families which there is a history of breast cancer, scientists have isolated and identified two gene linked to breast cancer. A person who has these modified genes (namely BRCA-1 and BRCA-2) has an 85% lifetime risk of developing breast cancer, as well as a significantly higher risk of ovarian cancer. BRCA (breast cancer) gene, a tumor suppressor gene, is involved in the maintenance of genome integrity. Mutation in BRCA-1 is characterized by predisposition to breast, ovarian, prostate and colon cancers, while mutation in BRCA-2 involves in others (66).

1.2.3 Treatment

A patient may have just one method of treatment or participation. Different method of treatment may be given at the same time or one after another.

1. Surgery is the common treatment for breast cancer. Clinical trials have proved that surgery provide the long-term survival rates for most types of early breast cancer. However, neither option guarantees that cancer will not recur.

2. Chemotherapy for breast cancer is usually a combination of drugs which may be given by mouth or by injection (75).

3. Radiation therapy (radiotherapy) is the used of high-energy rays to kill cancer cells and stop their growth. The treatments are 5 days a week for 5 to 6 weeks.

4. Hormonal therapy is used to keep cancer cells from getting the hormones they need to grow.

1.2.4 Side effects of breast cancer treatments

It is hard to limit the effect of therapy so that only cancer cells are killed. Because treatment often damages healthy cells and tissue, it can cause unpleasant side effects. The side effects of cancer treatment vary, depending on the type of treatment. Also, each woman reacts differently.

1. Side effects of breast cancer surgery which the imbalance of removal of a breast can cause discomfort in a woman's neck and back. The skin in the breast area also may be tight and the muscles of the arm and shoulder may feel stiff. The patients may have numbness and tingling in the chest, underarm, shoulder and arm. These feelings usually go away within a few weeks or months, but some women may have permanent numbness. Removing the lymph nodes under the arm slows the flow of lymph. In some women, this fluid builds up in the arm and hand causes swelling (lymphedema). Women need to protect the arm and hand on the treated side from injury, even long after surgery.

2. Side effects of breast cancer chemotherapy that the side effects of chemotherapy depend mainly on which drugs the patient receives and vary from patient to patient. In general, anticancer drugs affect rapidly dividing cells. These include blood cells, which fight infection, cause the blood to clot, and carry oxygen to all parts of the body. When blood cells are affected by anticancer drugs, women are more likely to get infections, bruise or bleed easily, and have less energy. Cells in hair roots and cells that line the digestive tract also divide rapidly. As a result, women may lose their hair and may have other side effect, such as nausea, vomiting or mouth sores.

Usually the doctor can suggest diet changes or medication to ease these problems. Most side effects of chemotherapy gradually go away during the recovery period or after treatment stops (76,77). The long-term side effects are quite rare, but there have been causes in which the heart is weakened and second cancers such as leukemia (cancer of the blood cells) have occurred. Also, some anti-breast cancer drugs can damage the ovaries. If the ovaries fail to produce hormones, the women may have symptoms of menopause, such as hot flushes and vaginal dryness. Her periods may become irregular or may stop and she may not be able to become pregnant. However, some women may still be able to get pregnant during treatment. Because the effects of chemotherapy on an unborn child are not known, it is important for a woman to talk to her doctor about birth control before treatment begins. After treatment, some women regain their ability to become pregnant, but in women over the age of 35 or 40, infertility is likely to be permanent (58).

3. Side effects of radiotherapy. Patients are likely to become very tired during radiation therapy, especially in the later weeks of treatment. Resting is important, but doctor usually advice patients to try to stay as active as they can. It is also common for the skin in the treated area to become red, dry, tender and itchy. There may be permanent darkening or “bronzing” of the skin in the treated area. This area should be exposed to the air as much as possible, but protected from sunlight. Patients should avoid wearing clothes that rub the treated area and cause irritation, patients may want to wear loose-fitting cotton clothes. The radiation therapist or nurse will give advice about keeping the skin clean. Patients should not use any lotion or cream on their skin without checking with the doctor.

4. Side effects of breast cancer radiotherapy. The radiation oncologist will explain the possible side effects of radiation therapy for breast cancer including uncommon side effects that may involve the heart, lungs and ribs. One of the common side effects is fatigue, especially in the later weeks of treatment and for sometime afterward. Resting is important, but doctors usually advise their patients to try to stay reasonably active, matching their activities to their energy level. For most women, the breast will look and feel about the same after radiation therapy. Occasionally, the treated breast may be firmer. Also, it may be larger (due to fluid buildup) or smaller

(because of tissue changes) than it was before. For some women, the breast skin is more sensitive after radiation treatment and for others, it is less sensitive (59).

5. Side effects of hormonal therapy which can cause a number of side effects. They depend largely on the specific drug or type of treatment and they vary from patient to patient (60).

2. Monoclonal antibody

In 1975, Köhler and Milstein were studying mechanisms of gene expression and chose the synthesis of antibodies by lymphoid cells. They reported the successful fusion between and antibodies-producing spleen cell and immortal myeloma cell. They called a hybridoma cell which are cell lines made by fusion lymphocytes from an immunized donor with myeloma cells (78). Monoclonal antibodies (MAb) produced from hybridoma cells are a significant part of the biotechnology boom that began in the mid 1970s. It was quickly recognized by immunologists and other scientists that there were far-reaching implications and tremendous opportunities of this technology (79,80). Monoclonal antibodies have two very important advantages over conventional polyclonal antibodies:

1. High specificity.

MAb have the advantage of recognization a single epitope or single protein. Thus, there can be little or no background signal in the absence of the protein under investigation.

2. The ability to be produced in an unlimited supply and with a constant specificity and affinity.

These characteristics have led to a number of applications for monoclonal antibodies, including cell-surface, diagnostic testing (ELISA, RIA, IRMA, etc.), tumor-associated antigen studies, histocompatibility studies, studies of infectious diseases, immunotherapy (radioimmunotherapy, chemotherapy, etc.) and purification of antigenic materials.

2.1 Monoclonal antibody in cancer therapy

Monoclonal antibodies (MAbs) offer the possibility of selective delivery of antineoplastic agents to the tumor site, thereby reducing systemic exposure and toxicity to normal tissue components. The ideal of attaching cytotoxic agents to

molecules specific for tumor cells was first postulate by Paul Ehrlich in 1906 (81) that uses the monoclonal antibody as a vehicle to transport the cytotoxic agent specifically to target tumor cells expressing the appropriate tumor associated antigen (TAA).

The toxicity and immunogenicity of new MAb constructs needs to be evaluated. Monoclonal antibodies have been shown to have clinical utility in the histologic assessment and classification of malignant diseases particularly for leukemias (anti-CD33) and lymphomas (anti-CD19 and anti-CD20) (82,86). The complete responses can resist for long time more that twelve months in both regimens. Monoclonal antibodies raised against tumor markers such as α-fetoprotein, carcinoembryonic antigen and CA-125, among others, have applicability in screening procedures and monitoring clinical disease states (83,84,85). Therapy is restricted by immunogenicity of the mouse constructs. Trials in solid tumor with humanized antibodies have started and initial results show reduced immunogenicity compared with their mouse counterparts. In early impressive results are the antibody rhu MAbHER2, which recognizes the $p185^{HER2}$ growth factor receptor in breast cancer patients. The cytotoxic chemotherapy when combined with the HER2 antibody show potential to kill breast cancer. Blocking growth factor receptor appears to potentiate cytotoxicity in other tumor systems. The variety of antigen systems, types of constructs and cell killing strategies will need considerable study before treatment regimens can be optimized (Table 5) (86).

Table 5 Monoclonal antibodies in clinical development for cancer therapy (87).

Cancer	Target Antigen	Antibody name	Product type	Sponsors	Trail status
Ovarian	CA 125	OvaRex	Murine	Altarex	Phase II/III
Breast	HER2/neu	Herceptin	Humanized	Genetech	FDA approved (1998) for metastizing tumor; Phase III for early tumors
Colorectal	17.1A cell surface antigen	Panorex	Murine	Glaxo Wellcome/ Centocor	Approved (1995) in Germany
Lung	Anti-idiotype CD3 epitope	BEC2	Murine	ImClone Sys	Phase III
Head and Neck	EGFR	IMC-C225	Chimeric	ImClone Sys	PhaseIII

2.2 Monoclonal antibodies combination with toxins or chemotherapeutic agent to control tumor cells

The cytocidal agent must be delivered to sensitive tumor target cells in a form that is fully active or that can be activated *in situ*. This criterion necessitates that the drug be either active when attached to monoclonal antibody or can be metabolized to an active form by intracellular mechanisms. Compounding this problem is the fact that monoclonal antibody-drug combination should be chemically stable with little or no release of free drug during systemic traversable on the bloodstream. Although the relative toxicities and maximal obtainable dosages are known for presently used chemotherapeutic agents, monoclonal antibody-drug combination should be reevaluated for possible adverse cross-reactivity and toxicities prior to embarking on clinical studies (88). The first reported successful treatment of a tumor with MAb used anti-idiotypic antibodies to target B-cell lymphomas whose surface immunoglobulin expressed the corresponding idiotype. The initial course of treatment usually leads to a remission, but the tumor always reappears in a mutant form that no longer binds to the antibody used for the initial treatment. This case represents a clear example of genetic instability enabling a tumor to evade treatment. This problem is circumvented by linking the antibody with toxin such as toxin from organisms or chemotherapeutic drugs and radioisotopes, producing a new form reagent called **an immunotoxin** (89,90)**.** The specificity of the MAb for a cell-surface antigen on the tumor concentrates the drug or toxin to the site of the tumor. After internalization, the drug or toxin is released in the endosomes and performs its cytostatic or cytotoxic effect. MAb linked to radionuclides concentrate the radioactive source in the tumor site. These approaches have the advantage of also killing neighboring tumor cells, because the released toxin, drug or radioactive emissions can affect cells adjacent to those that actually bind the antibody. Ultimately, combinations of toxin-, chemotherapeutic drug-, or radionuclide-linked MAb, together with vaccination strategies aimed at inducing T cell-mediated immunity, might provide the most effective cancer immunotherapy (89).

3. Medicinal plants

A lot of medicinal plants which traditionally used for thousands of years, the traditional medicine all over the world is nowadays revalued by an extensive activity of research on different plant species and their therapeutic principles. The therapeutic utilization of plants is part of universal human culture. Phytotherapy is considered and alternative to mitigate side effects due the indiscriminate use of synthetic drugs. In the last few years, several vegetable products capable of modifying the activity of mutagens and carcinogens in various test systems have been identified. These include extracts of plants as well as plant-derived products: pigments, vitamins, carotenes, phenolic, lactones, flavonoids, and tannins (91,92).

Thailand is in tropical area, which has an abundance of diverse plant resources and most of them can be used as remedy. Although Thai medicinal plants are still widely and legally used in traditional Thai medicine and are the important key in the search for new drug, especially to treat AIDS and cancer (93,94). Our study, two medicinal plants, namely *Phyllanthus amarus* and *Stephania venosa*, are investigated in chemotherapy and chemoprevention properties.

3.1 *Phyllanthus amarus*

The plants in genus *Phyllanthus* (Euphorbiaceae) have been widely used by traditional medical practitioners for the treatment of different types of diseases (95,96). Their extracts or infusions have long been used in folk medicine as diuretics, for intestinal infections, and for other disorders (97,98,99). Different species of *Phyllanthus* show inhibitory activity against some animal and human viruses (99,100,101,102). Furthermore, some of species of this genus inhibit the Epstein-Barr virus DNA polymerase (103), the reverse transcriptase of human immunodeficiency virus-1 (104,105), antipyretic, antibacterial (106), antiparasitic (107), anti-conceptive (108). In addition, antigenotoxic and/or antitumoral properties of some species of *Phyllanthus* have been reported (17,91,109,110,111,112). *Phyllanthus* is a widespread genus in most tropical and sub-tropical countries such as China, the Philippines, Indonesia, on the Malay Peninsula, Thailand, India, Nigeria, East and West Africa, the Caribbean and Central and South America (100,113,114). *Phyllanthus* genus is a very large genus consisting of approximately 550 to 750 species and is subdivided into 10

or 11 subgenera: *Botryanthus*, *Cicca*, *Co-nani*, *Emblica*, *Ericocus*, *Gomphidium*, *Isocladus*, *Kirganelia*, *Phyllanthodendron*, *Phyllanthus*, and *Xylophylla* (98,99).

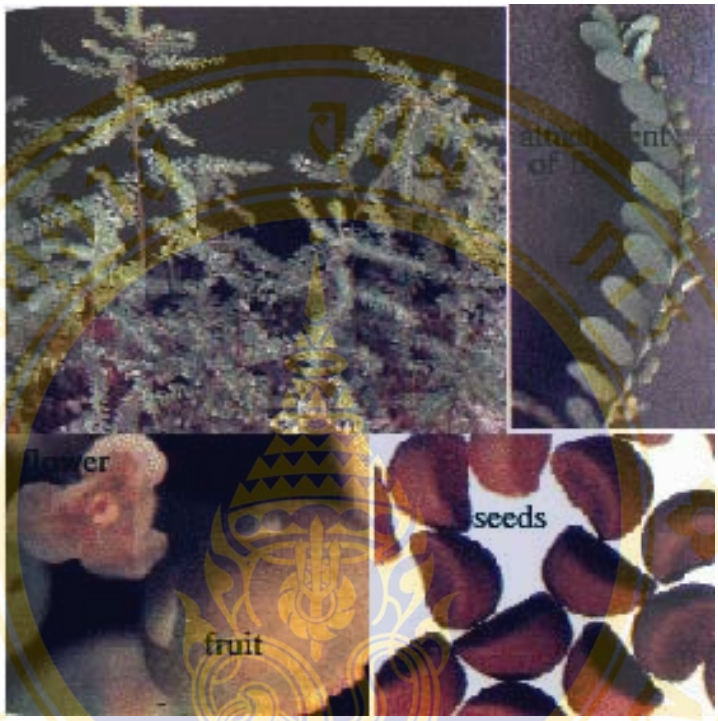


Figure 1 *Phyllanthus amarus* Schum and Thonn (115)

Phyllanthus amarus Schum and Thonn is a small tropical herb importance medicine widely used as a traditional medicine in India and else where including Thailand (116). In Thailand is called Look-tai-bai which is a small Euphorbiaceous herb, found easily throughout Thailand during the rainy season. *Phyllanthus amarus* is a tree of small or moderate size (about 10 to 50 cm) with a greenish-grey bark and small with whitish-yellowish flowers, sepals and green stripe along its length formed in axillary clusters. The feathery leaves are linear-oblong, with a rounded base and obtuse or acute apex. The tender fruits are like capsules, globular and flattened 2 to 3 mm in diameter which green, fleshy, globose and shining and change to light yellow or brick-red when mature at the angle of each little leaf of the branch. Each fruit carry two or three seeds (117,118,119).

3.1.1 Pharmacological active compounds

The major pharmacological active compounds are gallotannins (e.g. phyllanthusiin D, amariin, geraniin, corilagin (120,121). Lignins, such as phyllanthin, hypophyllanthin, were found to enhance the cytotoxic response mediators by vinblastine with multidrug-resistant cultured cells (122). Flavanoids, quercetin, astragalin, ellagitannins and hydrolysable tannin are shown to be present in this plant (16).

3.1.2 Pharmacological activity and clinical trial

Antibacterial activity:

Extracts of *Phyllanthus amarus* had been reported to have antibacterial (106,123).

Antiviral activity:

Phyllanthus amarus extracts can inhibit especially hepatitis B virus and related hepatitis viruses by inhibit DNA polymerase. Clinical trial studies of *P. amarus* at dosage up to 600 mg/ml for 30 days of dried whole plant were reported to decrease hepatitis B surface antigen (100,110,124,125).

Antimitotic activity:

25 µg/ml of aqueous extract from *Phyllanthus amarus* inhibits the activity of *cdc25* tyrosin phosphatase (16).

Antioxidative activity:

Crude extract of *Phyllanthus amarus* has been reported to provide antioxidant and/or antigenotoxic protection (91,112,126).

Anticarcinogenic activity:

Some of the hydrolysable tannins isolated from *P. amarus* has potent inhibitors P_{450} enzymes (121).

Antimutagenic activity:

The aqueous extract of *Phyllanthus amarus* indicates a wide range of antimutagenic activity against induction by 2-aminofluorene (AF2), 2-aminoanthracene (2AA) and 4-nitroquinolone-1-oxide (4-NQO) in *Salmonella typhimurium* strain TA98 and TA 100 and in *Escherichia coli* WP2 uvrA/ pKM101 (127).

3.1.3 Remedies for:

1. Research shows that *Phyllanthus amarus* acts primarily on the liver. It has been used since ancient times as a remedy for jaundice (128). Grind the leaves to a paste, make a small size dose and swallow it. As it is very bitter, have some sweetened milk or honey. Using 6 doses (twice a day) should cure for jaundice. It is extremely effective in the treatment of jaundice (129). The two most important traditional uses are:

   a. its action on kidney stones.
   b. its effect on liver diseases.

2. The whole plant has medicinal uses for liver tonics. (129).
3. *P. amarus* is also taken as a good tonic and diuretic in India
4. The decoction of *P. amarus* is a remedy for intermittent fevers and infections of the spleen and liver.
5. Due to its effects on liver, it is also used for digestive disorders as an anti-diabetic.

3.2 *Stephania venosa*

Figure 2 *Stephania venosa* (Blume) Spreng (130)

The genus *Stephania* of the family Menispermaceae comprises of about 45 species and 15 of which have been reported in Thailand (131). Several *Stephania* species have been used in traditional medicine to treat a variety of disease (132). The genus is also well known as an important source of isoquinoline alkaloids, one of the largest groups of natural products which display interesting pharmacological activity (133,134,135). The alkaloids of the Menispermaceae were reviewed by Tomita (136) in 1952. Since then the number of alkaloid occurrence reported has doubled and growth of interest in alkaloid biosynthesis. The genus *Stephania* could be a potential source of biologically active compounds which might be used as lead molecules for the development of new drugs. The leafy climber *Stephania venosa* is commnonly known in Thailand under the name of Sa-bu-leuad which means blood soap, due to the red color of its sap. The plant has a large exposed tuber (up 20-40 cm in diameter) with bitter taste. More than 20 isoquinoline alkaloids have been reported form *S. venosa* (19,137,138,), and some of which such as dehydrostephanine and dehydrocrebanine showed potent antiplasmodial activity.

3.2.1 Pharmacological active compounds

The pharmacological active compounds of *S. venosa* are tetrahydropalmatine, crebanine, palmatine (139). aporphines (140,141,142,143), oxoaporphines and protoberberines (143).

3.2.2 Pharmacological activity and clinical trial

Antimicrobial activity:

Extracts from *S. venosa* has protoberberine alkaloids that can be antimicrobial activity (141), and berberine in this plant has also antimicrobial activity on fungi, protozoa and bacteria (141).

Antiplasmodial activity:

Dehydrostephanine and dehydrocrebanine showed potent antiplasmodial activity (143).

Anticancer activity:

The berberine substance in berberine sulfate in this plant has anticancer activity (139,141).

Antileukemic activity:

The coralyne chloride (8-methylhexadehydroberberinium salt) has antileukemic activity on P-388 and L-1210 strain in mice (138,139).

3.2.3 Remedies for:

In Thai people used as a bitter tonic (134), suppress central nervous system (143) and kill human worm (143).

4. Apoptosis

Apoptosis or programmed cell death was first reported in 1972 by Kerr et al (144). Apoptosis is a physiological process that constitutes the natural fate of end-stage differentiated cells. It is essential for tissue patterning during embryonic development and for maintenance of tissue homeostasis of the adult organism. Since cell death is crucial in developing systems, the components of the cell death machinery are remarkably conserved through evolution (28,144). Apoptosis research has been the focus of intense research interest since the recognition of its active involvement in development and disease etiology. Most of the published works have focused on the mechanisms and regulation of apoptosis, the role of apoptosis in cancer etiology, and the induction of apoptosis by external stimuli including drugs. Relatively little is known about the kinetics and pharmacodynamics of drug-induced apoptosis.

4.1 Mechanisms and regulation of apoptosis

Apoptosis, a controlled physiological process of fundamental importance to all multicellular organism, occurs in a morphologically and biochemically distinct manner which ultimately leads to cell suicide. Apoptosis plays a central role in embryogenesis and normal adult tissue homeostasis by regulating the balance between cell death and cell proliferation. It is also important for eliminating cells with nonrepairable genotoxic injury. Deregulation of apoptosis is involved in the etiology of disease including degenerative diseases of the central nervous system, autoimmune diseases, viral infection, and cancer (145,146).

The apoptosis process involves a sequence of events including cell shrinkage, increased cytoplasmic density, chromatic condensation and segregation into sharply circumscribed masses, and the formation of membrane-bound smooth surface apoptotic bodies. Apoptosis is often accompanied by the activation of endogenous

Ca^{2+}- and Mg^{2+}- dependent endonucleases. These enzymes cleave DNA at internucleosomal sites, where DNA molecules are complexed with histone proteins, to produce fragments in multiples of approximately 185 bp. Apoptotic cells are phagocytosed from the midst of living tissue by neighboring cells or macrophages without eliciting an inflammatory reaction.

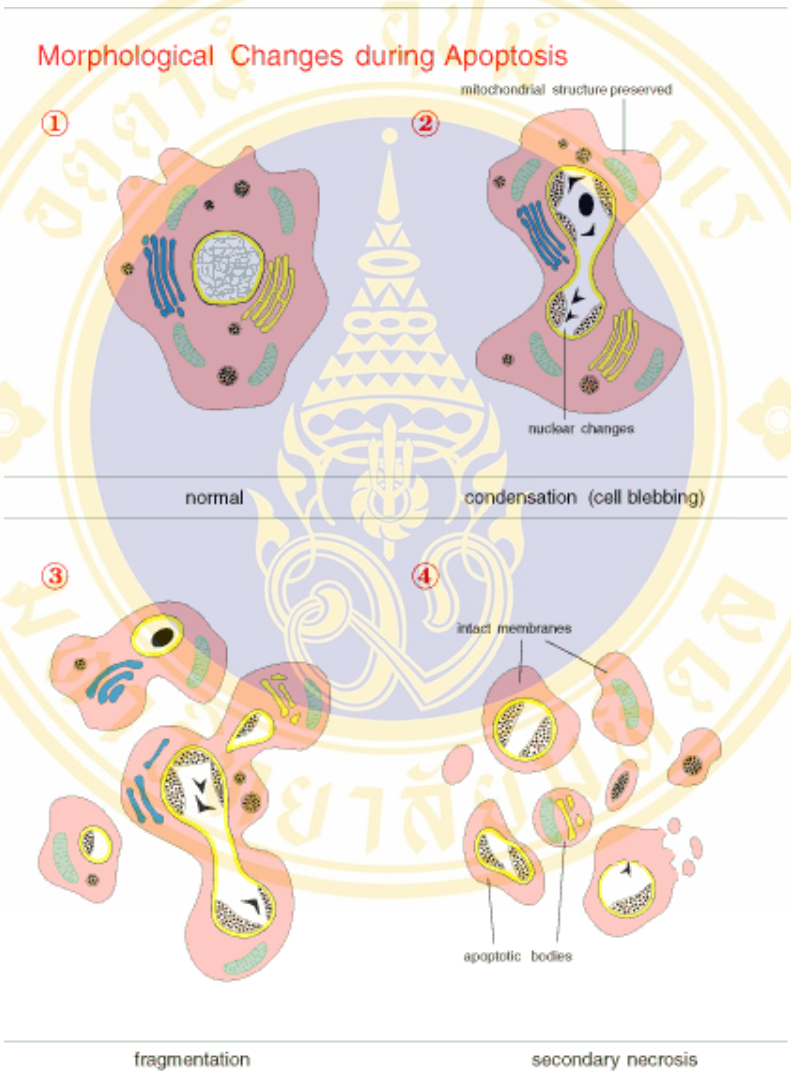


Figure 3 The pattern of morphological changes during apoptosis (147)

In spite of the rapid gain of knowledge in recent years, the biochemical mechanisms of apoptosis are far from being fully understood. Multiple genes and their protein products are involved in the induction and execution of apoptosis. Apoptosis is linked to cell cycle progression. *In vivo*, physiological apoptosis can be detected in

self-renewing tissues, such as intestinal crypts, epithelium of the adrenal cortex, differentiating spermatogonia, and germinal centers. Apoptosis become particularly evident in tissue after periods of rapid proliferation, such as mammary tissue following weaning, in the endometrium at uterus, during ovarian follicular atresia, and in malignant tumors. It has been suggested that apoptotic cells utilize the same proto-oncogene products and regulators of the cell cycle in a unique manner to induce a tightly controlled cell death. Several typical events of early cell cycle traverse are associated with apoptosis, e.g. unregulation of proto-oncogenes such as *c-myc*, *ras*, *c-fos*, *c-jun*, *cdc-2*, and phosphorylation of the protein product of the tumor suppressor retinoblastoma gene. Some of the molecules that induce apoptosis are also involved in the regulation of proliferation and differentiation. For example, the nuclear transcription factor *c-myc* which is classically associated with the promotion of cell growth has also been demonstrated to be a central mediator of apoptosis. Ceramide, a hydrophilic component of sphingolipids (especially spingomyelin) which induces differentiation, growth suppression and cell cycle progression, also induces apoptosis.

There are distinct cellular thresholds, or set points, for apoptosis induction and subsequent signaling. As a result, different cell types vary in their susceptibility to activate the apoptotic pathway. There are two general categories of apoptosis, i.e., primed apoptosis and unprimed apoptosis. Primed apoptosis is found in most cell types of normal or transformed hematopoietic lineages. In primed apoptosis, all of the effector molecules are expressed in the cell and the apoptosis program can be executed directly after it is initiated without the requirement of active gene transcription. In unprimed apoptosis, active gene transcription is required and the process occurs more slowly than primed apoptosis, cells successfully progress through one round of the cell cycle, but die in the subsequent cycle.

Apoptosis is controlled by multiple genes that have been evolutionarily conserved from the nematode *Caenorhabditis elegans* to mammals. These genes encode ligands and their receptors, and a number of signaling molecules which are linked to second messengers that bridge membrane events to transcription factors and gene expression. The resulting gene products act to either stimulate or block apoptosis.

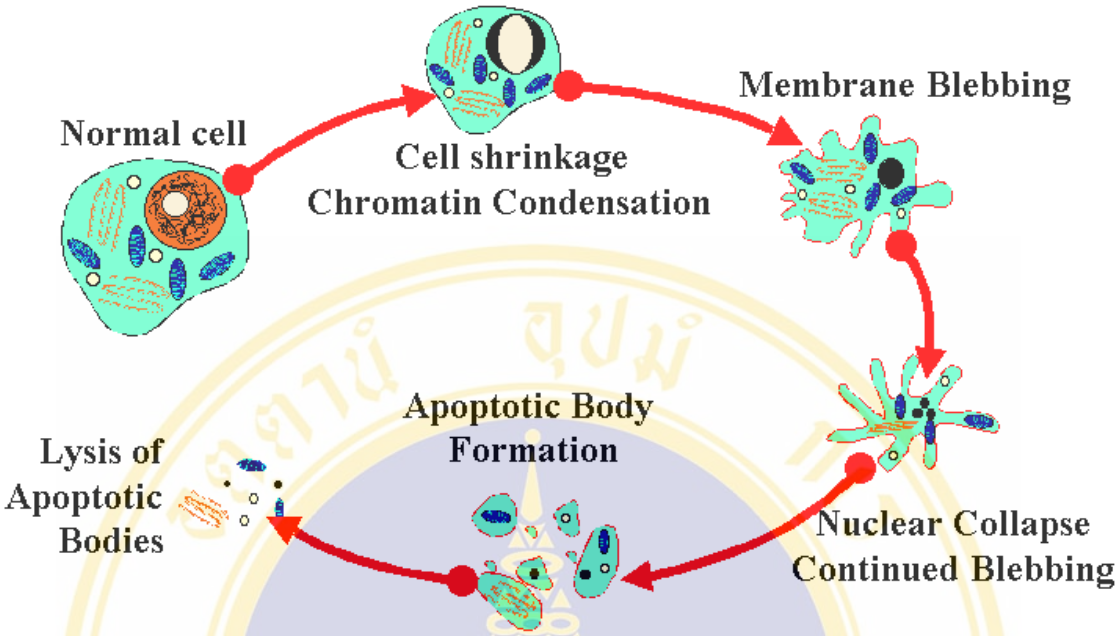


Figure 4 A model of molecular events and pathways of apoptosis (148)

4.2 Stages of apoptosis

Apoptotic signaling can be categorized in four stages (Figure 4).

1. The earliest stage, induction, describes how a cell interprets environmental or intrinsic cues to provoke the apoptotic response. Different insults, including drugs and irradiation that cause damage to DNA, drugs that cause damage to microtubules, ligands, binding to cell surface receptors, cytotoxic T cells, and growth factor withdrawal, can induce apoptosis.

2. During the second or detection stage, the apoptotic signal is detected and transduced to downstream effectors. Activation of the signaling molecules at this stage varies according to the type of stimuli

3. The third stage describes the effector of the apoptotic pathway, which involves a family of cysteine proteases (caspases) and endonucleases, as well as other positive and negative regulators of apoptosis. There is a preponderance of recent evidence indicating that the many signal transduction pathways converge at a common endpoint at effecter stage to elicit the apoptotic response.

4. The final and least understood stage of apoptosis, corpse disposal, is the stage where the apoptotic cells are phagocytosized and digested by neighboring cells or macrophages.

Table 6 The differences characteristic between apoptosis and necrosis (24).

Charecteristic	The differences between	
	Apoptosis	**Necrosis**
1. Nuclei	Pyknosis and karyorrhexis (dense condensation of chromatin)	Karyolysis, preceded by irregular chromatin clumping
2. Cytoplasmic organelles	Intact	Disrupted
3. Cell membrane	Apoptotic bodies blebbing	Blebbing and loss of integrity
4. Cell volume	Cells shrink	Cells swell
5. In tissues	Single cells affected	Groups of cells affected
6. Tissue response	None	Inflammation
7. Nuclear DNA damage	Nucleosomal and /or 50-300 kb fragments to ladders on gels	Random to smears on gels
8. Nuclear gene expression	Usually needed	Not needed
9. Mitochondrial DNA damage	Spared	Occurs early
10. Enzyme activity **10.1 Dnase** **10.2 Protease** **10.3 Tranglutaminase**	 Necessary Necessary Frequent	Not necessary
11. Membrane function	Intact	Loss of function
12. Cell internal milieu **12.1 pH** **12.2 Ca^{2+}** **12.3 Na^{+}/K^{+} pump**	 Slightly acidic (pH 6.4) Often increases May be intact	 Acidic Always increases Defective

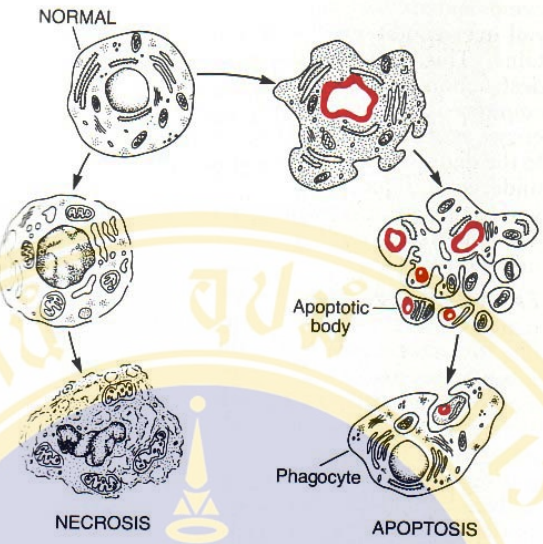


Figure 5 The different cell death pathway between necrosis and apoptosis (149)

4.3 Apoptosis as a predictive factor for cancer therapy

Generally, there are diseases with apoptosis. Investigation of the expression of protein regulating apoptosis as a predictive factor for cancer treatment is at its beginning. However three main points are already clear.

1. The expression of each of the protein regulating apoptosis is likely to have a different significance in different at organs, as recently pointed out for *bcl-2*. Indeed it has already been demonstrated that this protein has a predictive meaning in myeloid leukaemias and breast cancer.

2. It will be necessary to investigate not only the primary tumor, but, when possible, also the post-treatment residual tumor and the metastasis. These approach logical markers respond to treatment. This type of information could possibly lead us to choose both the best treatment for resistant clones and to identify in the primary tumors potentially resistant cells already present and treatment them immediately in an appropriate way.

3. It is likely that, in most type of tumor, to predict the sensitivity to treatment it will be necessary to assess the expression of more than one protein, possibly belonging to different pathways involved in both suppression and induction of apoptosis. The *bcl*-2-*p53* system in the first, even if incomplete, model available for such studies (59).

4.4 Methods for apoptotic determination

Apoptosis was originally distinguished from necrosis on the basis of its ultra structure (28). Electron microscopy still provides the most reliable method for distinguishing the two processes. In many cases, however, they can be identified using light microscopy. Apoptosis typically involves scattered individual cells in a tissue without local inflammatory reaction. Apoptotic cells are morphologically characterized by condensation of chromatic which is marginated against the nuclear envelop, condensation of cytoplasm, membrane blebbing, and apoptotic bodies. The advantage of morphological identification is that it is technically simple and quantitative. The disadvantages are that the evaluation is subjected to operator bias, and that it may be difficult to detect the early stages of apoptosis with little or no morphological changes. Futhermore, apoptotic cells may not be readily distinguished from other elements with condensed chromatin such as lymphocytes and cells undergoing mitosis (Table 6).

4.4.1 Light and/or fluorescent microscopy for apoptotic cells.

Light and/or fluorescent microscopy observed morphological changes in apoptotic cells. Both techniques are the simplest way in which apoptosis can be recognized in cell suspensions or tissue sections. Identification of the apoptotic cells by this method can then be confirmed by other techniques such as electron microscope, histochemical or biochemical studies (24).

4.4.2 Electron microscopy of apoptotic cells

The electron microscope is an important tool in the study of the complex morphological changes that occur in apoptosis. Early changes such as the formation of chromatin crescents and cytoplasmic condensation can easily be noted by transmission electron microscope (TEM). Fine details of the process, such as the disintegration of the nucleolus, or the deep convolutions of the nuclear membrane, can also be observed. TEM gives a bird’s eye view of the process occurring within a cell at the macromolecular level. Scanning electron microscopy (SEM) gives a 3-dimensional view of the apoptotic process, and displays the dramatic protuberances (blebbing) of the cell and nuclear membrane in the formation of apoptotic bodies. Electron microscopic studies have revealed the multi-step nature of the process of apoptosis and have been extensively described (24,150).

4.4.3 Biochemical detection of DNA fragments in apoptotic cells

Demonstration of DNA damage or release of products of DNA degradation is by itself insufficient to justify the description of the phenomenon as apoptosis. It is clear that an endonucleolytic pathway is activated in apoptosis and results in the cleavage of the cell genome, first into DNA fragments that vary from 300 kb to 50 kb in size, then into 180 bp multiples. Together with morphological changes characteristic of the process of apoptosis, the 180 bp periodicity of the DNA fragments visible on 1.5% - 2.0% agarose gels stained for DNA is a tell-tale sign of this mode of cell death. It is believed that DNA ladder formation by low molecular weight DNA fragments results from the nucleolytic cuts in linker DNA between nucleosome within chromatin; the DNA derived from one nucleosome and the linker region is about 180 bp long. Cuts which lead to oligosome-sized fragments result in multiples of 180 bp. The long-term stability of the extracted DNA also adds favor to these procedures because the DNA samples can be analyzed weeks after extraction. However, nucleosomal ladders are not essential for demonstration of apoptosis, since the process can occur without this type of DNA fragmentation (24,151).

4.4.4 Biochemical detection of DNA damage in apoptotic cells

In 1984, a single-cell gel electrophoresis method was developed that allowed measurement of DNA damage in individual cells (153). This technique was later modified and adapted for image analysis, providing greater sensitivity and resolution of subpopulations (152,154). In this method, single cells are embedded in agarose on a microscope slide, then lysed to remove proteins, exposed to low-voltage electrophoresis, and stained with a fluorescent DNA-binding dye. The amount of DNA that migrates is proportional to the number of DNA breaks present in the cell. This “comet” assay is versatible and able to detect DNA single-strand breaks, double-strand breaks, or cross-links in virtually any cell type, provided that a single-cell suspension can be obtained (152).

Correlation between DNA damage and cell killing

Recent results applying the comet assay to multicell tumor spheroids and murine tumors indicate that it may be possible to use initial DNA damage as a surrogate endpoint for cell killing by selected antitumor agents (155,156).

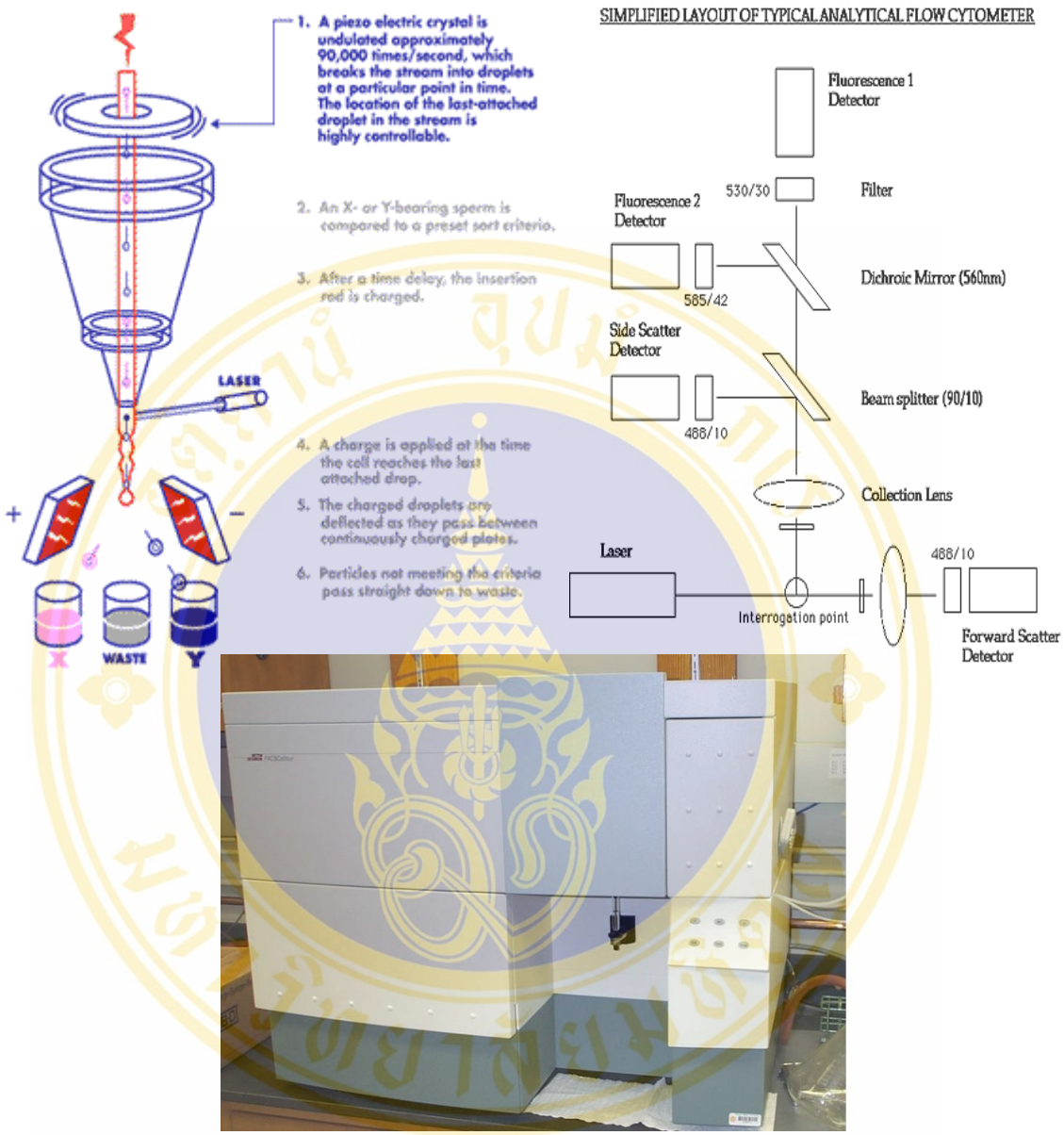


Figure 6 The principle and flow cytrometry for detect apoptosis and cell cycle analysis (157,158,159)

4.4.5 Analysis of apoptosis by flow cytometry

Flow cytometry allows one rapidly and accurately to measure individual cells in large cell populations. Because several cell attributes can be measured simultaneously and the data are recorded. Their multivariate analysis provides information on the relationship between these attributes. In contrast to biochemical analysis in bulk, cellular heterogeneity can be estimated and cell

populations with distinct characteristics can be discriminated. If need, cells with particular features can be electronically sorted. These advantages have all contributed to the fact that flow cytometry has become a methodology of choice in a variety of functional and structural assays of the cell, including cell viability. In the present, flow cytometry are widely in laboratory. In addition, characteristics of cell death are distinguished and mechanisms of cells at early and late stages of death are investigated remarkably such as activity of caspase, changes of phospholipid in plasma membrane by Annexin V, mitochondrial transmembrane potential by Rhodamine 123-PI and staining DNA fragments in apoptotic cells and others (Figure 6)(24).

A variety of methods exist to obtain information about cell proliferation, but assays which provide quantitative data about apoptosis lack specificity, are time consuming and usually require the destruction of cell integrity. Changes on the surface of apoptotic cells, such as the expression of thrombospondin binding sites, loss of sialic acid residues and exposure of phosphatidylserine have been difficult to recognize. Phosphatidylserine (PS), a negatively charged phospholipid, is normally predominantly present in membrane leaflets facing the cytosol. Surface exposure of PS has been reported for activated platelets and senescent erythrocytes and recently it was shown that cells undergoing apoptosis break up the phospholipid asymmetry of their plasma membrane to expose PS.

Annexin V was initially discovered as a vascular protein with strong anticoagulant properties. It appeared to belong to a multigene family of proteins defined by a repeated sequence motif, originally termed the endonexin loop. The annexins have the biological property of binding to phospholipids in a Ca^{2+}-dependent way. Annexin V binds preferentially to phospholipid species such as PS, which is normally absent in the outer leaflet of the plasma membrane and shows minimal binding to phospholipid species such as phosphatidylcholine and sphingomyeline, which are constitutively present in the outer leaflet of plasma membranes. When cell death occurs, PS is translocated to the outer layer of the membrane, i.e., the external surface of the cell. This occurs in the early phases of apoptotic cell death during which the cell membrane itself remains intact. Necrosis, on the other hand, is accompanied by loss of cell membrane integrity and leakage of cellular constituents into the environment. Therefore, we have reasoned that measurement of annexin V binding,

executed simultaneously with a dye exclusion test, would provide a perfect assay to detect apoptotic cells and to discriminate between apoptosis and necrosis (160,161).

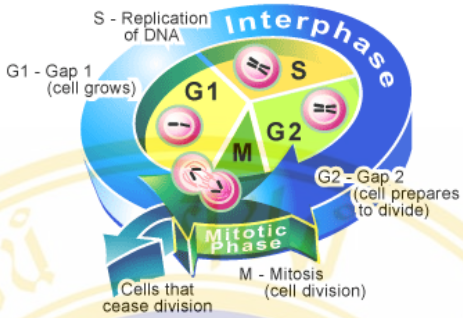


Figure 7 The cell cycle in each phase (169)

5. Cell cycle analysis by flow cytometry

There are a variety of methods for following changes in the cell cycle induced by drugs, radiation and environmental factors such as withdrawal of a growth factor. Equally, there is a battery of flow cytometric techniques for observing changes in cells undergoing apoptosis (162,163,164). Since some of the treatment that induce apoptosis are cell cycle specific and all of them in some ways will disrupt the cell cycle, an investigation of the relationship between the cell cycle and apoptosis can be of great value. The cell cycle phase from which apoptosis has been triggered can be directly measured. After some treatments, cells may progress through the cell cycle before undergoing apoptosis, in which case a different set of techniques will need to be employed. One of the features of apoptotic cells is extensive degradation of the DNA at the linkers between the nucleosomes. The result is a series of oligomers of DNA with a unit length of about 180 bp. If apoptotic cells are fixed in ethanol, and then resuspended in a buffer, such as PBS, the smaller fragments of DNA are extracted so that the apoptotic cells have a lower DNA content compared to the normal cells. Normally, the cells are stained with PI; an Argon-in laser producing blue light is used in the flow cytometer and red fluorescence from the PI/DNA is recorded (165,166). When the DNA histogram is measured, the apoptotic cells form a peak below the G1 peak. The amount of DNA extracted and, hence, the position of the G1 peak in the DNA histogram depends on the buffer used (35) and the cell type (167). Consequently, when working with a new cell line, preliminary work is needed to establish the best conditions for the assay.

Table 7 Chemotherapeutic drug which can arrest cell in different phase (168).

Chemotherapeutic drug: Cell Cycle Phase Specific	
G1 Phase	**G2 Phase**
Bleomycin	Bleomycin
Corticosteroids	Etoposide
Hormones	Taxol
L-Asparaginase	Vinorelbine
S Phase	**M Phase**
Cytarabine	Vinblastine
5-Fluorouracil	Vincristine
Hydroxyurea	Vindesine
Methotrexate	Vinorelbine
Thioguanine	
Chemotherapeutic drug: Cell Cycle-Specific (Phase Nonspecific)	
Busulfan	Dactinomycin
Carboplatin	Daunorubicin
Chlorambucil	Doxorubicin
Cisplatin	Idarubicin
Cyclophosphamide	Melphalan
Decarbazine	Liposomal Doxoubicin
Chemotherapeutic drug: Cell Cycle-NonSpecific	
Carmustine	Mechlorethamine
Lomustine	Semustine

6. Free radicals

6.1 Mechanisms of free radicals in normal condition

A free radical is defined as any species that has one or more unpaired electrons. Normally, >95% of the oxygen consumption by aerobic organisms is the result of enzymatic reduction to molecule of water (H_2O) in mitochondria by the terminal oxidase of the respiratory chain. When molecular oxygen is reduced by one electron, the product is superoxide radical ($O_2^{\bullet -}$). The addition of a second electron to

$O_2^{\bullet-}$ at physiologic pH gives rise to hydrogen peroxide (H_2O_2), an oxidizing species that has no unpaired electrons and thus is not a free radical. The one electron reduction of H_2O_2 yields H_2O and hydroxyl radical ($^{\bullet}OH$), the strongest oxidant produced in biological systems. Generation of $^{\bullet}OH$ from H_2O_2 is catalyzed by transition metals, particularly iron and copper. Finally, $^{\bullet}OH$ reduction produces a second molecule of H_2O. Together, $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ are known as reactive oxygen species (ROS) and are continuously produced by aerobically growing cells (170).

Another free radical species, nitric oxide ($^{\bullet}NO$), was described in the course of defining mechanisms of endothelial control of vascular blood flow. Nitric oxide is formed in mammalian cells by enzymatic oxidation of the amino acid L-arginine to citrulline and $^{\bullet}NO$, via the action of constitutive and inducible nitric oxide synthases (171). Nitric oxide is now appreciated to be an important mediator of diverse physiologic process including neurotransmission, regulation of blood pressure, inhibition of platelet aggregation, and as an effector of immune responses. Excess production of $^{\bullet}NO$ can be cytotoxic, a reaction we now appreciate to largely depend on the formation of secondary highly reactive species derived from the reaction of $^{\bullet}NO$ with ROS. For example, the rapid and almost diffusion-limited reaction between $^{\bullet}NO$ and $O_2^{\bullet-}$ yields peroxynitrite ($ONOO^-$) a potent oxidizing species. Nitric oxide and the secondary species derived from it are known as reactive nitrogen species (RNS) (172).

6.2 Relative between reactive species and diseases

Until relatively recently, we now also appreciate the more subtle contributions of reactive species in maintaining tissue homeostasis, e.g., the regulation of gene expression, neurotransmittor actions, control of blood flow and the orchestration of inflammatory signaling reactions. Many evidences have shown that oxidative stress, defined as an imbalance between oxidants and antioxidants in favor of the former. This condition of oxidative stress was considered purely from a toxicologic perspective, as ROS and RNS react with and modify the structure and function of biomolecules including proteins, lipids, sugars and nucleic acids.

Lipid. The principal targets of lipid oxidation are plasma and organelle membranes, along with plasma lipoproteins. The initiation of lipid oxidation is frequently caused by a free radical-mediated abstraction of a hydrogen atom from a

methylene (-CH_2-) carbon of polyunsaturated fatty acids. Reaction of O_2 with lipid radical intermediates yields a reactive peroxyl radical that can in turn abstract a hydrogen atom from neighboring unsaturated lipids, forming a hydroperoxide and an alkyl radical. Alkyl radicals can in turn combine again with O_2 to regenerate another lipid peroxyl radical, thus autocatalytically initiating a new round of oxidation (173). The oxidation of lipoproteins has been proposed as the inciting event that mediates and accelerates vascular disease such as arterial lesion development (174).

DNA. Low levels of oxidative damage to DNA bases occur in all aerobic cells, with these levels significantly increasing and becoming an etiologic factor in cancer induced by smoking, chronic inflammatory diseases and endogenous oxidant production (i.e., increased mitochondrial oxygen radical production). Cigarette smoke contains high levels of RNS and phagocytic cells recruited to sites of chronic infection abundantly generate ROS and RNS. Oxidative damage to nucleic acids includes adducts of both base and sugar group modifications, single and double strand breaks in the DNA "backbone", and cross-links between DNA and other molecules (175). For example, elevated DNA oxidation has been measured during early *Helicobacter pylori* infection (stomach cancer) (176), smoking, asbestos and exposure to diesel exhaust particles (lung cancer) (177,178) and benzene (leukemia) (179), among others.

Protein. Proteins present a diverse spectrum of molecular targets for oxidative damage (180). Besides the amino acid residues of proteins, oxidizable prosthetic groups such as metal-sulfur clusters contribute to the sensitivity of proteins to damage by reactive species (181). A principal amino acid target for oxidative modification in proteins is the reactive sulfhydryl of cysteine residues. For example, oxidation of cysteine (mediated by $^{\cdot}OH$ or $ONOO^-$) leads to enzyme inactivation and subsequent impairment of glycolysis in cells. Moreover, the modified tyrosine product has been identified in atherosclerotic vessels, acute and chronic lung injury, acute and chronic kidney rejection, central nervous system tissue from Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis patients (182).

In addition, oxidative stress is an important contributing factor in several human diseases, including arthritis, vasculitis, glomerulonephritis, stroke, intestinal ischemia, AIDS, gastric ulcers, hypertension, hemochromatosis, amyotrophic lateral

sclerosis, muscular dystrophy, alcoholism, smoking-related diseases, aging process and many others (170).

6.3 The relationship between apoptosis and oxidative stress

The normal cells undergo a suicide process called apoptosis. As note aboved, diseases of insufficient cell death (such as cancer) are promoted when cells develop resistance to apoptosis. Too much apoptosis contributes to disease marked by excess cell death (such as heart disease and AIDS).

The generation of potentially toxic compounds is a necessary consequence of life in an oxygen environment. As the result of free electrons in the cell combining with oxygen, reactive molecules are formed. These reactive molecules can react with and damage proteins, membranes or DNA. Cells are protected against such damage by a battery of molecules collectively called the antioxidant defense. Vitamins E and C, for example, are part of antioxidant defense. Oxidative stress occurs in the cell when the antioxidant defense cannot keep pace with the rate at which reactive oxygen species are generated. In mechanism of apoptosis, oxidative stress was playing a role. Specifically, we observed a weakened antioxidant defense in cells treated to undergo apoptosis. The hypothesis that oxidative stress is important in the mechanism of apoptosis leads to several predictions. First it should be possible to increase a cell's resistance to apoptosis by increasing its resistance to oxidative stress. Conversely, cell with weakened antioxidant defense should be more sensitive to apoptosis. Second, cells dying by apoptosis should have oxidative damage to their proteins, membranes or DNA. Finally, it should be possible to identify proteins involved in the mechanism of apoptosis that sense and are regulated by oxidative stress (183).

6.4 Role of nitric oxide in carcinogenesis and tumor progression

Nitric oxide (NO) is a short-lived (half-life 3-30s) colorless gas that is moderately soluble in water (up to 2 mM/L) but highly soluble in organic solvents (184,185,186). Its lipophilic nature means that it can diffuse between cells very easily. NO is generated from the terminal guanido nitrogen atom of L-arginine by various NADPH-dependent enzymes called NO synthases (NOS). The three main isoforms are neuronal (n) NOS, inducible (i) NOS, and endothelial (e) NOS. Generally, nNOS and eNOS (187,188,189,190) are expressed constitutively in neurons and endothelial cells, respectively, though they can also be expressed by other cells. Activation of these two

isoforms depends on calcium ions and calmodulin, resulting in NO production in low concentrations (usually nanomolar). Expression of iNOS, by contrast, typically requires induction by bacterial products alone or with inflammation-associated cytokines in many cell types, particularly macrophages. Activation of iNOS does not require calcium ions and calmodulin; activation of this enzyme leads to production of high concentration of NO (generally micromolar), which may be sustained for a long period (191).

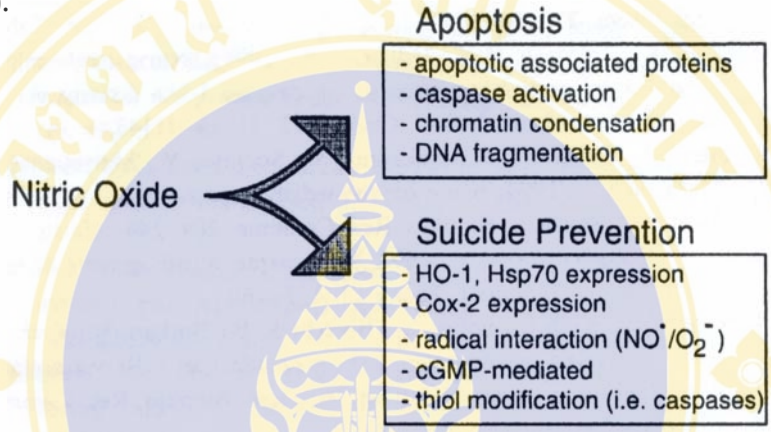


Figure 8 The dual role of NO during apoptotic cell death. Main components that characterize NO-mediated apoptosis are indicated in the upper part of the figure while the lower part indicates NO-associated transducing pathways that signal cell protection (195).

The toxicity of NO is influenced by the existing biological milieu. Relative rates of NO formation, its oxidation and reduction, the combination with oxygen, superoxide, and other biomolecules will determine the signaling pathway of nitric oxide. In some cellular systems activation of the iNOS grnerated sufficient amounts of NO to promote cell death that is defined by typical morphological and biochemical features which resemble apoptosis. Apoptosis in response to NO donors or endogenous NO generation is accompanied by an early accumulation of the tumor suppessor protein *p53*, caspase activation, and DNA fragmentation. These alteration are attenuated by *Bcl-2* gene transfer, which blocked distal to *p53* accumulation and proximal to caspase activation.

Not all cellular systems which show iNOS upregulation after cytokine treatment enter the death pathway which is exemplified for rat mesangial cells

(192,193). Antagonistic and/or protective principle must exist (Figure 8). An antagonistic principle becomes evident by a balanced rate of $O_2^{\bullet-}$ production which redirects cell destruction to cell protection. Besides adverse effects of NO the molecule also signals cell protection. $^{\bullet}$NO-derived protection can be divided into mechanisms that upregulate cell protective proteins such as heat shock protein or cyclooxygenase-2 and into processes that are transmitted by thiol modification, i.e., caspase inactivation and elevated levels of cyclic GMP.

6.5 Antioxidants for cancer prevention and treatment

Antioxidant is molecules that would avidly react with annihilate reactive species before they could inflict oxidative damage to vital components, such as DNA or cell membranes. The result was hundreds of kinds of such antioxidant molecules, especially in plants. Among the successful of these molecules are the water-soluble antioxidant, i.e., ascorbic acid (vitamin C) and the lipid-soluble antioxidant, i.e., α-tocopherol (vitamin E). There are many researches done on antioxidants, which show effectiveness in prevention and treatment of cancer. Antioxidants protect against chemotherapy toxicity and local toxic effects of tumors or surrounding tissues. Antioxidants act to inhibit or reverse oxidation. Free radicals are potentially carcinogenic, and are created by healthy cell metabolism, diseased cells, carcinogen, treatment with chemotherapy, treatment with radiation, cancer. Action of free radicals are damage DNA, cause mutations, damage proteins, damage lipids, damage carbohydrates, alter function of proteins, lipids, carbohydrates, and create more free radicals (194). A wide range of chemicals, both naturally-occur-ring and synthetic, can protect against the development of cancer. These include coumarins, diterpenes, dithiolethiones, idoles, isothiocyanates, lactones, organosulphides and phenols. Compounds with cancer chemopreventive properties have been subdivided into blocking agents and suppressing agents on the basis of the stage during carcinogenesis at which they act. Blocking agents prevent carcinogens from modifying DNA and causing mutations. This is usually achieved by increasing the expression of detoxication and antioxidant enzymes in target tissue, though alterations in the pharmacokinetic of xenobiotics may also serve to protect against tumorigenesis.

CHAPTER III
MATERIALS AND METHODS

1. Materials

1.1 Cell lines

Human ovarian cancer cell line (SKOV3) and human breast cancer cell line (SKBR3) in this study were cultured and grown as monolayer.

1.1.1 Human ovarian cancer cell line (SKOV3)

Human ovarian cancer cell was obtained from American Type Culture Collection (ATCC HTB77) which was isolated from ascitic fluid of a Caucasian female 64 years old patient with an ovarian adenocarcinoma tumor.

1.1.2 Human breast cancer cell line (SKBR3)

Human breast cancer cell was obtained from American Type Culture (ATCC HTB30) which was isolated from pleural effusion of a Caucasian female 43 years old patient with a breast adenocarcinoma tumor.

1.1.3 OVS1 hybridoma cell line

OVS1 hybridoma cell was kindly provided by Prof. Dr. Neelobol Neungton, Department of Biochemistry, Faculty of Medicine, Siriraj Hospital, Mahidol University. OVS1 hybridoma cells, produced OVS1 monoclonal antibody (OVS1 MAb), was obtained by fusing of NS1/1-Ag4-1 murine myeloma cells and spleenocytes from BALB/C mouse immunized with fresh human ovarian mucinous cystadenocarcinoma.

1.2 Thai medicinal plant extracts

1.2.1 80% Ethanol crude extract from *Phyllantus amarus*

1.2.2 80% Ethanol crude extract from *Stephania venosa*

1.2.3 Acetone crude extract from *S. venosa*

1.2.4 Ether crude extract from *S. venosa*

1.2.5 Petroleum ether crude extract from *S. venosa*

1.2.6 Ethyl acetate crude extract from *S. venosa*

1.2.7 Silica gel column fraction number 12-17 from *S. venosa*

1.2.8 Silica gel column fraction number 18-19 from *S. venosa*

1.2.9 Silica gel column fraction number 20-23 from *S. venosa*

These extracts were prepared and deposited by Assoc. Prof. Dr. Omboon Luanratana, Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University (Figure 9).

1.3 Culture media

Chemicals and Reagents	Catalog No.	Company
RPMI 1640 medium	T121-01	Biochrom
Fetal calf serum	S0215	Biochrom
Trypsinase	L2133	Biochrom
L-glutamine	25030-149	Gibco BRL
Sodium bicarbonate	478537	Carlo Erba
Streptomycin	M7185	M & H
Penicillin G	E911015	GPO, Thailand
Sterile water	1590	GPO, Thailand

1.4 Chemical reagent for purification of OVS1 monoclonal antibody

Chemicals and Reagents	Catalog No.	Company
Protein A Sepharose 4B	17-0780-01	Amersham
Sodium dihydrogen phosphate	471	Univar
Disodium hydrogen phosphate	1235	Unilab
Sodium citrate	106430	Merck
Tris	22664	USB

1.5 Chemical reagents for identification of OVS1 monoclonal antibody

1.5.1 Chemical reagents for measuring the quantity of OVS1 MAb

Chemicals and Reagents	Catalog No.	Company
Micro BCA protein assay		
Reagent MA	1851630	Pierce
Reagent MB	23235	Pierce
Reagent MC	1851650	Pierce

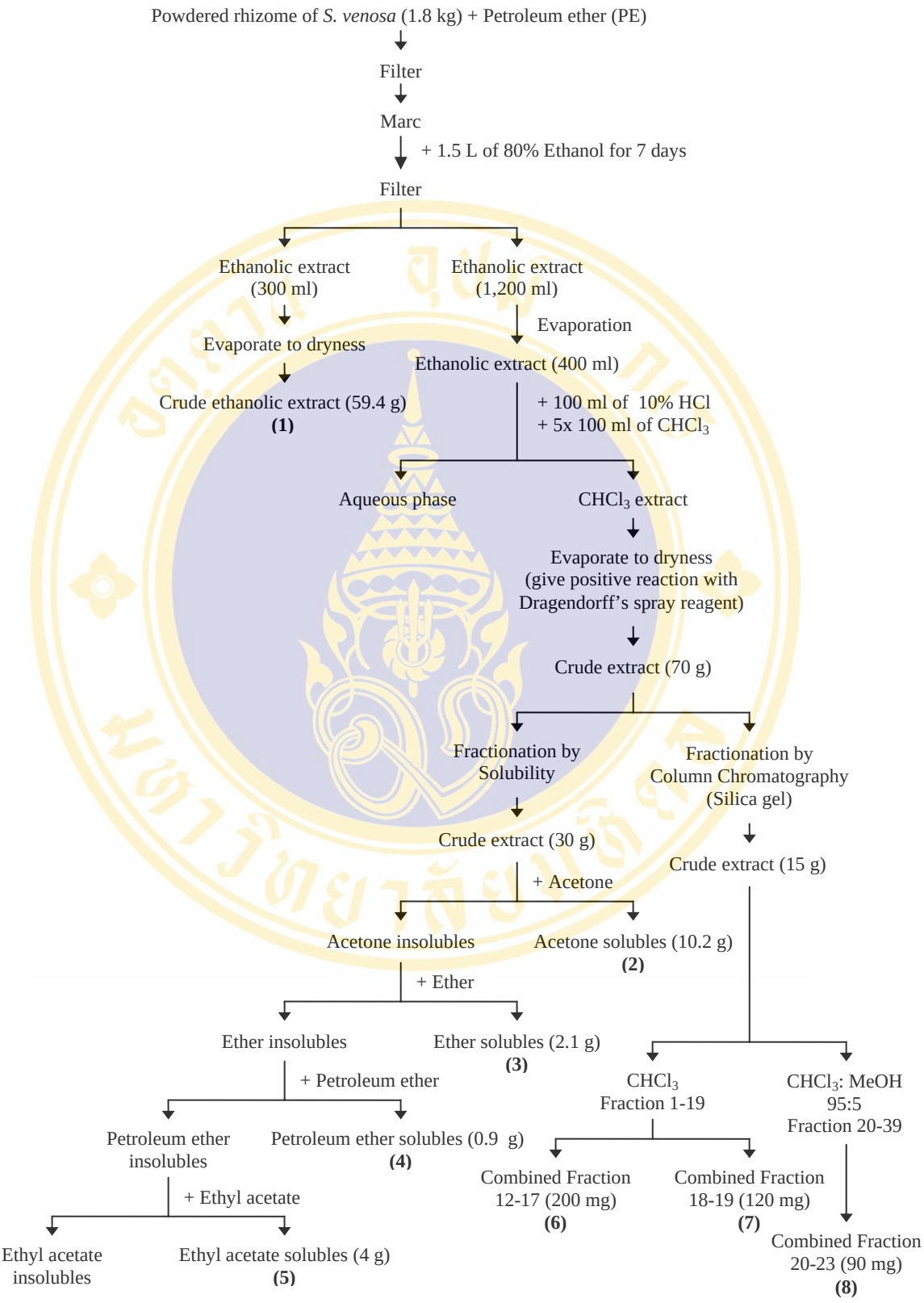


Figure 9 The Preparation of test samples from *S. venosa* extract.

1.5.2 Chemical reagents for determination of OVS1 purity by SDS-PAGE

Chemicals and Reagents	Catalog No.	Company
Acrylamide PAGE	17-1302-01	Pharmacia
Methylene bis-acrylamide	17-1304-01	Pharmacia
Ammonium persulfate	17-1311-01	Pharmacia
Sodium dodecyl sulfate	75819	USB
Tris	22664	USB
Glycine	16407	USB
Glycerol	104094	Merck
Bromphenol blue	108122	Merck
TEMED	161-0800	Bio-Rad
Kaleidoscope polypeptide standard	85611	Bio-Rad

1.6 Chemical reagents for cell viability evaluation by MTT test

Chemicals and Reagents	Catalog No.	Company
MTT	M2128	Sigma
Hanks's balanced salt solution	H1387	Sigma
Isopropanol	UN1219	BDH

1.7 Chemical reagents for determination of cell morphological changes

Chemicals and Reagents	Catalog No.	Company
Acetone	10003	BDH
Hoechst 33342	B2261	Sigma
Propidium iodide	P4170	Sigma
Methanol	Me0315	Scharlau

1.8 Chemical reagents for determination of DNA fragmentation by DNA ladder assay

Chemicals and Reagents	Catalog No.	Company
Absolute ethanol	10107	BDH
Chloroform	100776B	BDH
Agarose gel	50000	SeaKem
Proteinase K	V302B	Promega
RNase	0675	Amresco

Chemicals and Reagents	Catalog No.	Company
EDTA	0105	Amresco
Sodium chloride	479687	Carlo Erba
Isoamyl alcohol	9038-03	Baker Analyzed
Ethidium bromide	102225	Merck
Sample loading buffer	83068	Bio-Rad

1.9 Chemical reagents for determination of DNA damage by Comet assay

Chemicals and Reagents	Catalog No.	Company
Agarose gel	50000	SeaKem
Low melting agarose gel	V2111	Promega
Tris	22664	USB
Sodium chloride	479687	Carlo Erba
EDTA	0105	Amresco
Proteinase K	V302B	Promega
Triton X-100	22686	USB
DMSO	A3534	Labscan
Sodium hydroxide	480507	Carlo Erba
Ethidium bromide	3011E	Research Organics

1.10 Chemical reagents for determination of phasphatidylserine by Annexin V assay

Chemicals and Reagents	Catalog No.	Company
Annexin V-FITC kit test	65874	Becton Dickinson

1.11 Chemical reagents for determination of DNA content for cell cycle analysis

Chemicals and Reagents	Catalog No.	Company
Sodium dihydrogen phosphate	471	Univar
Disodium hydrogen phosphate	1235	Unilab
Citric acid	244.1000	Merck
Triton X-100	22686	USB
RNase	0675	Amresco
Propidium iodide	P4170	Sigma

1.12 Chemical reagents for determination of reactive oxygen species by fluorescence probe

Chemicals and Reagents	Catalog No.	Company
DCFH-DA	D 6883	SIGMA
α-Tocopherol (vitamin E)	T 1157	SIGMA
Hydrogen peroxide (H_2O_2)	107298	Merck

1.13 Chemical reagent for determination of nitric oxide by Griess reagent

Chemicals and Reagents	Catalog No.	Company
Sodium nitrite	S3421	Sigma
Sodium nitroprusside	S-0501	Sigma
Sulfanilamide	S9251	Sigma
N-(1-naphtyl) ethylendiamine	N5889	Sigma
Phosphoric acid	UN1805/2796	Mallinckrodt

1.14 Equipments and instruments used in this study

Name	Company
Microplate reader	Molecular Devices
Inverted microscope	Olympus
Fluorescence inverted microscope	Zeiss
Transilluminator	LKB
FACSort flow cytometry	Becton Dickinson
Antibody purification system	Bio-Rad
Luminescence spectrometer	Perkin Elmer
Centrifuge	Clements PTY
Vortex mixer	Labnet
Water bath	Heto
Autoclave	Tomy Seiko
CO_2 incubator	Shel-lab, Flufrance
Biohazard laminar air flow	Flufrance
Spectrophotometer	Shimatzu
Plate shaker	Werk Janke & Kunkel
SpeedVac concentrator	Savant
Gel drying system	E-C Apparatus

2. Methods

2.1 Culture of SKOV3 and SKBR3 cell lines

SKOV3 and SKBR3 were continuously grown in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum. Ten milliliter of 100 μg/ml streptomycin and 100 units/ml of penicillin G were added in each liter of RPMI 1640 medium for antibiotic reagent. The culture cells were stored in humidified atmosphere of 5% CO_2 at 37°C.

2.2 Culture of OVS1 hybridoma cells

OVS1 hybridoma cells were continuously grown in RPMI 1640 medium supplemented with 5% (v/v) fetal calf serum, 10 mM glucose, 1.2 mM pyruvic acid and 0.7 mM L-glutamine. Ten milliliter of 100 μg/ml streptomycin and 100 units/ml penicillin G was added in each liter of RPMI 1640 medium for antibiotic reagent. The culture cells were stored in humidified atmosphere of 5% CO_2 at 37°C.

2.3 Preparation of OVS1 monoclonal antibody from OVS1 hybridoma cells

The supernatant from OVS1 hybridoma cells was harvested when OVS1 cell lines secreted a high titer of OVS1 monoclonal antibody (OVS1 MAb) that the color of medium was changed from a pink color to yellow color. The cell pellets were removed from supernatants by centrifugation at 4°C and 5,000 rpm for 10 min. Supernatant consisted of OVS1 MAb was then filtrated throughout cellulose acetate filter membrane (pore size 0.45 μm) for separating bacterial contaminant. The clear yellow supernatant of OVS1 MAb was collected and stored at 4°C before purification.

2.4 Purification of OVS1 monoclonal antibody

Immunoaffinity purification is a highly biospecific, reversible chromatographic separation techniques that can be used to purify proteins (200). OVS1 MAb containing IgG_1 MAb, was purified by using Protein A Sepharose 4B affinity chromatography.

The protein A Sepharose 4B column at bed volume about 6 ml was washed with 0.05 M phosphate buffer saline (PBS) pH 8.0 before loading OVS1 MAb supernatant. OVS1 MAb supernatant was applied into column. Unbound proteins from supernatant after were removed by 0.05 M PBS pH 8.0. Bound protein of OVS1 MAb was eluted by 0.01 M sodium citrate pH 3.0 about 3 volumes of column. One milliliter

of OVS1 MAb fraction was collected into each tube containing 100 µl of 2 M Tris-HCl pH 9.0 for pH neutralization of the eluant. The neutral eluant was measured the absorbance at 280 nm. The high absorbance of OVS1 MAb fractions were pooled in the same dialystic bag. The pooled eluant was dialyzed with 0.05 M PBS pH 7.4. Dialysed OVS1 MAb was concentrated by SpeedVac concentrator (Savant) and then stored in aliquot portion at -80°C before further experiments (12,13).

2.5 Measurement of OVS1 monoclonal antibody concentration by Micro BCA protein assay kit

The reagent of Micro BCA protein assay kit (PIERCE) were a high sensitive reagent for the spectrophotometric determination of protein concentration in diluted solutions. Bicinchoninic acid (BCA) was utilized as a chelating agent for Cu^{1+} forming a color complex in the presence of protein (196). This reagent kit was a pre-formulated reagent system that was supplied in three separate parts.

1. Micro reagent A (MA): contains sodium carbonate, sodium bicarbonate and sodium tartrate in 0.2N sodium hydroxide.
2. Micro reagent B (MB): contains 4% BCA in water.
3. Micro reagent C (MC): contains 4% cupric sulfate pentahydrate in water.

Working reagent in ratio of 50 parts MA to 48 parts MB to 2 parts MC should be prepared freshly and sufficiently. These reagents were stable for 24 h. One hundred microliters of various concentrations of standard or unknown protein samples were added into each microtiter well. Working reagent was added and mixed. All wells were incubated at 60^{o}C for 60 min and measured the absorbance at 560 nm by microplate reader (Thermomax, Molecular Devices) (196). The graph was plotted between the value of optical density (Y-axis) and various concentrations of standard protein (X-axis). The concentrations of unknown proteins were calculated from curve (196,197).

2.6 Determination of OVS1 monoclonal antibody by SDS-PAGE method

SDS-PAGE was served to identify the purity and evaluated the molecular weight of OVS1 MAb. This method was performed as originally described by Laemmli and modified by Bio-Rad Mini-Protean 3 Electrophoresis Cell Technique. The slab gel contains 7.5% acrylamide for separating gel and 4.0% acrylamide for stacking gel. The elements of both gels were demonstrated in Table 8.

Table 8 Components per one gel of polyacrylamide gel (Laemmli's system) (203).

Components	7.5% separating gel	Components	4.0% stacking gel
Distilled water	2,425 µl	Distilled water	1,590 µl
1.5M Tris-HCl pH 8.8	1,250 µl	0.5M Tris-HCl pH 6.8	625 µl
10% SDS	50 µl	10% SDS	25 µl
30% Acrylamide-bis stock solution	1,250 µl	30% Acrylamide-bis stock solution	250 µl
TEMED	2.5 µl	TEMED	2.5 µl
10% Ammonium persulfate	25 µl	10% Ammonium persulfate	25 µl
Total volume	5,000 µl	Total volume	2,517 µl

Samples were treated with sample buffer (Appendix A) in a ratio of 1:1 and boiled for 5 min prior to loading. SDS-PAGE running was performed at constant current of 100 volts until the tracking dye reached the bottom of gel. Kaleidoscope prestained standard (Bio-Rad) was served as protein markers. It contained individually color proteins that consisted of six polypeptides with molecular weights ranging from 18.4 to 216 kD (Table 10) (199). After running, the protein bands on gel were visualized by Coomassie blue staining for the proper time and then destained in destaining solution (Appendix A) until its clear background and the sharp bands occur.

SDS-PAGE was frequently used to determine the molecular weight of protein since protein migration is generally proportional to the mass of the protein. A standard curve was plotted between the semi-log graph of the known protein molecular weights (Y-axis) and their R_f (X-axis).

- **Calculation of R_f value**

$$R_f \text{ value} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

A standard curve was generated from proteins that known molecular weight and the molecular weight of the interesting proteins could be extrapolated from this curve. Therefore, the distances of OVS1 MAb polypeptide bands were measured to calculate the molecular weights of OVS1 MAb compared with the standard curve of protein marker (200,201).

2.7 Culture conditions of SKOV3 and SKBR3 cell lines

Both SKOV3 and SKBR3 cell lines were continuously cultured and grown as monolayer. These lines were cultured in RPMI 1640 medium containing 5% (v/v) fetal calf serum, incubated in humidified atmosphere at 37^{o}C and 5% CO_2. Streptomycin (100 μg/ml) - penicillin G (100 units/ml) were used to prevent the contamination of other particles. Trypsinase enzyme at concentration of 0.01% trypsin in EDTA/PBS pH 7.4 was used to detach cells from surface. After trypsinization, cells were washed with RPMI 1640 medium without fetal calf serum and centrifuged about 5 min to remove trypsin and other wastes. Cell pellets were resuspended with culture medium until became single cells, then distributed thoroughly in flask. SKOV3 and SKBR3 could attach on surface again after incubation about 2 to 3 h at optimum conditions. In each experiment, cells were counted at a suitable density by inverted microscope on hemocytometer (202).

2.8 Preparation of stock solution of medicinal plant extracts

Each medicinal plant extract from *P. amarus* and *S. venosa* was dissolved in RPMI 1640 medium containing 5% (v/v) fetal calf serum and 10% (v/v) dimethyl sulfoxide (DMSO) as 1,000 μg/ml stock solution and stored at 4^{o}C until further experiments within 1 month. Stock solution were diluted with culture medium to obtain the tested concentrations before treating to cells in each experiment. The concentration of DMSO in medicinal plant solutions at tested concentrations should not effect against cell growth and viability (22,203).

2.9 Antiproliferative assay

2.9.1 Cell viability evaluation by MTT test

Cytotoxicity assay was a conventional method to assess the number of viable cells growing in microtiter plate. The assay presented the changing of colorimetric formation in wells and measuring by automatic microplate reader as described by Mosmann (21). The only living cells could reduce the yellow tetrazolium salt MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] to insoluble blue formazan crystal by intracellular succinate dehydrogenase. Therefore, this procedure could be evaluated the inhibitory dose of medicinal plants on cancer cells (21,22,204).

Fifty microliters of 1 x 10^4 cells in culture medium were plated into each well of 96-well plate containing 50 μl of various concentrations of samples ranging from 0 to 200 μg/ml and incubated at 37°C, 5% CO_2, for 48 h. After incubation, media were then removed by carefully inverting, flicking or blotting the plates and incubated with 50 μl of 1,000 μg/ml of MTT in Hank's balanced salt solution (HBSS) at 37°C for 2 h. MTT solutions were leaved and 50 μl of isopropanol were added into each well. Plates were then gently shaken to dissolve formazan crystal by plate shaker (IKA-Werk Janke & Kunkel) at 25°C for 5 min and the intensity of the colored formazan derivative were determined by a microplate reader (Thermomax, Molecular Devices) at 590 nm (21,22,23,24,204). The results were shown in line graph between the percentage of cell viability (Y-axis) and the concentrations of each sample (X-axis).

- **Calculation of the percentage of cell viability**

$$\text{The percentage of cell viability} = \frac{\text{O.D. of treated cells}}{\text{O.D. of untreated cells}} \times 100$$

The ED_{50} could calculate from this curve. It was defined as the 50% reduction of the absorbance or 50% of the percentage of cell viability compared with untreated cells in MTT assay (204).

2.10 Apoptotic assay

2.10.1 Determination of morphological changes

This procedure was used to observe morphological changes of cells after treated with plant extracts. Two fluorochromes, bisbenzimide dye (Ho33342) and propidium iodide (PI), were utilized to stain cells. Ho33342 was the membrane-permeant dye which could label nuclei of all cells such as living and apoptotic cells (29). But PI, the membrane-impermeant dye, could label nuclei of dead cells that were late apoptotic and necrotic cells (30). However, PI can label nuclei of living and early apoptotic cells after treated with acetone:methanol for breaking cell membrane thus PI can permeabilize to nucleus (205).

Cells were plated at density of 2 x 10^5 cells per a 35 x 10 mm cell culture dish and incubated for 12 h until cell attachment. Cells were treated with proper concentrations of plant extracts and OVS1 MAb which followed the ED_{50} value of cytotoxic MTT assay at culture condition for 48 h. Three condition-criteria were established to observe the morphological changes:

1). Control or treated cells were not stained and took a series of pictures by phase contrast inverted microscope.

2). Cells were stained with Ho33342 and observed by fluorescence inverted microscope (Axiovert 25, Zeiss).

3). PI was used to visualize the nuclear changes of cells at the same of Ho33342 and observed by fluorescence inverted microscope (Axiovert 25, Zeiss).

To improve the accuracy of this method, the measurement amount of apoptotic cells needed the connection of computer and specific program with the fluorescence microscope.

2.10.1.1 Observation of cell by phase contrast microscope

After cells were treated with plant extracts and OVS1 MAb, media were removed and treated cells were washed once time with HBSS. They were observed by phase contrast inverted microscope (Axiovert 25, Zeiss) at 400 magnification. Membrane blebbing and apoptotic body can be observed but nuclear shrinkage and fragmentation were not determined.

2.10.1.2 Benzimidazole Ho33342 staining

After incubation of cells with plant extracts and OVS1 MAb, media were removed and cells were washed once time with HBSS. Five hundred microliters of 1 μg/ml of Ho33342 were utilized to stain cells and incubated at 37°C for 30 min in the dark. Living and apoptotic cells (nuclear fragmentation and shrinking) were visualized by blue filter of fluorescence inverted microscope (Axiovert 25, Zeiss) at 400 magnification (12,13,29,204).

2.10.1.3 Propidium iodide (PI) staining

Media were removed from dishes after treated with extracts and cells were washed once time with HBSS and permeabilized with acetone:methanol (1:1) at -20°C for 10 min. Cells were washed once time with HBSS to get rid of acetone and methanol then 5 μg/ml of PI in HBSS were used to stain the nuclear changes of cells. PI solution was incubated with cells at 37°C for 30 min in the dark. Nuclear changes of cells were detected by green filter of fluorescence inverted microscope (Axiovert 25, Zeiss) at 400 magnification (12,13,30,205).

2.10.2 Determination of DNA fragmentation by DNA ladder assay

The presence of oligonucleosome fragmentation of DNA can demonstrate apoptotic cell after treated with plant extracts (24).

Cells were cultured at density of 3 x 10^6 cells per 5 ml RPMI 1640 medium containing 5% (v/v) fetal calf serum in 60 x 15 mm cell culture dish at 37°C and 5% CO_2 for 24 h. Cells treated with various concentrations of plant extracts and untreated cells were incubated at the same culture condition for 24 h. After incubation, cells were detached from plate by gently scraping and packed the cell pellets by centrifugation at 14,000 rpm for 5 min. Media were removed and cells were washed once time with 0.01 M PBS pH 7.4. A hundred microliters of lysis buffer (Appendix A) were added in each tube to break cell membrane. Cell pellets were resuspended with lysis buffer and then incubated at 50°C for 1 h. After that, 10 μl of 20 mg/ml of proteinase K were mixed with cells in lysis buffer and continuously incubated at 50°C for 30 min. Cell suspensions in lysis buffer were then added with 3 μl of 10 mg/ml of RNase enzyme and incubated at 50°C for 2 h. Additionally, chloroform:isoamyl alcohol (24:1) were added into each tube to remove proteins and other wastes from DNA solution by centrifugation at 14,000 rpm for 15 min. DNA fragments could be

dissolved in upper supernatant which were collected and stored at 4^{o}C before gel eletrophoresis (24,201).

Solution of DNA fragment was mixed with loading dye (Bio-Rad) in the suitable ratio and dropped into each well of 1.5% agarose gel and ran electrophoresis at 100 volts. Ladder of DNA fragment was stained with 0.05% ethidium bromide in water for about 30 min and destained a few minute to eliminate excess ethidium bromide with distilled water until ladders were sharp and background of gel was clear. Ladder of DNA fragment can be observed by transilluminator (202).

2.10.3 Determination of DNA damage by the comet assay

The comet assay originally introduced by Ostling and Johansen (31,152). The comet assay has been widely used to detect DNA damage in cells exposed *in vitro* and *in vivo* to variety of physical or chemical agents. The severe DNA damage is a common endpoint in many detecting system for detect DNA damage. The comet assay has proven to be an extremely sensitive and reliable method of choice in several studies (32,153,154).

Five hundred microliters of cells at density of 1 x 10^5 cells per each well of 24-well plate were cultured and incubated at the same culture condition for 24 h. After cell attachment, cells in each well were treated with various concentrations of plant extract for 48 h. Cells were detached by 0.01% trypsin in EDTA/PBS pH 7.4 and washed once time with 0.01 M PBS pH 7.4. Four hundred fifty microliters of 1% normal melting agarose (NMA) were applied as base layer on microscope slide and covered immediately with a coverslip. The treated cells were resuspended in 200 µl of 0.75% low melting agarose (LMA) which temperature not more than 37°C and applied to the second layer on the base layer at the same slide and covering again with a coverslip. Low melting agarose was allowed to solidify on ice. Both of LMA and NMA were dissolved in PBS. The coverslip was removed after solidify of agarose. The agarose slide was placed into an alkaline lysis solution (Appendix A) overnight at 4°C and then transferred to electrophoresis by soaking in cold alkaline electrophoresis buffer (Appendix A) for 30 min to allow the DNA to unwind before applying a current at 256 mA, 25 volt for 30 min. The agarose slide was washed three times for 5 min with neutralization buffer (Appendix A) and stained with 60 µl of 20 µg/ml ethidium bromide per slide. The slide was recovered with a coverslip at 4°C in humidified box

and kept in the dark for 30 min. DNA damage was observed by green filter of fluorescence inverted microscope (Axiovert 25, Zeiss).

2.10.4 Determination of phosphatidylserine by Annexin V-FITC assay

The membrane phosphatidylserine (PS) of early apoptotic cells was translocated from the inner to the outer plasma membrane leaflet. Annexin V was a protein which had a high affinity for PS (33). Annexin V-FITC kit (Becton Dickinson) was specifically utilized to measure the amount of apoptotic cells in each extract. Viable cell was not stained, late apoptotic and necrotic cells were stained with PI in test kit. Annexin V-FITC can only bind cells which was in early apoptotic stage.

Five hundred microliters of cells at density of 2×10^5 cells per each well of 24-well plate were cultured and incubated at the general culture condition for 24 h. Cells in each well were treated with various concentrations of each extract for 48 h. After incubation, cells were detached by 0.01% trypsin in EDTA/PBS pH 7.4 and washed once time with 0.01 M PBS pH 7.4. The cell pellet was added with 100 μl of cool binding buffer. Then, 5 μl of 5 μg/ml of Annexin V and 5 μl of 50 μg/ml of PI were added and cell pellets were gently resuspended and mixed with these reagents thoroughly. Each tube was incubated at 25^oC for 15 min in the dark. Four hundred microliters of cool binding buffer were added into each tube and slightly mixed again after incubation. The quantitative analysis of ratio of living cells, early apoptotic cells, and late apoptotic and necrotic cells in each extract were analysed by flow cytometry (FACSort, Becton Dickinson) (33,160).

2.11 Cell cycle analysis

2.11.1 Determination of DNA content on cell cycle analysis

DNA content of cell was detected by DNA stain. Generally, propidium iodide (PI) is the most commonly used dye for DNA content and cell cycle analysis. PI can be used to stain whole cells or isolated nuclei. This dye intercalates into the major groove of double-stranded DNA and produces a highly fluorescent adduct that can be excited at 488 nm with a broad emission centre around 600 nm that facilitates its use on the benchtop cytometers. PI can also bind to double-stranded RNA so is necessary to treat the cells with RNase for optimal DNA resolution (34,162).

Five hundred microliters of cells at density of 5 x 10^6 cells were cultured in each well of 24-well plate and incubated for 24 h for cell attachment. After, cells were treated with various concentrations of plant extracts at various time. Cells were harvested every 8 h and washed twice time with 0.01 M PBS pH 7.4. The cell pellets were collected by centrifugation at 5,000 rpm for 5 min. Cell pellets were resuspended and fixed by transferring into 1.5 ml of 70% cool ethanol in eppendroff on ice. Cells were stored in the fixative at -20°C for further test. Cells were centrifuged and thoroughly decanted ethanol and then added with 50 µl DNA extraction buffer (Appendix A). Cells were incubated in water bath at 37°C for 30 min on the shaker. The cell pellets from centrifugation were resuspended in 1 ml of cell dye for cell cycle analysis (Appendix A) at room temperature for 15 min in the dark. Cells were measured fluorescence for PI detection at red wavelengths in a flow cytometer (FACSort, Becton Dickinson).

2.12 Antioxidative assay

2.12.1 Determination of reactive oxygen species by fluorescence probe

Reactive oxygen species (ROS), i.e., hydroxy radical ($^{\bullet}OH$), superoxide radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2), were created from cancer cells after treating with inducer. Several protocols were used to detect them. DCFH-DA, 2′,7′-dichlorodihydro fluorescein diacetate, was served as fluorescence probe to estimate the level of cytosolic ROS. Cytoplasmic esterase could cleave DCFH-DA to H_2DCF that was finally converted to DCF, a fluorescent molecule, by cytosolic peroxide. The intensity of DCF, which was measured by luminescence spectrophotometer, correlated with the level of cytosolic ROS. Since the level of ROS without inducer in both untreated and treated cells were produced in low level which cannot detected by luminescence spectrophotometer. Therefore, H_2O_2 was used as an inducer that could increase the production of ROS in cells that ROS level can be detected.

Five hundred microliters of 2 x 10^5 cells per well were plated into 24-well plate at 37^{o}C, 5% CO_2 for 24 h until cells attached. Cells were treated with various concentrations of plant extracts for 48 h, then trypsinized and washed once time with 0.01 M PBS pH 7.4. Five hundred microliters of 10 µM DCFH-DA in PBS

were added into each tube. Cells were resuspended and incubated at 37^{o}C and 5% CO_2 for 30 min in the dark and then washed by centrifugation at 5,000 rpm for 5 min. Five hundred microliters of 4 mM H_2O_2 were loaded into each tube and incubated at the same condition. The level of cytosolic ROS in each tube was measured by microplate luminescence spectrophotometer (Perkin Elmer) with excitation and emission at 485 and 530 nm respectively. The results were expressed as the percentage of relative amount of cytosolic ROS (Y-axis) compared with each concentration of plant extract (X-axis).

- **Calculation of the percentage of ROS production**

$$\text{ROS production (\%)} = \frac{\text{Fluorescence intensity of treated cells}}{\text{Fluorescence intensity of untreated cells}} \times 100$$

2.12.2 Determination of nitric oxide by Griess reaction

The Griess reaction provides a simple method to determine the cellular production of nitric oxide ($^{\bullet}$NO) by measuring its stable product, nitrite, in the cell culture supernatant (180,181). Cells synthesize nitric oxide by utilizing different isoforms of the enzyme nitric oxide synthase (NOS) which convert L-arginine to L-citrulline and NO. In the presence of oxygen NO decomposes to nitrite (NO^{-}_{2}) and nitrate (NO^{-}_{3}). This assay relies on a diazotization reaction that was originally described by Griess in 1879. The Griess reagent system is based on the chemical reaction, which uses sulfanilamide and N-1-napthylenediamine dihydrochloride (NED) under acidic (phosphoric acid) conditions added to cell culture supernatants converts nitrite into a purple azo dye which can be quantified photometrically and thus used as a parameter for the NO synthesis of cultured cells (185,186,187,188,189). This system detects NO_2^- in variety of biological and experimental liquid matrixes. The nitrite sensitivity is dependent on the matrix (43,44,45).

Cells were plated at density of 5 x 10^5 cells per each well of 24-well plate and incubated until cell attachment. Cells in each well were treated with 10 μM Sodium nitroprusside (SNP) and various concentrations of each extract for 24 h. The 500 μl of supernatant were mixed with 500 μl of freshly prepared Griess reagent (1 volume of 0.1% naphthylethylene diamine dihydrochloride in distilled water plus 1 volume of 1% p-aminobenzene-sulfonamide in 5% phosphoric acid). The mixtures

were protected from light for 10 min at room temperature. The optical density were measured at 540 nm (Thermomax, Molecular Devices) comparing with a standard curve of known concentrations of sodium nitrite.

- **Calculation of the percentage of NO production**

$$\text{NO production (\%)} = \frac{\text{Fluorescence intensity of treated cells}}{\text{Fluorescence intensity of untreated cell}} \times 100$$

2.13 Statistical analysis

Each experiment was performed at least in triplicate. Results were expressed as the mean value ± SD. Statistical analysis was performed using an Anova statistic by SPSS version 10. *P* values < 0.05 were considered significantly different from control group (209).

CHAPTER IV
RESULTS

1. Preparation of OVS1 monoclonal antibody from OVS1 hybridoma cells

OVS1 hybridoma cells were cultured in RPMI 1640 medium containing 10% (v/v) fetal calf serum and growth factors supplements (as discussed in Chapter III). The supernatant was harvested when OVS1 hybridoma cells secreted a high titer of OVS1 monoclonal antibody (MAb). The pink color of supernatant was changed to yellow color which presumed the high quantity of immunoglobulin (IgG). The supernatant was collected, after cultured OVS1 hybridoma cells, by centrifugation and filtration to remove the cell pellets and debris. The OVS1 MAb supernatant was stored at 4°C until further purification.

2. Purification of OVS1 monoclonal antibody

OVS1 MAb was purified from collected OVS1 MAb supernatant by using Protein A Sepharose 4B affinity chromatography.

The first step, OVS1 MAb supernatant was loaded into column (Figure 10, no.1). Unbound protein was washed through column by 0.05 M PBS pH 7.4. OVS1 MAb containing mouse IgG had affinity to bind with protein A, therefore, OVS1 MAb was not removed from column. Unbound protein was washed out until the curve decreased to baseline which indicated the complete removal of nonspecific proteins (Figure 10, no.2). Then, bound OVS1 MAb was eluted by 0.1 M sodium citrate buffer pH 3 and a peak curve of OVS1 MAb polypeptide was obtained (Figure 10, no.3). After, OVS1 MAb was eluted completely and the absorbance curve became baseline again (Figure 10, no.4). The column was washed with 0.05 M PBS pH 7.4 until column was equilibrated with this buffer.

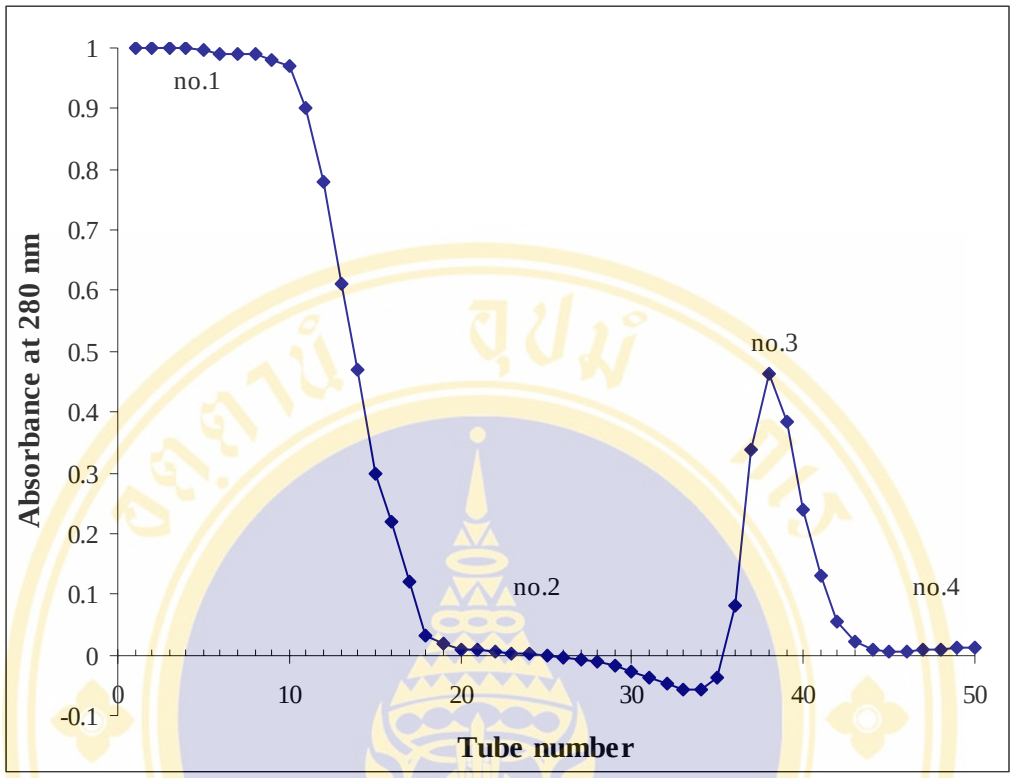


Figure 10 Purification of OVS1 MAb by Protein A Sepharose 4B affinity chromatography.

no.1 OVS1 MAb supernatant was loaded into column.

no.2 Unbound proteins were removed through column.

no.3 OVS1 MAb was eluted by 0.1 M sodium citrate buffer pH 3.0.

no.4 Column was washed after elution and equilibrated with 0.05 M PBS pH 7.4.

The tubes no. 36 through 43 (Figure 10) which gave high absorbance at 280 nm were pooled and dialyzed against 0.05 M PBS pH 7.4. The OVS1 MAb pooled fractions were concentrated by SpeedVac concentrator (Savant) and stored in aliquot volumes at -80°C until further experiments.

3. Measurement of OVS1 monoclonal antibody concentration by Micro BCA protein assay kit

Micro BCA protein assay kit or Bicinchoninic acid assay was a highly sensitive reagent for measuring the amount of diluted protein. The correlativity of bovine serum albumin (BSA) concentration and absorbance at 560 nm were plotted as a standard curve. The approximate concentration of OVS1 MAb obtained from the standard curve (Figure 11) and the concentrations from five purify-experiments were shown in Table 9.

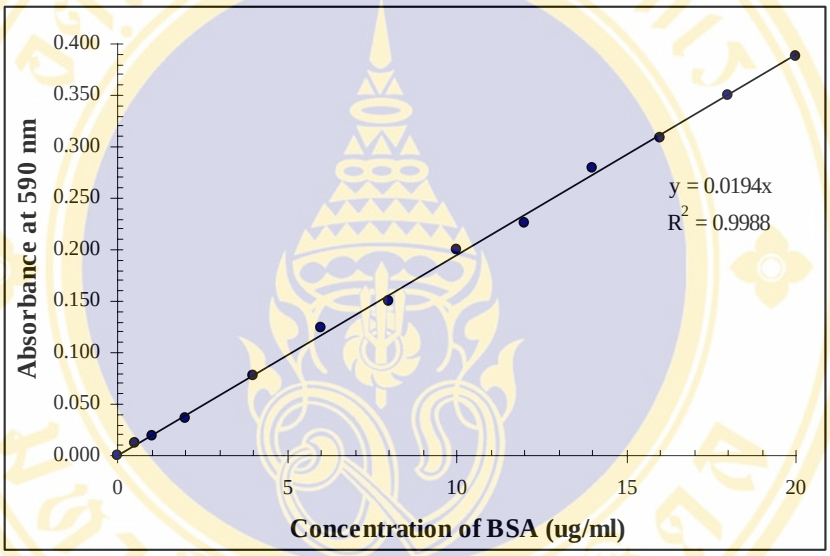


Figure 11 The standard curve of bovine serum albumin (BSA) by Micro BCA kit test.

Table 9 The concentrations of OVS1 MAb in each lot.

OVS1 MAb Lot No.	Concentrations of OVS1 MAb (μg/ml)	Volume (ml)	Total amount concentration (μg)
1	2142.0 ± 2.14	1.4	2998.8
2	1428.0 ± 3.37	1.3	1856.4
3	1464.0 ± 1.85	1.0	1464.0
4	1071.0 ± 0.96	1.2	1285.2
5	928.2 ± 2.02	1.2	1113.8

4. Determination of OVS1 monoclonal antibody by SDS-PAGE method

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) at reducing condition was performed to detect the purity and molecular weight of purified OVS1 MAb. The patterns of protein bands of OVS1 MAb supernatant before and after purification were demonstrated in Figure 12. Supernatant after purification contained purified OVS1 MAb with major proteins at reduced heavy and light chains positions, approximately 50,000 and 25,000 D, respectively. This IgG can bind specifically with SKOV3 and SKBR3 cell lines by immunobinding-ELISA assay. Purified OVS1 MAb was confirmed its specificity by double sandwich ELISA as reported by Nuttavut (13). Molecular weights and R_f values of Kaleidoscope protein standard marker were shown in Table 10 and were expressed in the standard semi-log curve in Figure 13.

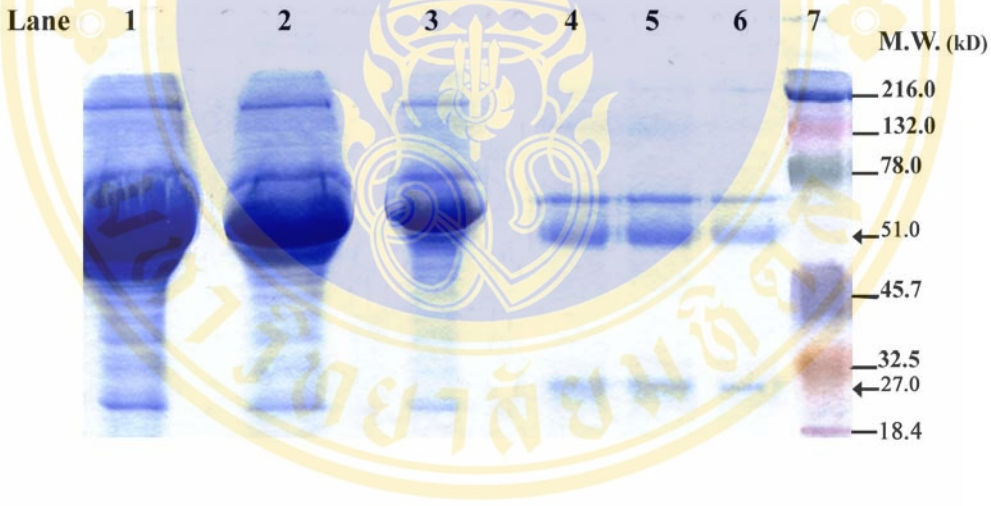


Figure 12 Polyacrylamide gel electrophoresis of OVS1 monoclonal antibody. Slab gel contained 7.5% and 4.0% acrylamide gel of separating and stacking gels, respectively.
Lane 1-3: OVS1 MAb supernatant before purification
Lane 4-6: OVS1 MAb supernatant after purification
Land 7: Kaleidoscope protein standard marker

Table 10 The relations of molecular weight and R_f value of Kaleidoscope protein Standard.

Band No.	Type of Protein	Color	Molecular weight (D)	R_f value
1	Myosin	Blue	216,000	0.157
2	B-galactosidase	Magenta	132,000	0.255
3	BSA	Green	78,000	0.392
4	Carbonic anhydrase	Violet	45,700	0.647
5	Soybean trypsin inhibitor	Orange	32,500	0.843
6	Lysozyme	Red	18,400	0.980

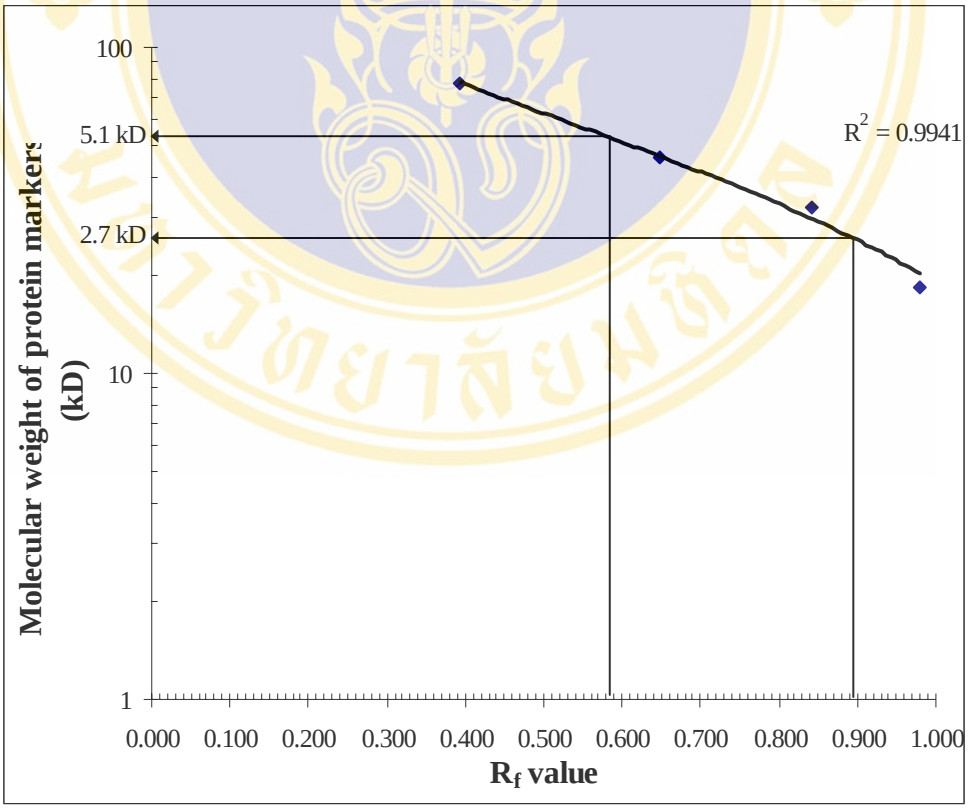


Figure 13 The standard semi-log graph was showed between molecular weight of each polypeptide of Kaleidoscope standard with their R_f values.

The estimated molecular weight of OVS1 MAb gained from the standard semi-log curve of molecular weights of known proteins and their R_f values of Kaleidoscope standard. Molecular weight of OVS1 MAb was extrapolated from the curve showing that OVS1 MAb composed of 2H (heavy) chains of 51,000 D each and 2L (light) chains of 27,000 D each. The report of Abbas et al. (210) and Stites et al. (211) demonstrated molecular weigh of heavy chain of IgG was 50 through 70 kD and molecular weight of light chain of IgG was 23 through 24 kD.

Table 11 Molecular weights of OVS1 MAb polypeptides from the graph.

Band No.	R_f value	molecular weight (D)
1	0.588	51,000
2	0.901	27,000

Chemotherapeutic activities of extracts from medicinal plants

5. Antiproliferative assay

5.1 Cell viability evaluation by MTT test

The MTT assay is a reduction of tetrazolium bromide to formazan product by the mitochondrial enzyme succinate-dehydrogenase by living but not by dead cells. MTT assay is potentially very useful for assaying cell survival and proliferation (12).

MTT method was performed to demonstrate the percentage of cell viability and ED_{50} of SKOV3 and SKBR3 after treated with various concentrations of OVS1 MAb and each extract from medicinal plants. The effects were obtained in triplicate from each experiment (Table 12-14, Figure 14-18).

5.1.1 The antiproliferative assay of OVS1 MAb against SKOV3 and SKBR3 cell lines

Table 12 The percentage of cell viability after treated with OVS1 Mab.

Concentrations of OVS1 Mab (μg/ml)	The percentage of cell viability	
	SKOV3 cells	SKBR3 cells
0.00	100.00 ± 6.78	100.00 ± 8.74
0.01	98.67 ± 5.79	99.65 ± 5.21
0.05	95.39 ± 3.42	96.82 ± 3.63
0.50	90.46 ± 4.78	89.73 ± 9.17
1.25	82.43 ± 5.70	79.85 ± 5.56
2.50	72.86 ± 7.33	70.92 ± 6.89
5.00	58.55 ± 6.78	65.34 ± 5.72
12.50	20.82 ± 3.95	25.76 ± 5.78
25.00	-5.78 ± 3.47	-12.43 ± 7.86
50.00	-7.86 ± 2.43	-16.78 ± 1.78
ED_{50} (μg/ml)	**6.71 ± 1.32**	**7.91 ± 1.45**

1. SKOV3 cells against OVS1 Mab. **2. SKBR3 cells against OVS1 MAb.**

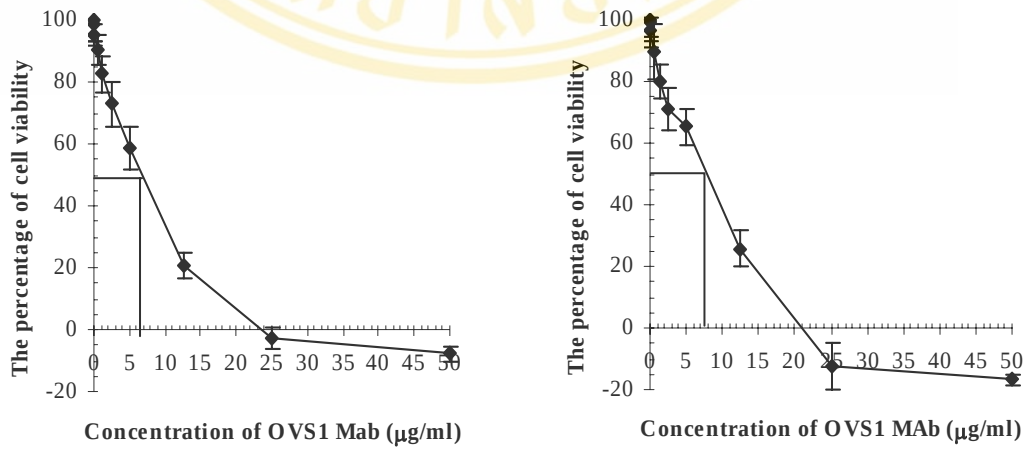


Figure 14 MTT test results of **SKOV3** and **SKBR3** cells treated with **OVS1 MAb**. The ED_{50} were **6.71 ± 1.32** and **7.91 ± 1.45** μg/ml respectively.

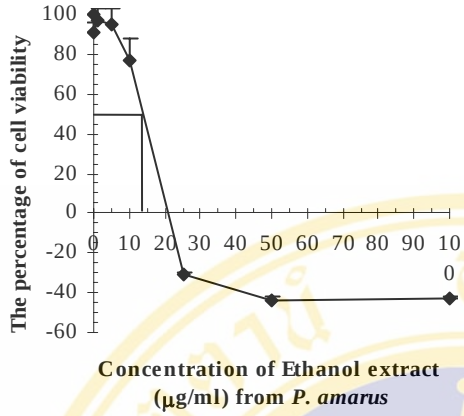
5.1.2 The antiproliferative assay of medicinal plant extracts against SKOV3 cells

Table 13 The percentage of cell viability of **SKOV3** after treated with extracts.

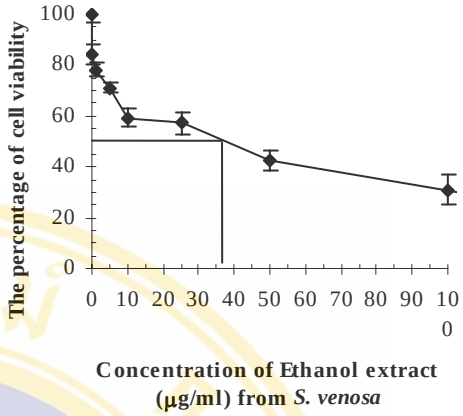
Concentration of medicinal plant	The percentage of viability cells of SKOV3 after treated with samples								
		S. venosa							
	P. amarus EtOH	EtOH	Acetone	Ether	Petroleum ether	Ethyl acetate	F. 12-17*	F. 18-19*	F. 20-23*
0	100 ± 6.23	100 ± 3.37	100 ± 4.89	100 ± 4.50	100 ± 4.68	100 ± 2.88	100 ± 5.05	100 ± 3.39	100 ± 4.39
0.1	90.72 ± 5.47	84.30 ± 3.96	95.31 ± 3.33	83.33 ± 8.40	75.13 ± 2.11	78.95 ± 2.87	74.62 ± 6.27	74.44 ± 5.52	70.64 ± 4.21
1	96.68 ± 6.27	78.33 ± 2.39	89.02 ± 6.81	76.86 ± 8.80	69.26 ± 5.15	76.04 ± 5.70	65.15 ± 2.93	58.59 ± 5.04	63.07 ± 5.27
5	94.71 ± 8.78	70.99 ± 2.01	35.13 ± 6.19	54.53 ± 6.61	53.71 ± 7.44	44.83 ± 5.38	35.04 ± 2.41	23.40 ± 2.37	33.30 ± 2.90
10	76.64 ±11.16	59.22 ± 3.58	19.45 ± 1.89	39.48 ± 9.23	44.39 ± 5.40	29.04 ± 3.08	33.14 ± 3.67	16.51 ± 3.41	27.25 ± 1.39
25	-30.95 ± 1.48	57.17 ± 4.51	12.70 ± 1.77	-3.56 ± 2.62	13.47 ± 2.62	-0.36 ± 3.95	-12.03 ± 1.76	-14.19 ± 1.94	-9.28 ± 1.38
50	-43.82 ± 1.54	42.83 ± 3.99	-3.20 ± 1.71	-26.86 ± 3.45	-17.96 ± 1.61	-24.50 ± 5.08	-21.12 ± 1.86	-16.93 ± 1.36	-25.13 ± 1.85
100	-43.22 ± 1.63	31.06 ± 5.86	-8.24 ± 2.30	-29.45 ± 2.62	-26.25 ± 2.92	-25.59 ± 4.45	-20.83 ± 2.42	-16.51 ± 1.28	-26.34 ± 2.76
200	-42.54 ± 1.48	5.12 ± 3.03	-7.09 ± 0.79	-29.94 ± 2.71	-26.60 ± 2.99	-26.68 ± 5.38	-19.22 ± 2.24	-16.60 ± 1.21	-27.14 ± 2.15
ED_{50} (µg/ml)	13.62 ± 0.96	35.11 ± 3.36	3.91 ± 0.48	6.67 ± 2.69	7.03 ± 6.61	4.38 ± 0.69	3.01 ± 0.24	1.95 ± 0.52	3.13 ± 1.59

***** Fraction 12-17, 18-19 and 20-23 were obtained after fractionation by silica gel column chromatography of *S. venosa*.

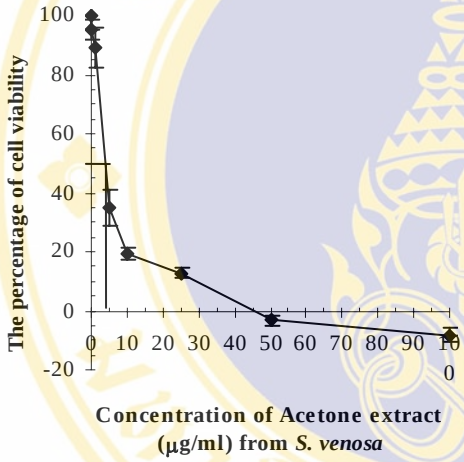
1. 80% Ethanol extract of *P. amarus*



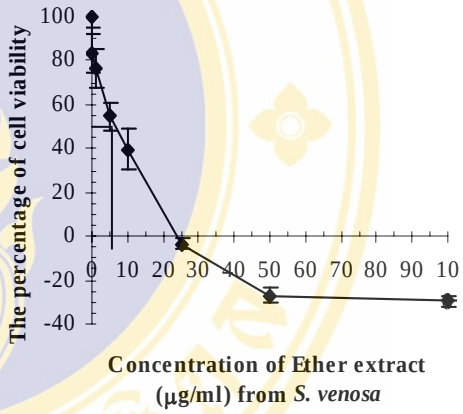
2. 80% Ethanol extract of *S. venosa*



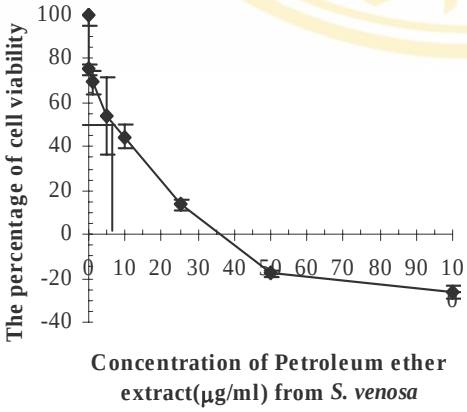
3. Acetone extract of *S. venosa*



4. Ether extract of *S. venosa*



5. Petroleum ether extract of *S. venosa*



6. Ethyl acetate extract of *S. venosa*

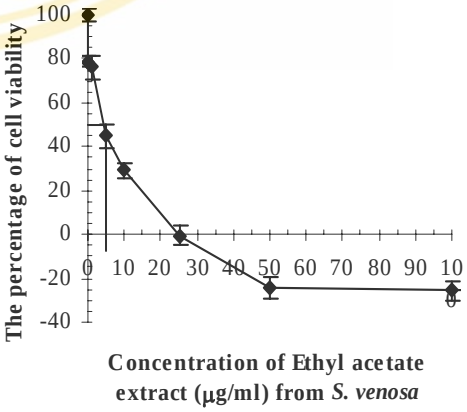
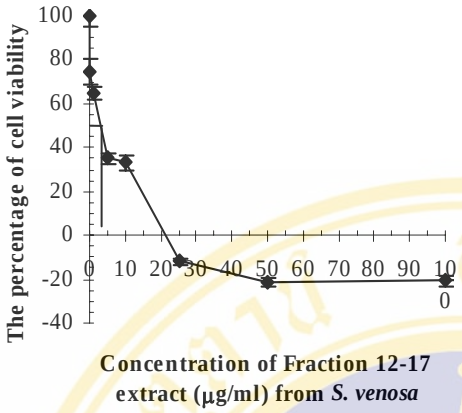
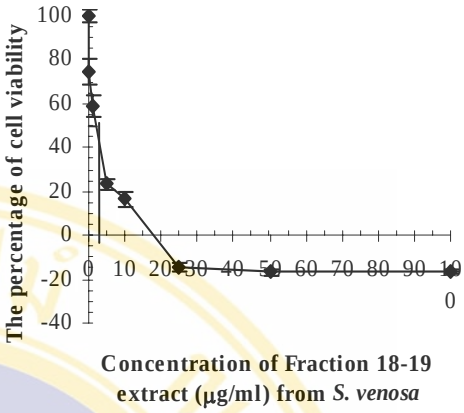


Figure 15 MTT test results of **SKOV3** cells treated with medicinal plant extracts from ***P. amarus*** and ***S. venosa***. The ED_{50} were determined from the MTT curve.

7. Fraction 12-17 extract of *S. venosa*



8. Fraction 18-19 extract of *S. venosa*



9. Fraction 20-23 extract of *S. venosa*

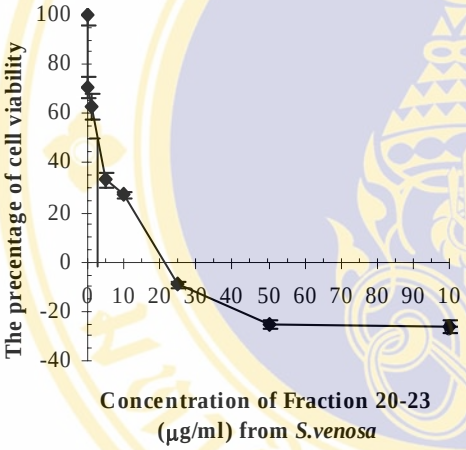


Figure 16 MTT test results of **SKOV3** cells treated with medicinal plant extracts from ***S. venosa***. The ED_{50} were determined from the MTT curve.

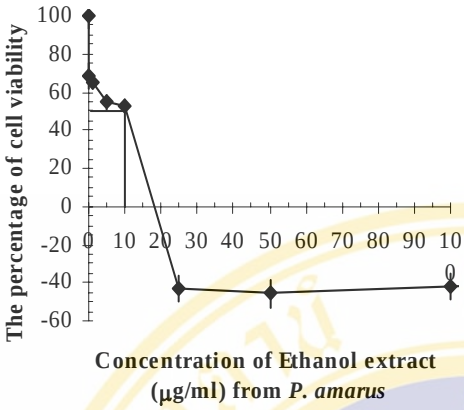
5.1.3 The antiproliferative assay of medicinal plant extracts against SKBR3 cells

Table 14 The percentage of cell viability of **SKBR3** after treated with extracts.

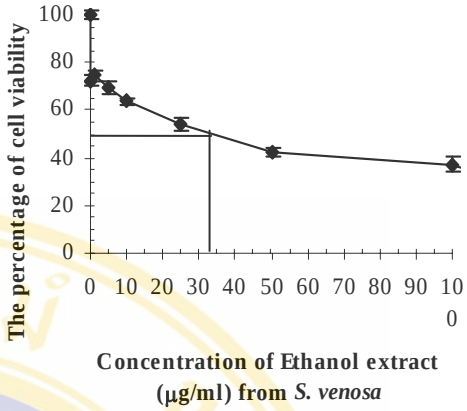
Concentration of medicinal plant	The percentage viability cells of SKBR3 after treated with samples								
		S. venosa							
	***P.amarus* EtOH**	**EtOH**	**Acetone**	**Ether**	**Petroleum ether**	**Ethyl acetate**	**F. 12-17***	**F. 18-19***	**F. 20-23***
0	100 ± 6.88	100 ± 2.01	100 ± 4.07	100 ± 9.65	100 ± 4.90	100 ± 1.11	100 ± 3.81	100 ± 7.01	100 ± 5.57
0.1	68.68 ± 6.51	72.51 ± 1.95	102.14 ± 5.16	75.56 ± 3.59	77.95 ± 7.54	75.95 ± 3.20	70.86 ± 8.59	70.06 ± 4.60	72.57 ± 5.15
1	65.26 ± 3.10	74.39 ± 2.39	89.00 ± 2.30	65.84 ± 6.67	67.50 ± 5.97	70.38 ± 9.97	70.25 ± 2.75	61.25 ± 2.68	70.23 ± 4.11
5	54.47 ± 3.97	69.35 ± 2.52	48.86 ± 1.57	45.39 ± 3.78	53.77 ± 4.62	50.13 ± 1.96	44.57 ± 3.22	38.38 ± 7.77	54.05 ± 2.03
10	52.63 ± 3.65	63.58 ± 1.49	43.00 ± 2.05	20.20 ± 6.69	39.07 ± 6.33	30.89 ± 1.77	35.63 ± 4.68	28.93 ± 6.41	43.48 ± 3.73
25	-43.16 ± 6.82	54.10 ± 3.08	19.14 ± 0.99	4.24 ± 7.21	35.78 ± 8.76	7.59 ± 6.64	18.68 ± 3.66	-2.66 ± 5.02	34.57 ± 3.47
50	-45.79 ± 7.57	42.41 ± 1.91	3.86 ± 1.50	-27.68 ± 6.69	27.85 ± 8.64	-12.15 ± 5.67	-22.34 ± 3.37	-19.74 ± 4.75	-29.90 ± 2.63
100	-42.11 ± 7.04	27.08 ± 3.11	-6.00 ± 2.40	-42.14 ± 9.58	-1.16 ± 6.73	-24.05 ± 4.85	-23.76 ± 3.08	-20.02 ± 4.84	-31.14 ± 1.92
200	-34.74 ± 1.20	-10.09 ± 1.31	-6.57 ± 3.11	-44.89 ± 8.14	-11.22 ± 7.38	-24.81 ± 3.92	-22.94 ± 2.90	-19.56 ± 4.68	-31.14 ± 2.92
ED_{50} (µg/ml)	10.44 ± 0.66	39.67 ± 0.60	5.24 ± 0.44	4.07 ± 0.75	6.42 ± 1.65	4.90 ± 2.43	4.19 ± 0.44	2.80 ± 1.67	7.03 ± 1.06

***** Fraction 12-17, 18-19 and 20-23 were obtained after fractionation by silica gel column chromatography of *S. venosa*.

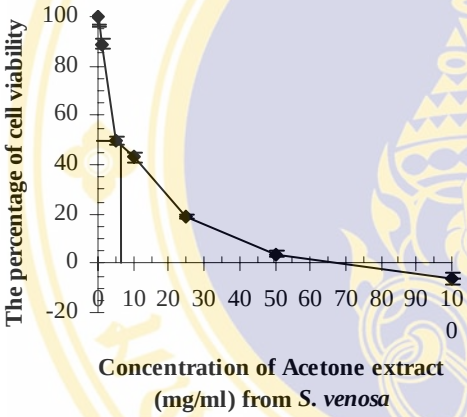
1. 80% Ethanol extract of *P. amarus*



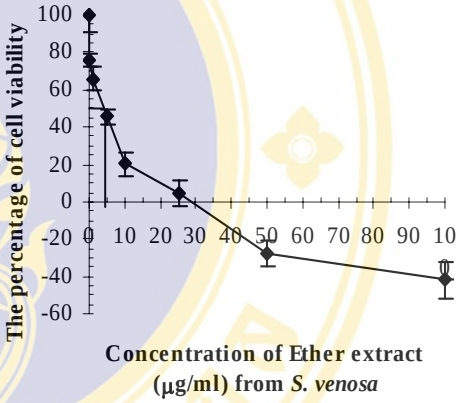
2. 80% Ethanol extract of *S. venosa*



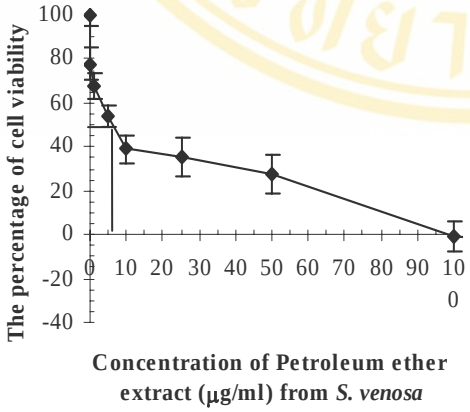
3. Acetone extract of *S. venosa*



4. Ether extract of *S. venosa*



5. Petroleum ether extract of *S. venosa*



6. Ethyl acetate extract of *S. venosa*

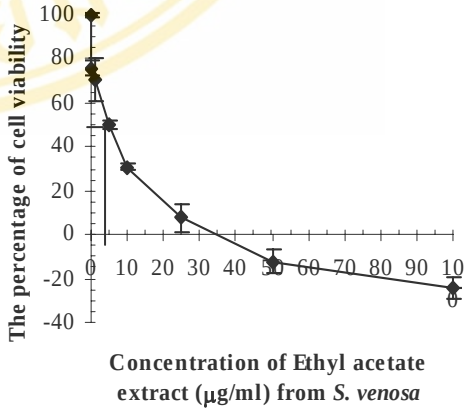
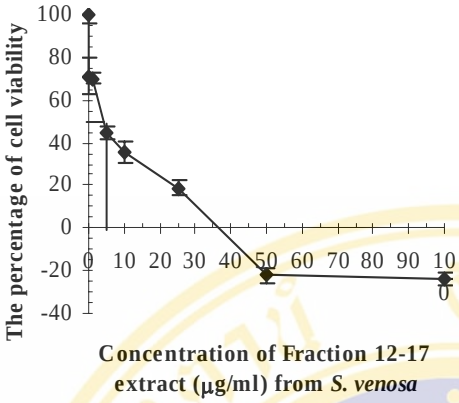
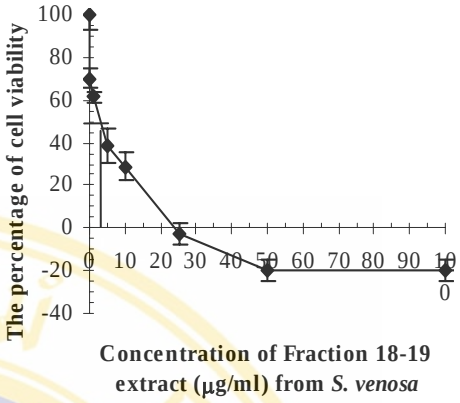


Figure 17 MTT test results of **SKBR3** cells treated with medicinal plant extracts from ***P. amarus*** and ***S. venosa***. The ED_{50} were determined from the MTT curve.

7. Fraction 12-17 extract of *S. venosa*



8. Fraction 18-19 extract of *S. venosa*



9. Fraction 20-23 extract of *S. venosa*

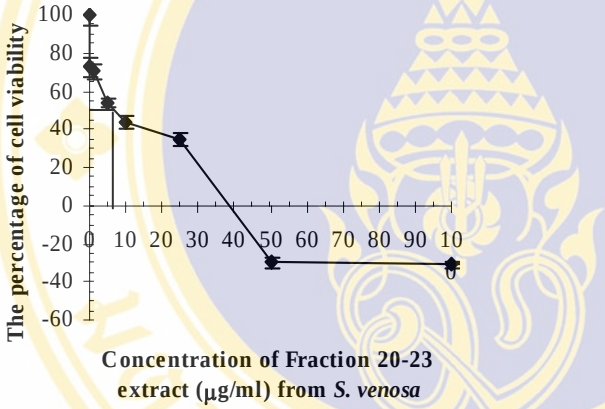


Figure 18 MTT test results of **SKBR3** cells treated with medicinal plant extracts from ***S. venosa***. The ED_{50} were determined from the MTT curve.

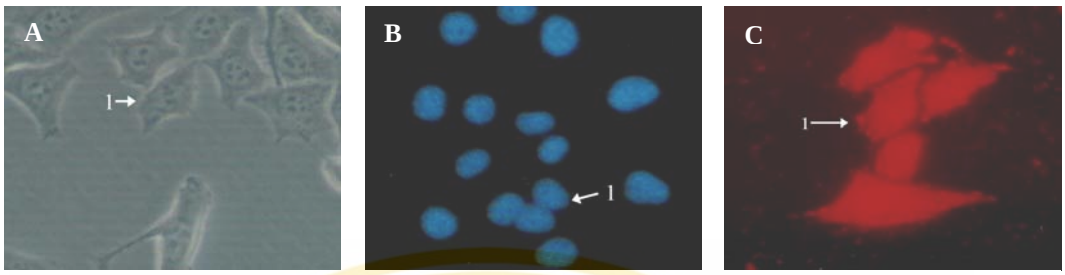
6. Apoptotic assay

6.1 Determination of morphological changes

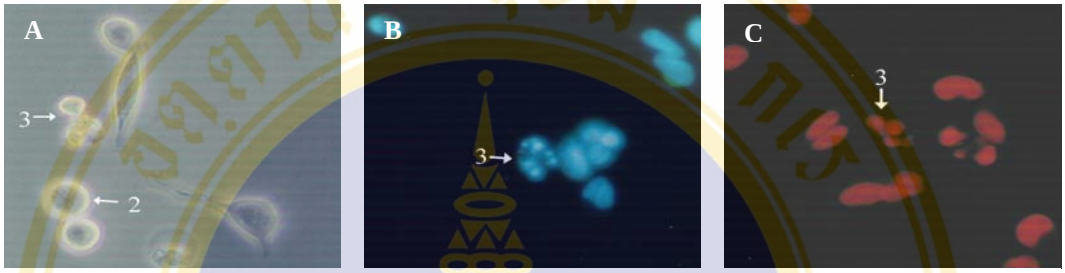
Both cultured cell lines were detected for apoptotic cell death after treated with the medicinal plant extracts and stained with fluorescence dyes. Apoptotic cells were observed by phase contrast inverted fluorescence microscope. Benzimidazole Ho33342 dye, a blue fluorochrome membrane-permeable, could pass through membrane of the cells and stained chromosome and nucleus and showed blue nucleus under fluorescence light. Propidium iodide, impermeant-dye, could permeate into cells after treated cell membrane with solution of acetone:methanol for detect nuclear changes. Red cells and nucleus were detected under fluorescence light. This method was the simplest method for classification of morphological change cells.

Morphological changes were shown distinguishing between different stages of typical necrosis and apoptosis. Necrosis cells were propidium iodide (PI) positive (red), showing generally swollen and round nuclei without any nuclei condensation. Early apoptosis cells were not stained by PI but exhibited classical nuclei alteration such as condensation and fragmentation visualized by Ho33342 stain (blue). During, the late stage of apoptosis, cell progressively lost their cell membranes integrity and PI became positive. These cells showed red-orange apoptotic nuclei due to transition of fluorescence color from blue to red PI fluorescence plus quenching of the Ho33342 fluorescence by energy transfer to PI.

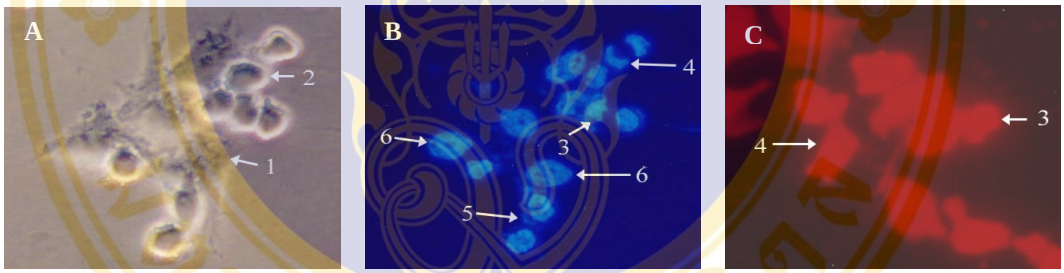
The effects of OVS1 MAb and medicinal plant extracts on SKOV3 and SKBR3 were expressed in Figure 19 and 20, respectively.



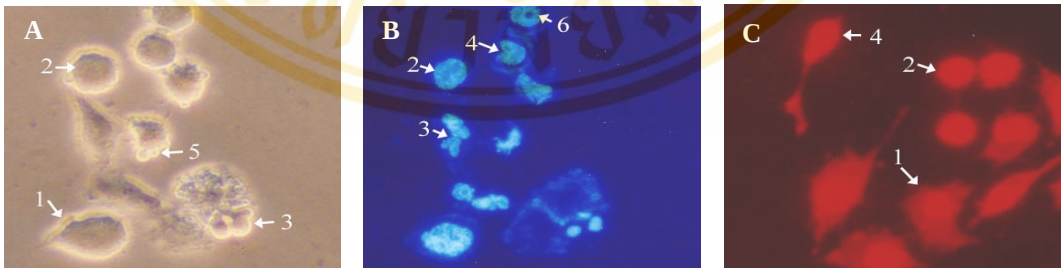
Untreated SKOV3



Cells were treated with **10** μg/ml of **OVS1 MAb**



Cells were treated with **30** μg/ml of **80% Ethanol extract** from ***P. amarus***



Cells were treated with **80** μg/ml of **80% Ethanol extract** from ***S. venosa***

Figure 19 Morphological characteristic of untreated **SKOV3** cells and **SKOV3** cells after treated with samples.

A: Cells were observed by phase contrast inverted microscope.

B: Cells were stained with Ho33342.

C: Cells were stained with PI.

Observation on SKOV3

1: SKOV3

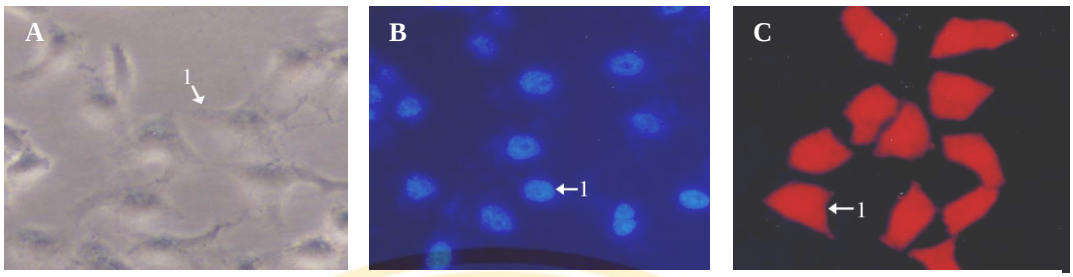
2: Apoptotic bodies

3: DNA fragmentation

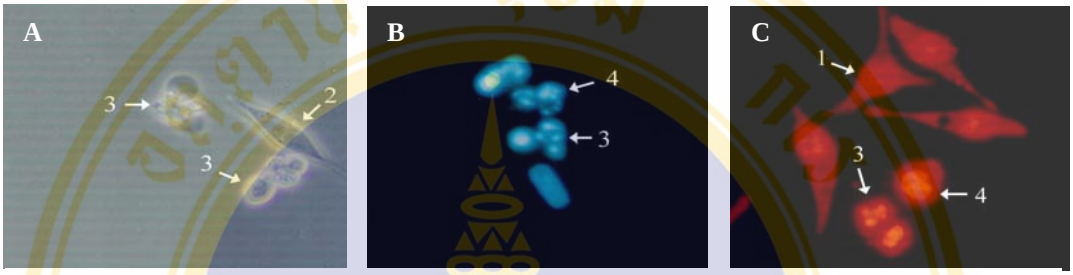
4: Nuclear shrinkage

5: Membrane blebbing

6: Vacuoles inside cell

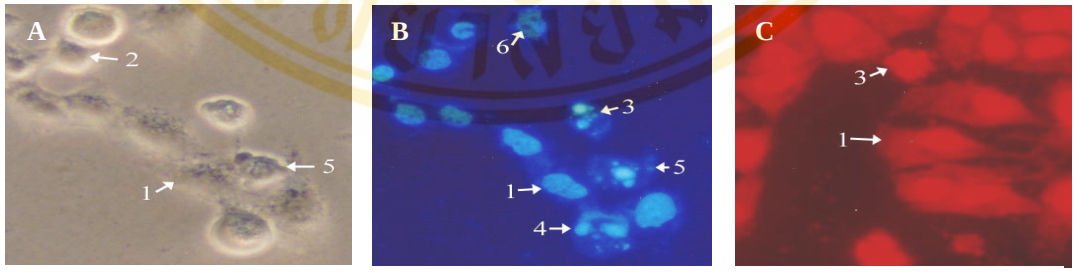


Untreated SKBR3



Cells were treated with **10** μg/ml of **OVS1 MAb**

Cells were treated with **30** μg/ml of **80% Ethanol extract** from ***P. amarus***



Cells were treated with **80** μg/ml of **80% Ethanol extract** from ***S. venosa***

Figure 20 Morphological characteristic of untreated **SKBR3** cells and **SKBR3** cells after treated with samples.

A: Cells were observed by phase contrast inverted microscope.

B: Cells were stained with Ho33342.

C: Cells were stained with PI.

Observation on SKBR3

1: SKBR3
2: Apoptotic bodies
3: DNA fragmentation
4: Nuclear shrinkage
5: Membrane blebbing
6: Vacuoles inside cell

6.2 Determination of DNA fragmentation by DNA ladder assay

The famous method for confirmation of apoptosis is DNA ladder assay. The hallmark of apoptosis is DNA fragmentation while DNA is splited up to small pieces about 180-200 bp of DNA. The fragmentation DNA is called DNA ladder that can be detected by gel electrophoresis of isolated DNA from apoptotic cells. Both treated cell lines, with each medicinal plant extract at various concentrations, were shown DNA ladder in following Figures 21-26.

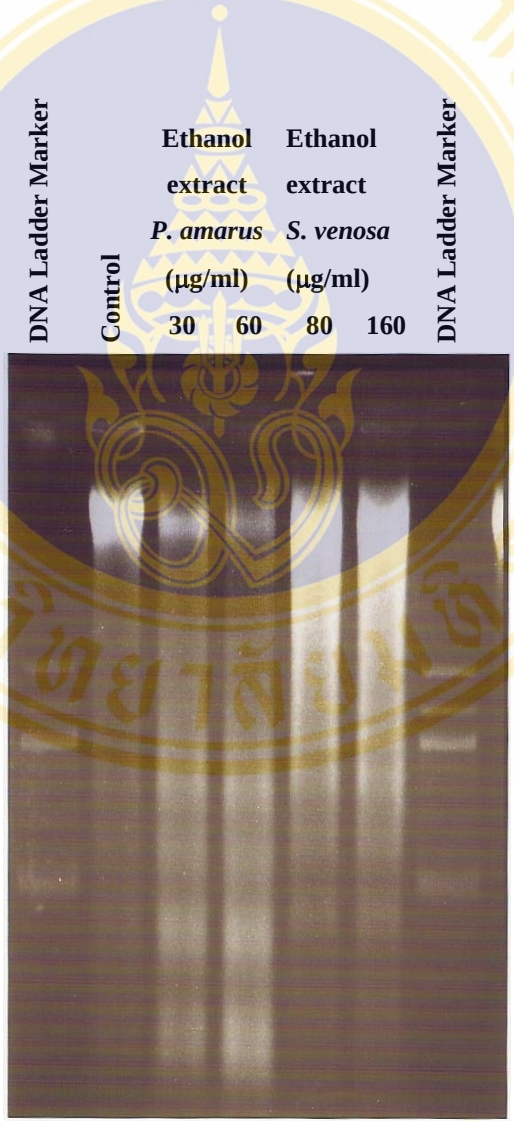
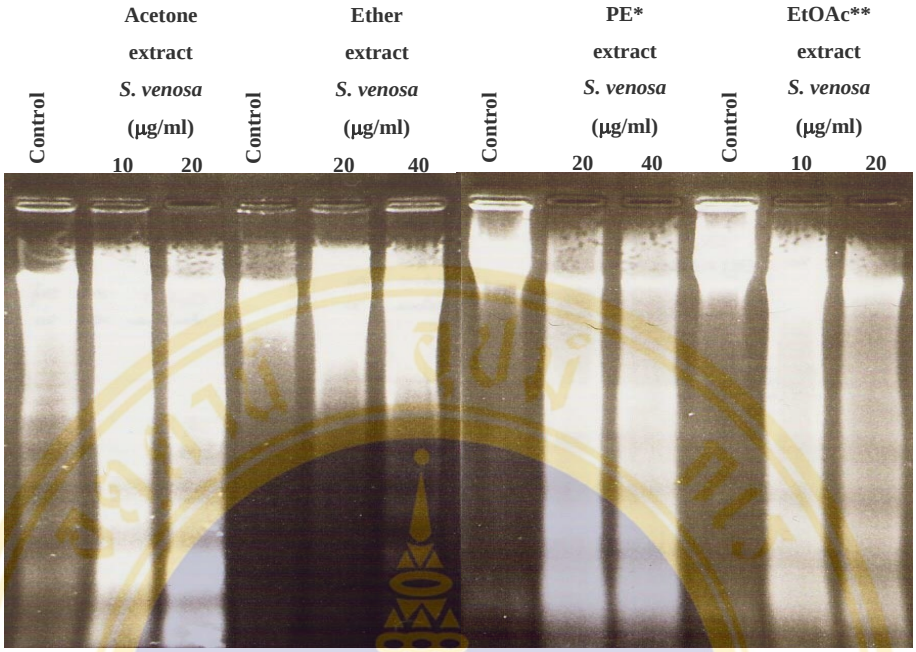


Figure 21 DNA ladder appearance of **SKOV3** cells after treated with **80% Ethanol extract** from ***P. amarus*** and ***S. venosa***.



***PE:** Petroleum ether

****EtOAc:** Ethyl acetate

Figure 22 DNA ladder appearance of **SKOV3** cells after treated with **Acetone, Ether, Petroleum ether** and **Ethyl acetate extract** from ***S. venosa***.

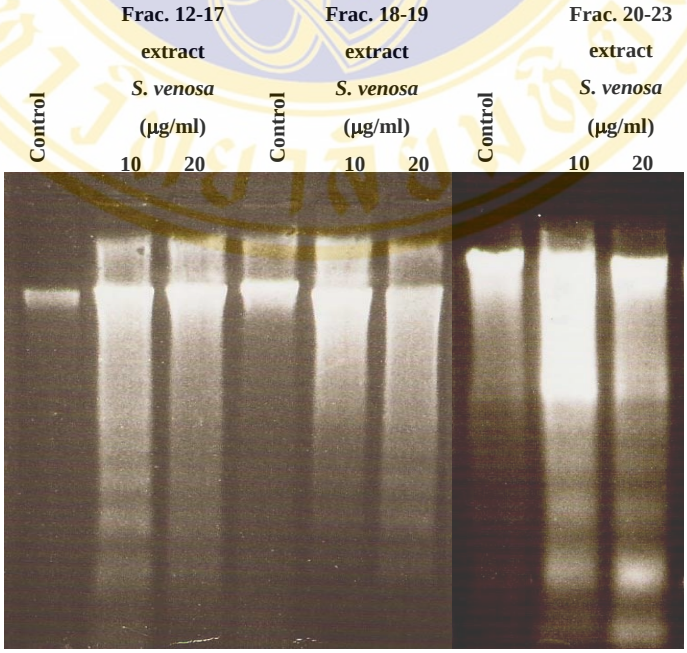


Figure 23 DNA ladder appearance of **SKOV3** cells after treated **with Fraction 12-17, 18-19** and **20-23 extract** from ***S. venosa***.

Figure 24 DNA ladder appearance of **SKBR3** cells after treated with **80% Ethanol extract** from ***P. amarus*** and ***S. venosa***.

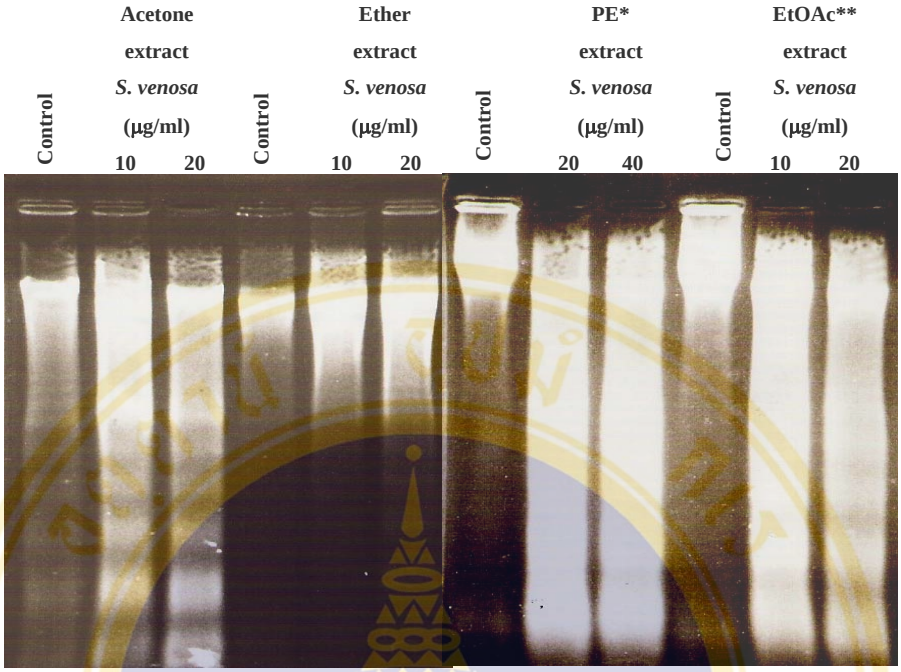


Figure 25 DNA ladder appearance of **SKBR3** cells after treated with **Acetone**, **Ether, Petroleum ether** and **Ethyl acetate extract** from ***S. venosa***.

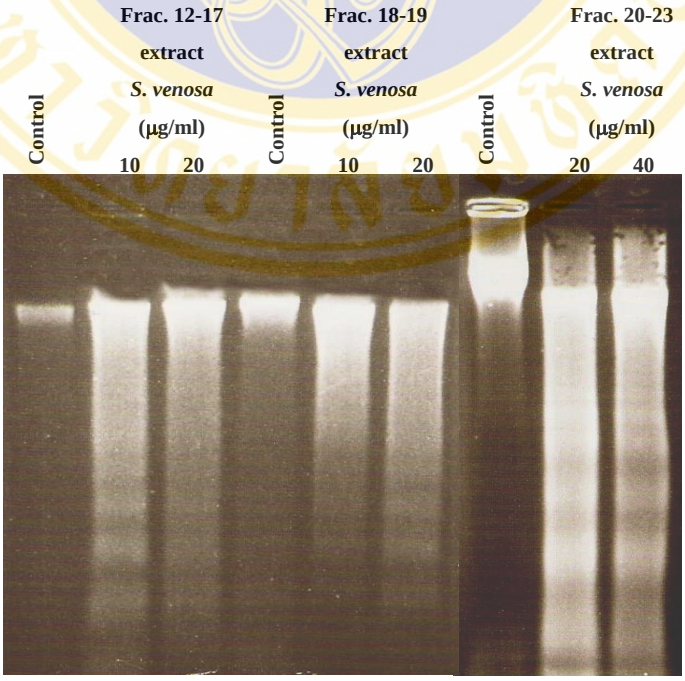


Figure 26 DNA ladder appearance of **SKBR3** cells after treated **with Fraction 12-17, 18-19** and **20-23 extract** from ***S. venosa***.

6.3 Determination of DNA damage by the comet assay

The comet assay is a simple, sensitive and rapid method for measuring DNA strand break in individual cell. The comet method can separate the treated cells so that undamaged cells retained their original profile. The appearances of comets were measured to determine the extent of DNA damage that depending on the dose of medicinal plant extracts. The results of comet assay on both cancer cells after treated with medicinal plant extracts were presented in Figure 27-28 and 29-30, respectively.

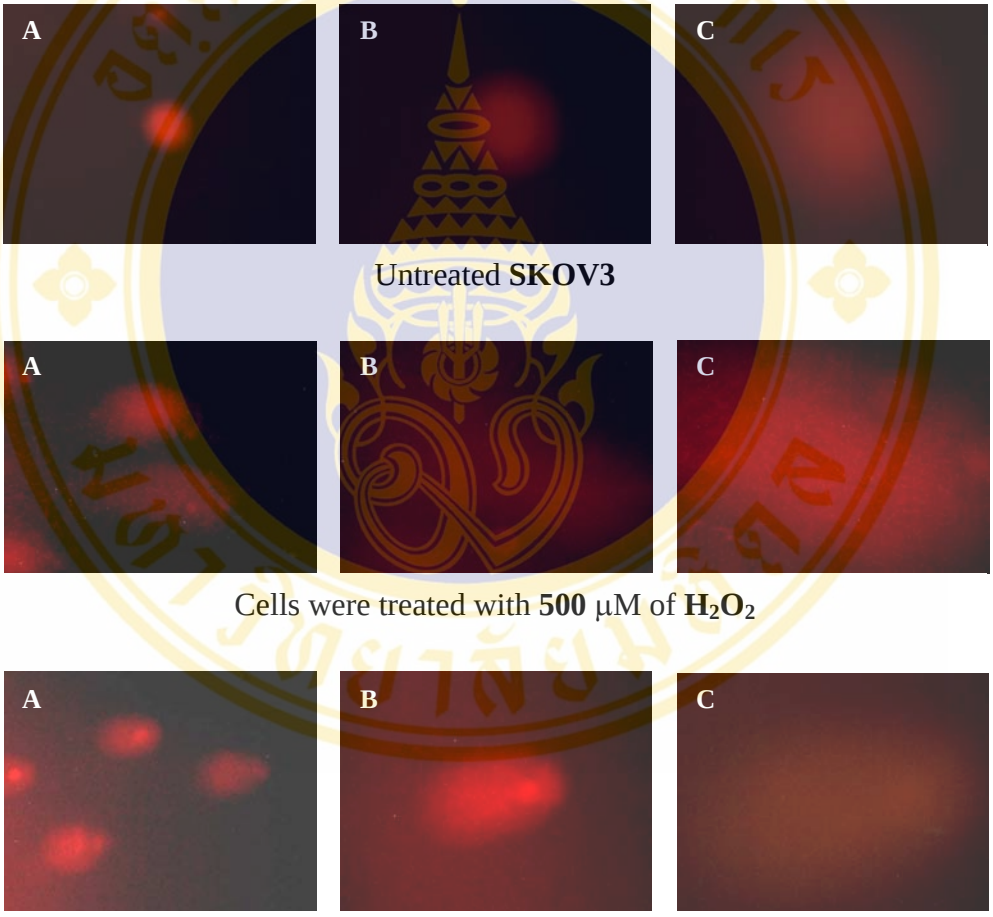


Figure 27 Comet assay to detect the DNA damaged features of **SKOV3** cells.

A: 10x magnification.

B: 20x magnification.

C: 40x magnification.

Cells were treated with **80** μg/ml of **80% Ethanol extract** from ***S. venosa***

Cells were treated with **10** μg/ml of **Acetone extract** from ***S. venosa***

Cells were treated with **10** μg/ml of **Fraction 18-19 extract** from ***S. venosa***

Figure 28 Comet assay to detect the DNA damaged features of **SKOV3** cells.

A: 10x magnification.

B: 20x magnification.

C: 40x magnification.

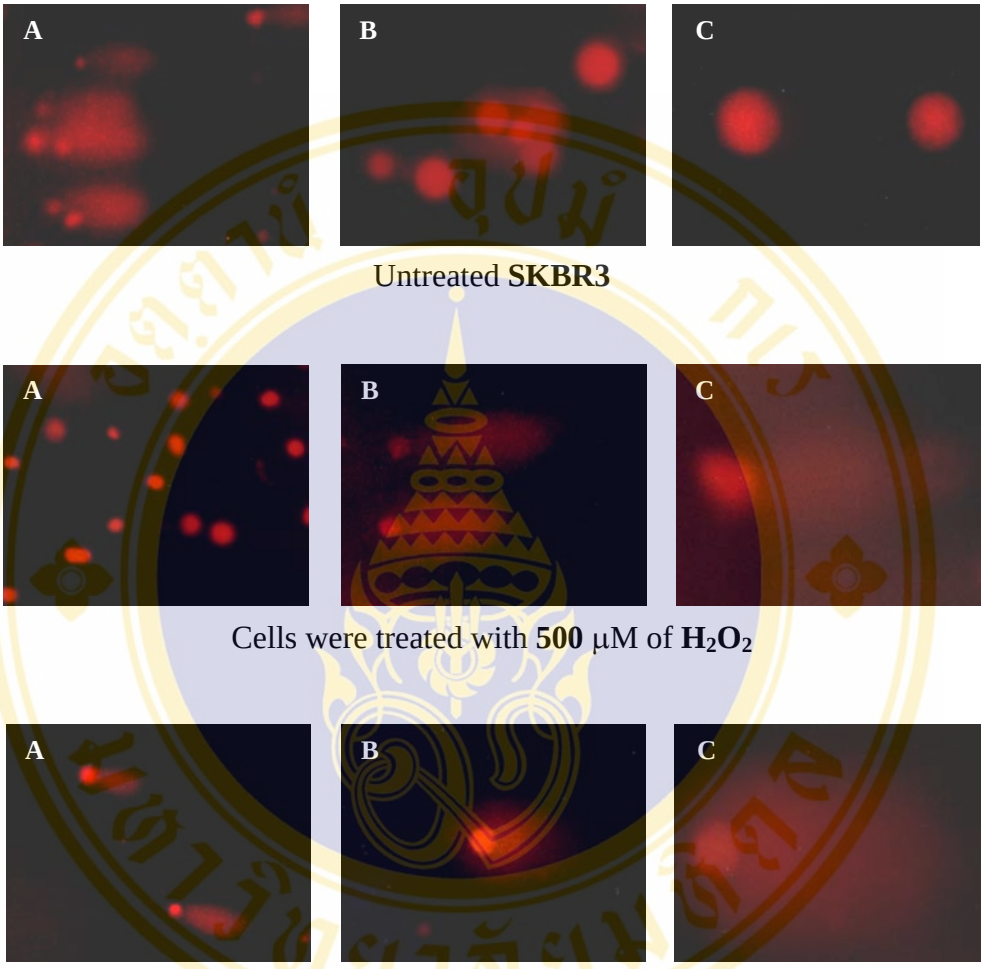
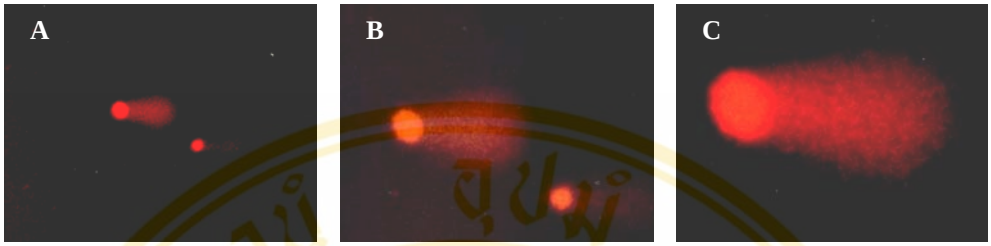


Figure 29 Comet assay to detect the DNA damaged features of **SKBR3** cells.

A: 10x magnification.

B: 20x magnification.

C: 40x magnification.



Cells were treated with **80** μg/ml of **80% Ethanol extract** from ***S. venosa***

Cells were treated with **10** μg/ml of **Acetone extract** from ***S. venosa***

Cells were treated with **10** μg/ml of **Fraction 18-19 extract** from ***S. venosa***

Figure 30 Comet assay to detect the DNA damaged features of **SKBR3** cells.

A: 10x magnification.

B: 20x magnification.

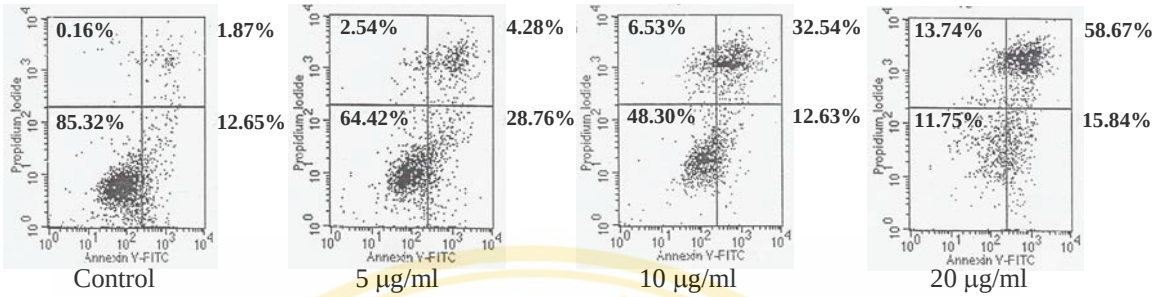
C: 40x magnification.

6.4 Determination of phosphatidylserine by Annexin V-FITC assay

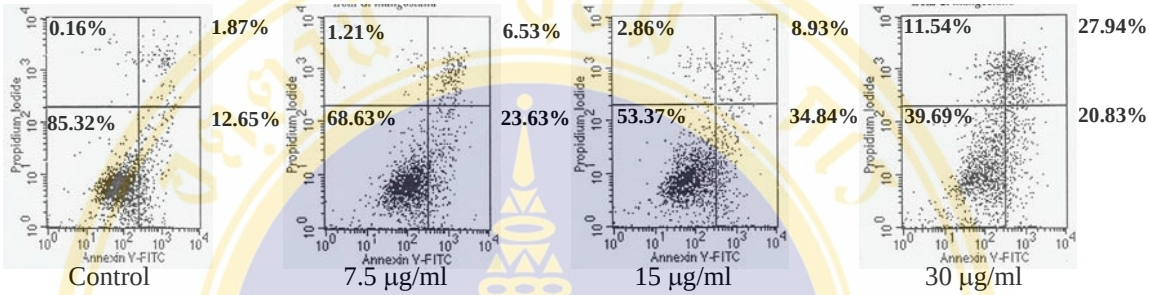
The early apoptotic cells change on plasma membrane according to the phosphatidylserine (PS) translocation from the inner to the outer at plasma membrane leaflet region. Annexin V-FITC assay has a high affinity for PS and is specific method to classify the early apoptotic cells, which are undergoing apoptosis, by flow cytometric analysis.

In flow cytometry analysis, viable cells expressed Annexin V-FITC and PI negative in Lower-Left (LL) quadrants; in early apoptotic cells were binding Annexin V-FITC positive and PI negative in Lower-Right (LR) quadrants; the late apoptosis were binding both Annexin V-FITC and PI positive in Upper-Right (UR) quadrants; and the dead cells, apoptotic and debris cells were binding Annexin V-FITC negative and PI positive in Upper-Left (UL) quadrants.

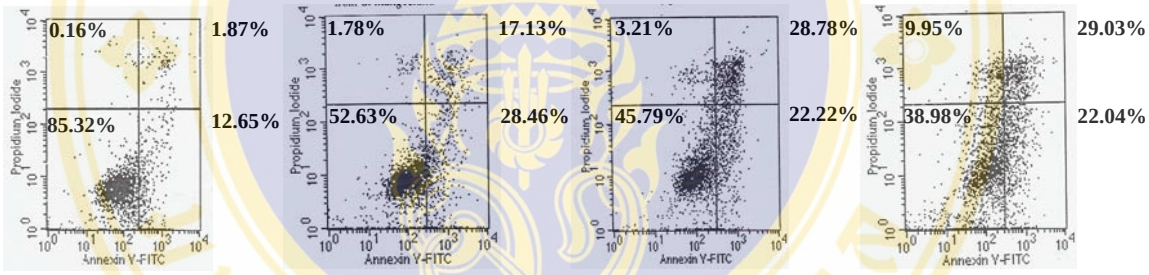
The effect of OVS1 MAb on SKOV3 and SKBR3 expressed significant in a dose-dependent manner. The effect of 80% Ethanol extract from *P. amarus* against SKOV3 and SKBR3 demonstrated significant following in a dose-dependent manner on both cancer cell at $0.5xED_{50}$, ED_{50} and $2xED_{50}$ (Figure 31 and 32). The 80% Ethanol, Acetone and Fraction 18-19 extract from *S. venosa* showed significant in a dose-dependent manner on both cancer cells, especially, Acetone extract from *S. venosa* (Figure 31 and Figure 32). The effects of Annexin V-FITC assay after treated with OVS1 MAb and medicinal plant extracts were expressed in Figure 31-32.

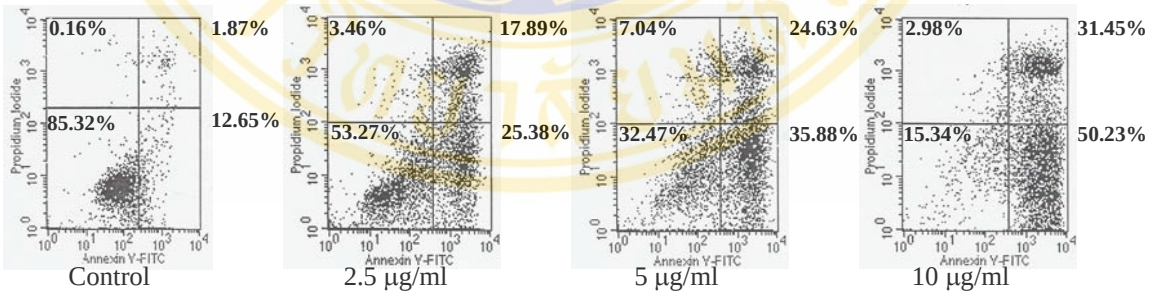


SKOV3 cells after treated with **OVS1 MAb**

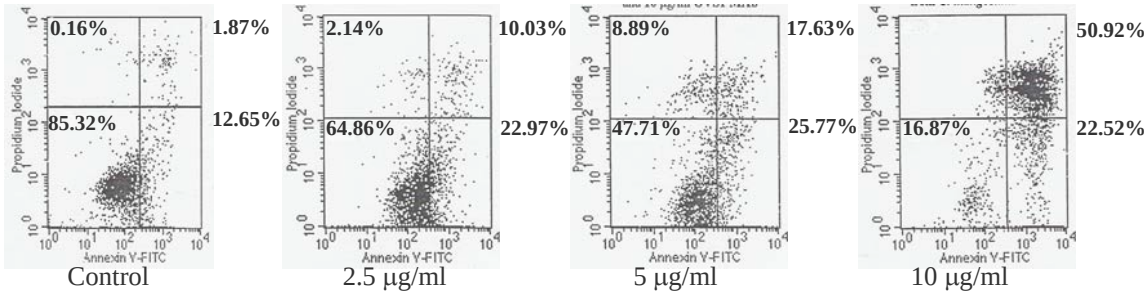


SKOV3 cells after treated with **80% Ethanol extract** from ***P. amarus***



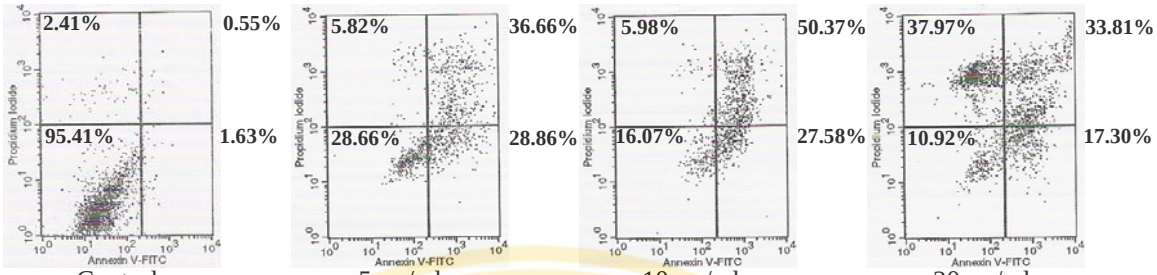


SKOV3 cells after treated with **Acetone extract** from ***S. venosa***



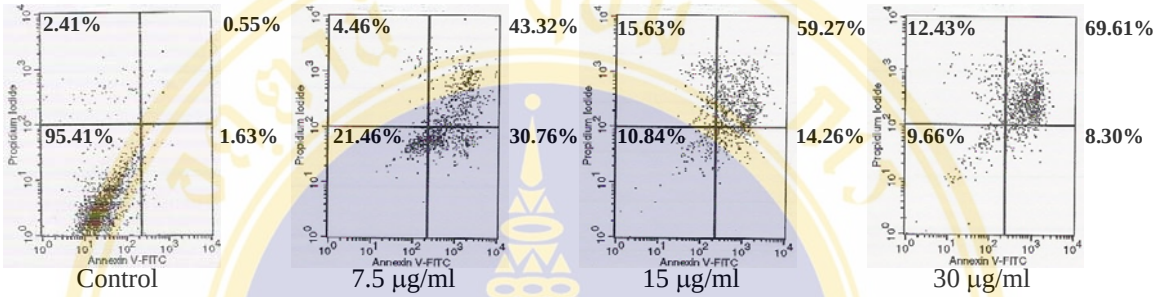
SKOV3 cells after treated with **Fraction 18-19 extract** from ***S. venosa***

Figure 31 Assessment of phosphatidylserine by Annexin V-FITC on **SKOV3**.



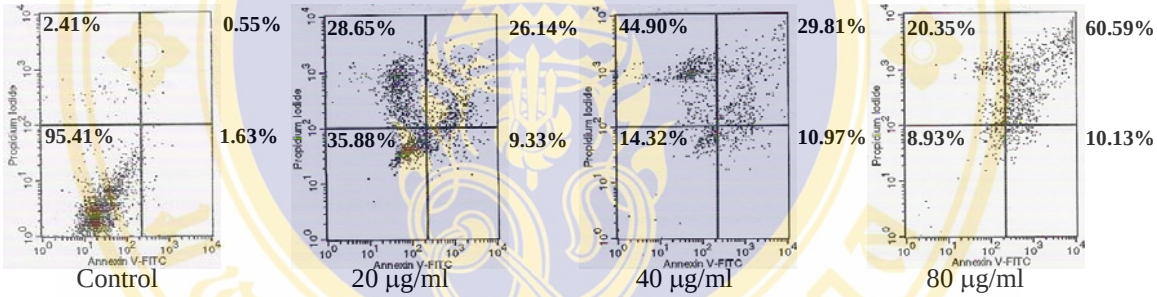
Control 5 µg/ml 10 µg/ml 20 µg/ml

SKBR3 cells after treated with **OVS1 MAb**



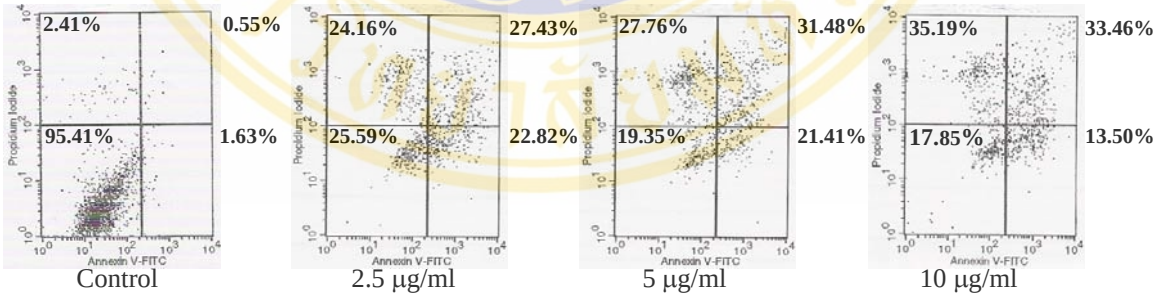
Control 7.5 µg/ml 15 µg/ml 30 µg/ml

SKBR3 cells after treated with **80% Ethanol extract** from ***P. amarus***



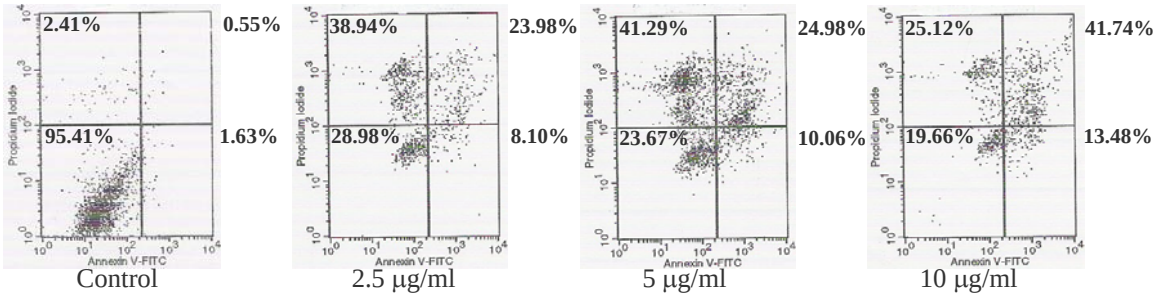
Control 20 µg/ml 40 µg/ml 80 µg/ml

SKBR3 cells after treated with **80% Ethanol extract** from ***S. venosa***



Control 2.5 µg/ml 5 µg/ml 10 µg/ml

SKBR3 cells after treated with **Acetone extract** from ***S. venosa***



Control 2.5 µg/ml 5 µg/ml 10 µg/ml

SKBR3 cells after treated with **Fraction 18-19 extract** from ***S. venosa***

Figure 32 Assessment of phosphatidylserine by Annexin V-FITC on **SKBR3**.

7. Cell cycle analysis

7.1 Determination of DNA content on cell cycle analysis

DNA content and distribution in normal cells, different stages of cells and cell death can measure by flow cytometry. Cells were fixed in ethanol before being subjected to mild extraction of low-molecular weight DNA. Samples were then stained with Propidium iodide (PI) in the presence of RNase A and analyzed by flow cytometry. Propidium iodide is an intercalating dye with a specific affinity for DNA. Its quantitation, in permeabilized cells, measures DNA content and is used to estimate the cell cycle distribution in populations of cells.

Flow cytometry often is called upon to distinguish diploid from aneuploid cells. Ploidy refers to DNA content and normal cells are usually diploid. In contrast, cancer cells often contain abnormal amounts of DNA reflecting genetic instability, and are therefore considered aneuploid. DNA aneuploidy has been found to be an independent prognostic factor, conferring a poor prognosis in a variety of tumors. Flow cytometry can also assess S-phase content. The DNA synthesis portion of the cell cycle, S-phase, is a reflection of cell proliferation (176).

Normal cells pass through the cell cycle, they proceed from the G0/G1 phase with a diploid (2N) amount of DNA, through the DNA synthesis (S) phase where the DNA can range from 2N to 4N. Cells then enter the G2-phase with a tetraploid (4N) amount of DNA until mitosis (M-phase) when they divide and return to a diploid state.

The impact of medicinal plant extracts and Taxol on the cell cycle was examined against SKOV3 and SKBR3 cell lines. The DNA content of both cell lines were demonstrated in Figure 34-36 and Figure 37-39, respectively. Our experiments, we used Taxol that is a chemotherapeutic drug arrest cell cycle in G2/M phase. In Figure 33 illustrated potential arrest G2/M on SKOV3 and SKBR3 cell lines at 0.5 μg/ml of dose at various time.

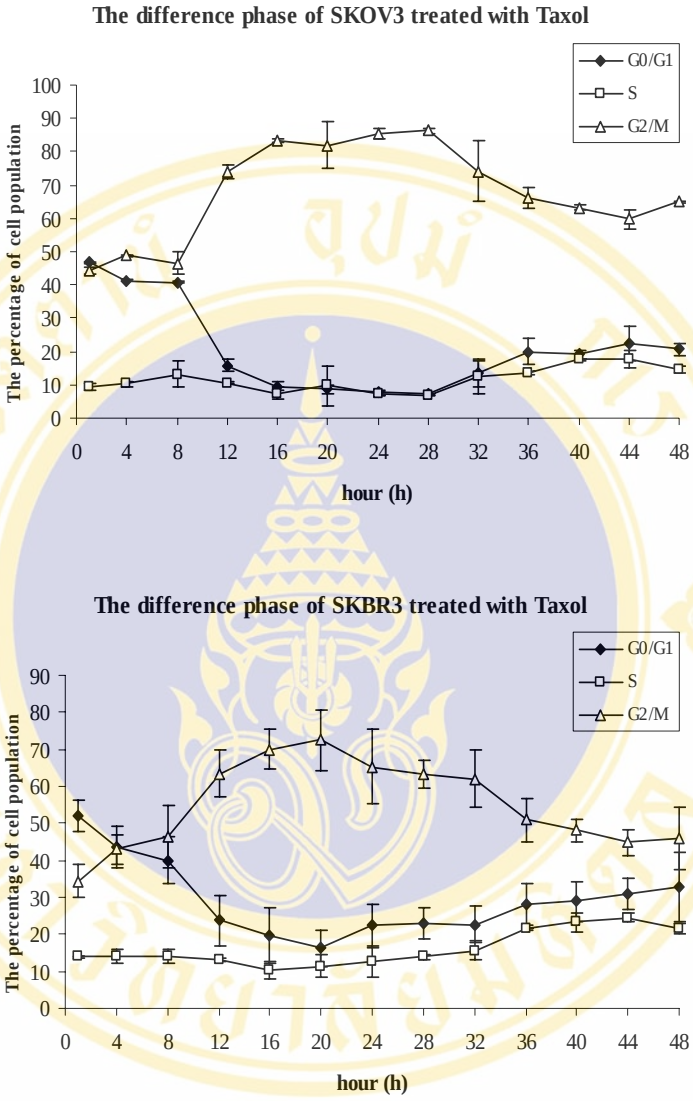


Figure 33 The effect of 0.5 μg/ml of Paclitaxel or Taxol treated on SKOV3 and SKBR3 showed the percentage of cell population in each times. The cells were harvested after incubation at various times. Taxol could arrest cancer cells at G2/M phase, began at 8 h.

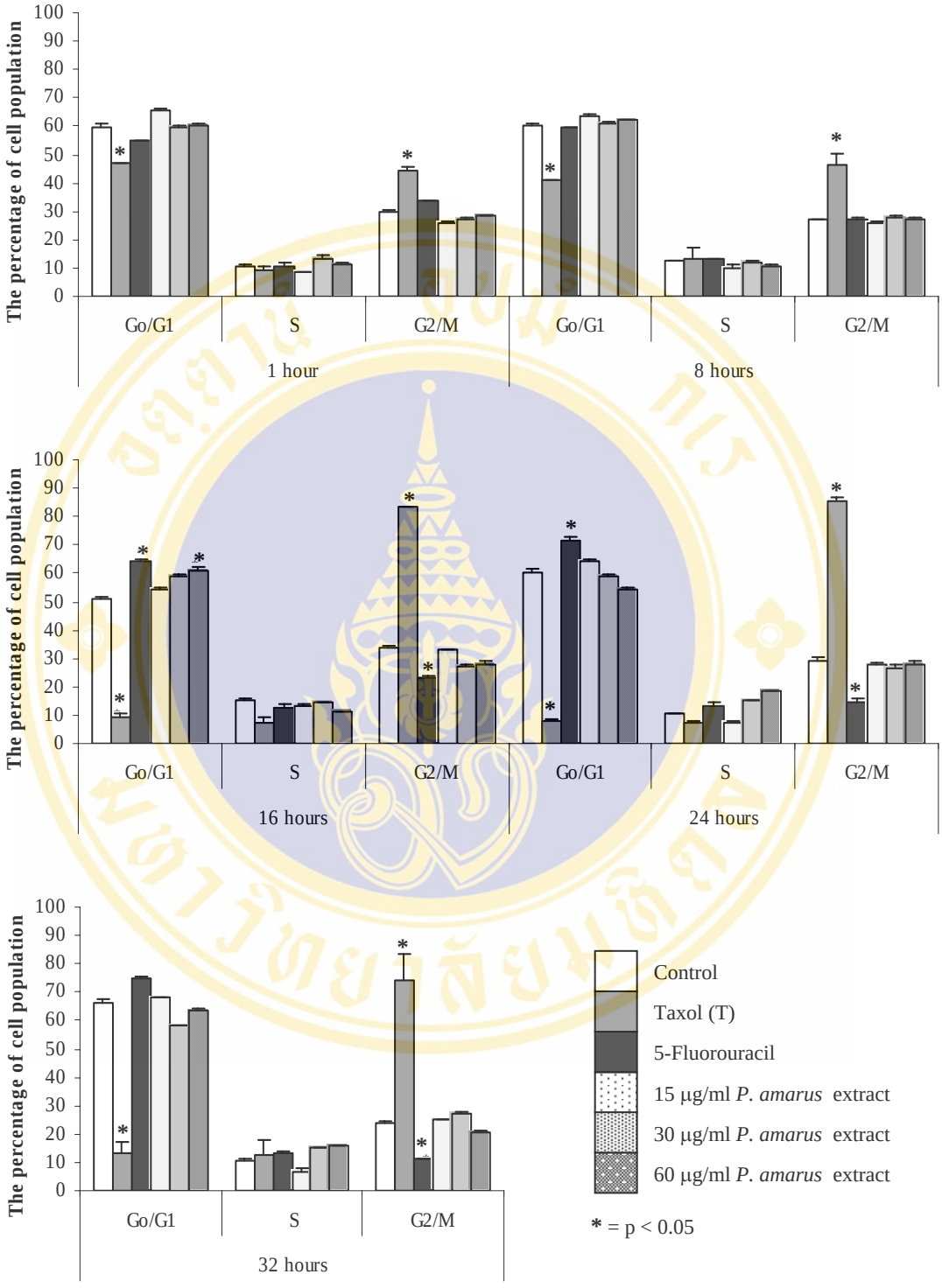


Figure 34 The percentage of **SKOV3** population treated with **80% Ethanol extract** from ***P. amarus*** at **15, 30** and **60** µg/ml respectively.

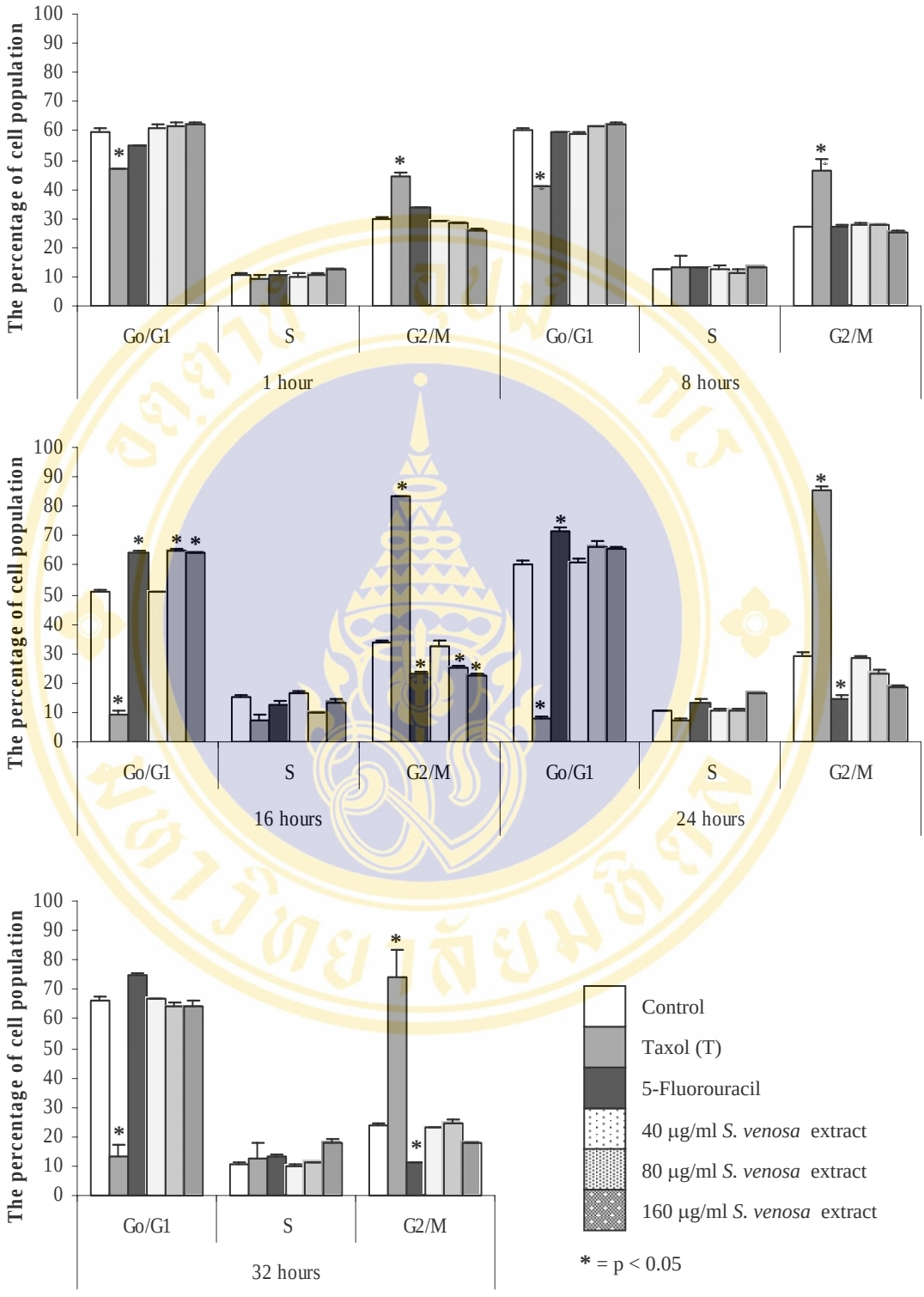


Figure 35 The percentage of **SKOV3** population treated with **80% Ethanol extract** from ***S. venosa*** at **40, 80** and **160** µg/ml respectively.

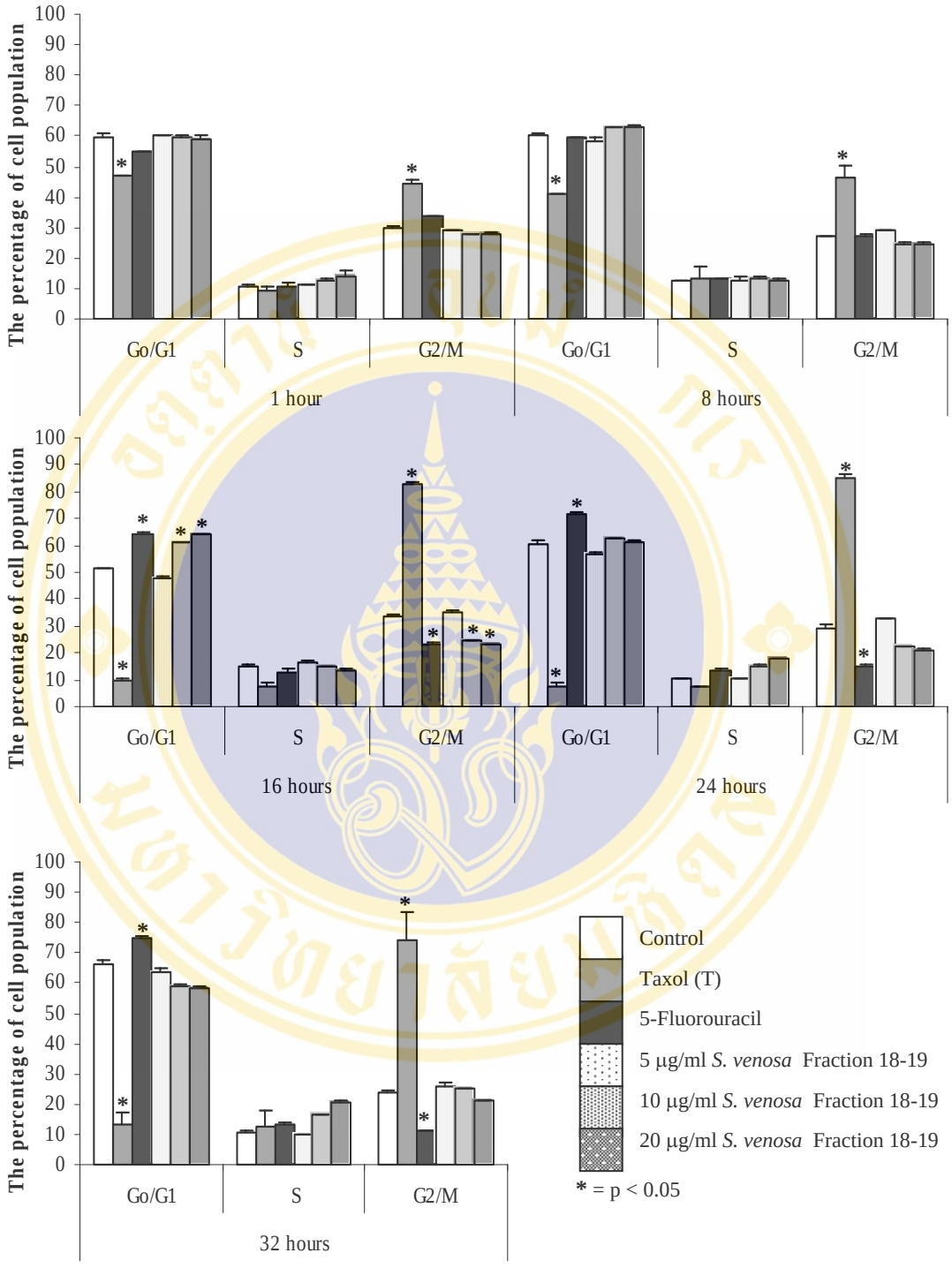


Figure 36 The percentage of **SKOV3** population treated with **Fraction 18-19 extract** from ***S. venosa*** at **5, 10** and **20** µg/ml respectively.

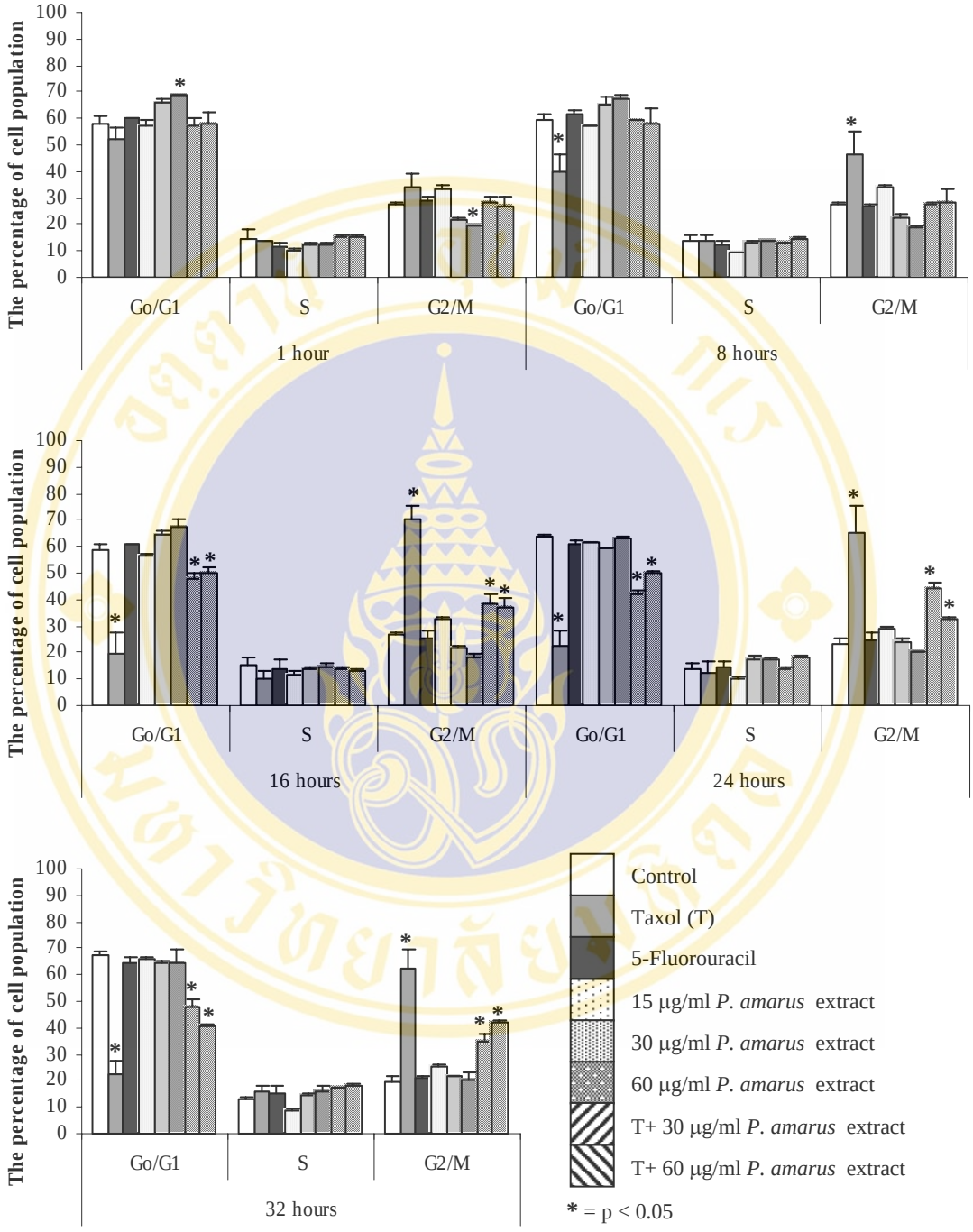


Figure 37 The percentage of **SKBR3** population treated with **80% Ethanol extract** from ***P. amarus*** at **15**, **30** and **60** µg/ml respectively. Cells were treated with Taxol for 16 hours before adding **80% Ethanol extract** from ***P. amarus*** at **30** and **60** µg/ml respectively (T+30, T+60 µg/ml of ***P. amarus***).

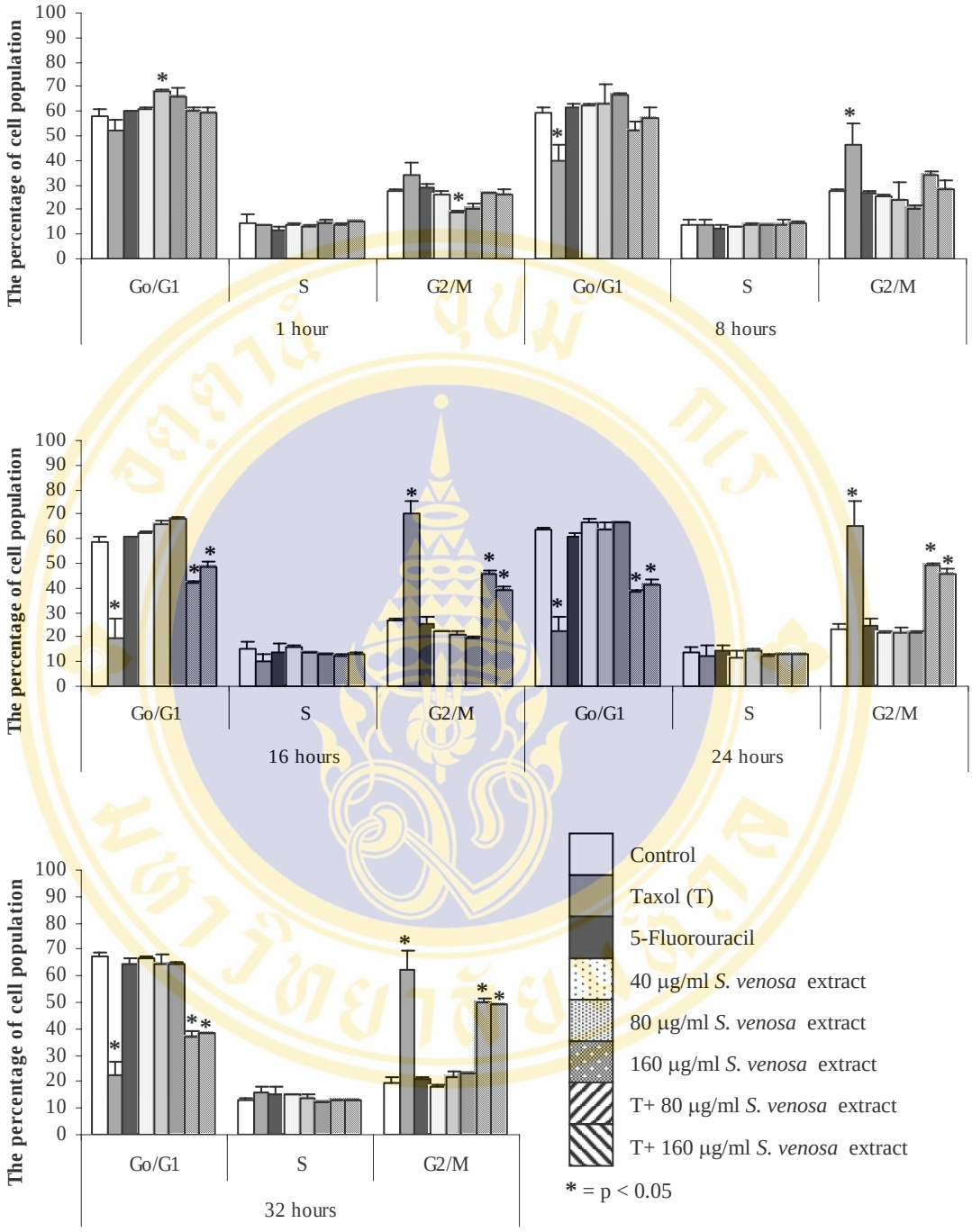


Figure 38 The percentage of **SKBR3** population treated with **80% Ethanol extract** from ***S. venosa*** at **40, 80** and **160** µg/ml respectively. Cells were treated with Taxol for 16 hours before adding **80% Ethanol extract** from ***S. venosa*** at **80** and **160** µg/ml respectively (T+30, T+60 µg/ml of ***S. venosa***).

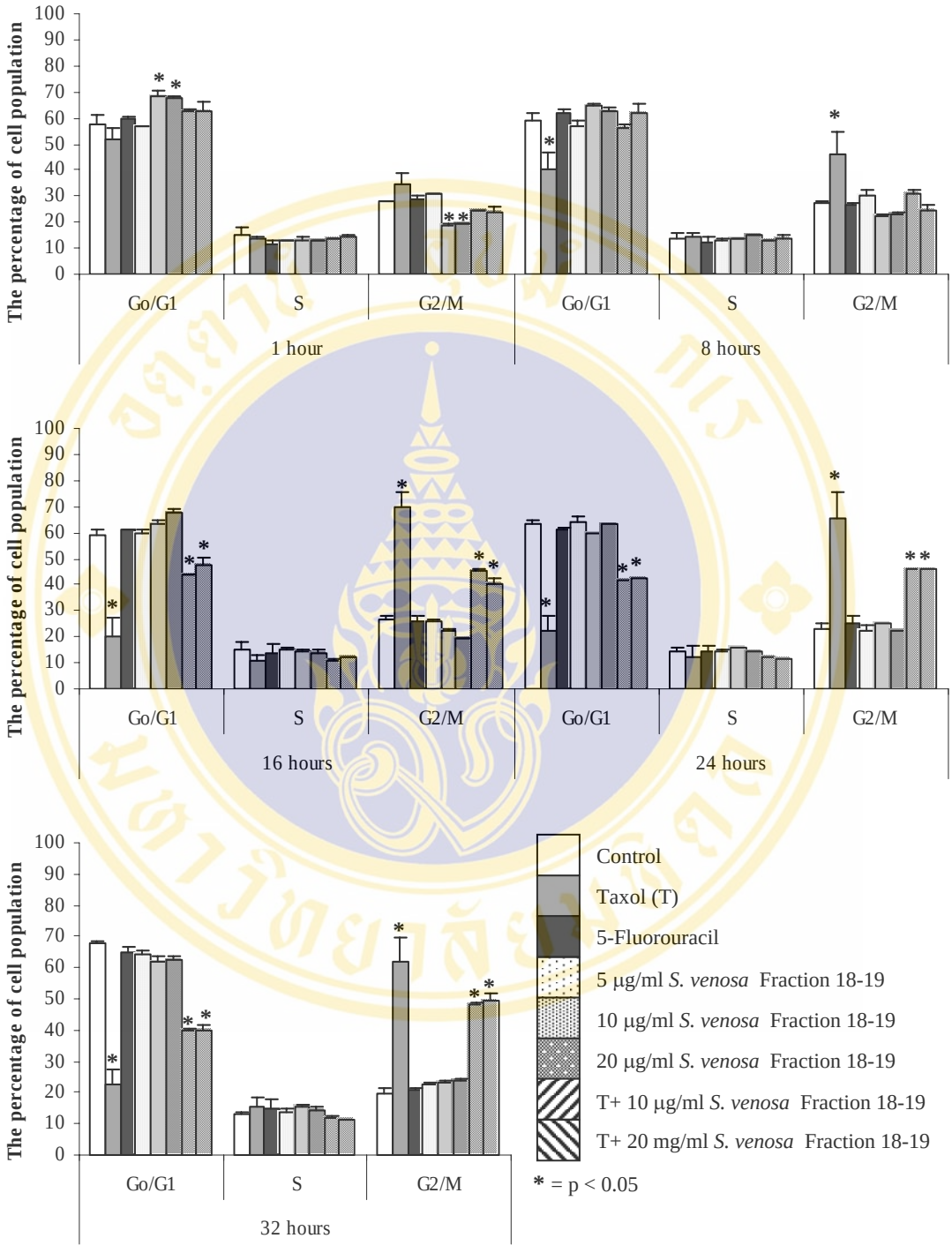


Figure 39 The percentage of **SKBR3** population treated with **Fraction 18-19 extract** from ***S. venosa*** at **5, 10** and **20** µg/ml respectively. Cells were treated with Taxol for 16 hours before adding **Fraction 18-19 extract** from ***S. venosa*** at **10** and **20** µg/ml respectively (T+30, T+60 µg/ml of ***S. venosa***).

Chemopreventive activities of extracts from medicinal plants

8. Antioxidative assay

8.1 Determination of reactive oxygen species by fluorescence probe

Reactive oxygen species (ROS) are constantly generated under normal conditions as a consequence of aerobic metabolism. When ROS overcome, the cells were altered to undergo apoptosis. Intracellular ROS was estimated by using a fluorescent probe, 2',7'-dichlorofluorescenin diacetate (DCFH-DA) readily diffuses through the cell membrane and is enzymatically hydrolyzed by intracellular esterase to form non-fluorescent 2',7'-dichlorofluorescein (DCFH), which is then rapidly oxidized to form highly fluorescent 2',7'-dichlorofluorescin (DCF) in the presence of ROS. The DCF fluorescence intensity is believed to be parallel to the amount of ROS formed intracellularly.

ROS was apart of apoptotic property therefore we were also to interest in antioxidative activity of medicinal plant extracts. In this assay, we selected H_2O_2 to induce oxidative stress and used DCFH-DA to serve as fluorescence probe to measure the changes of cytosolic ROS after treated with each extract. The effects of medicinal plant extracts on SKOV3 and SKBR3 were shown in following Figure 40 and 41, respectively.

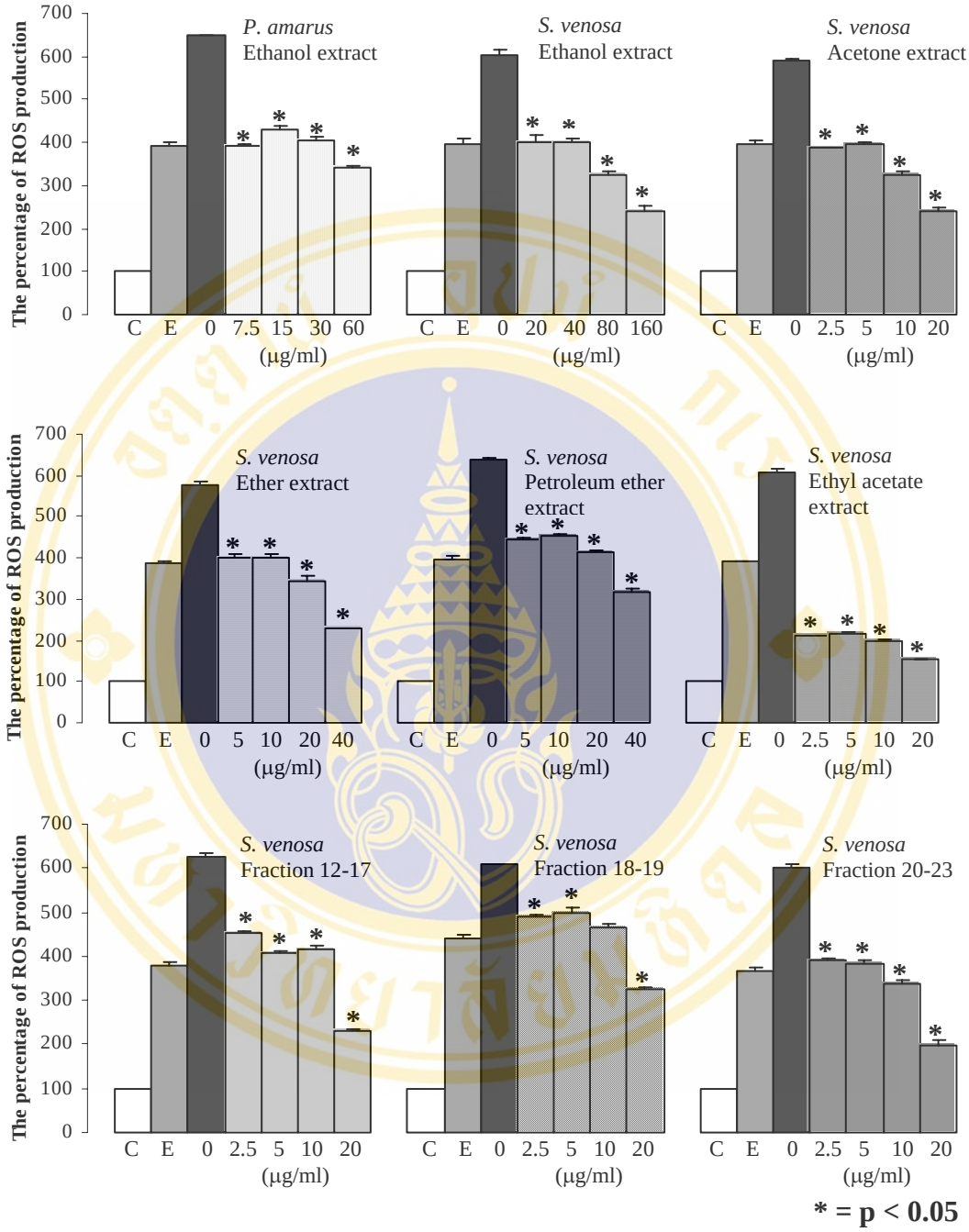


Figure 40 Inhibition of reactive oxygen species (ROS) production of **SKOV3** cells after treated with medicinal plant extracts.

C: Control; untreated SKOV3 cells.

E: SKOV3 treated with 4 mM of H_2O_2 and 100 μg/ml of Vitamin E.

O: SKOV3 treated with 4 mM of H_2O_2.

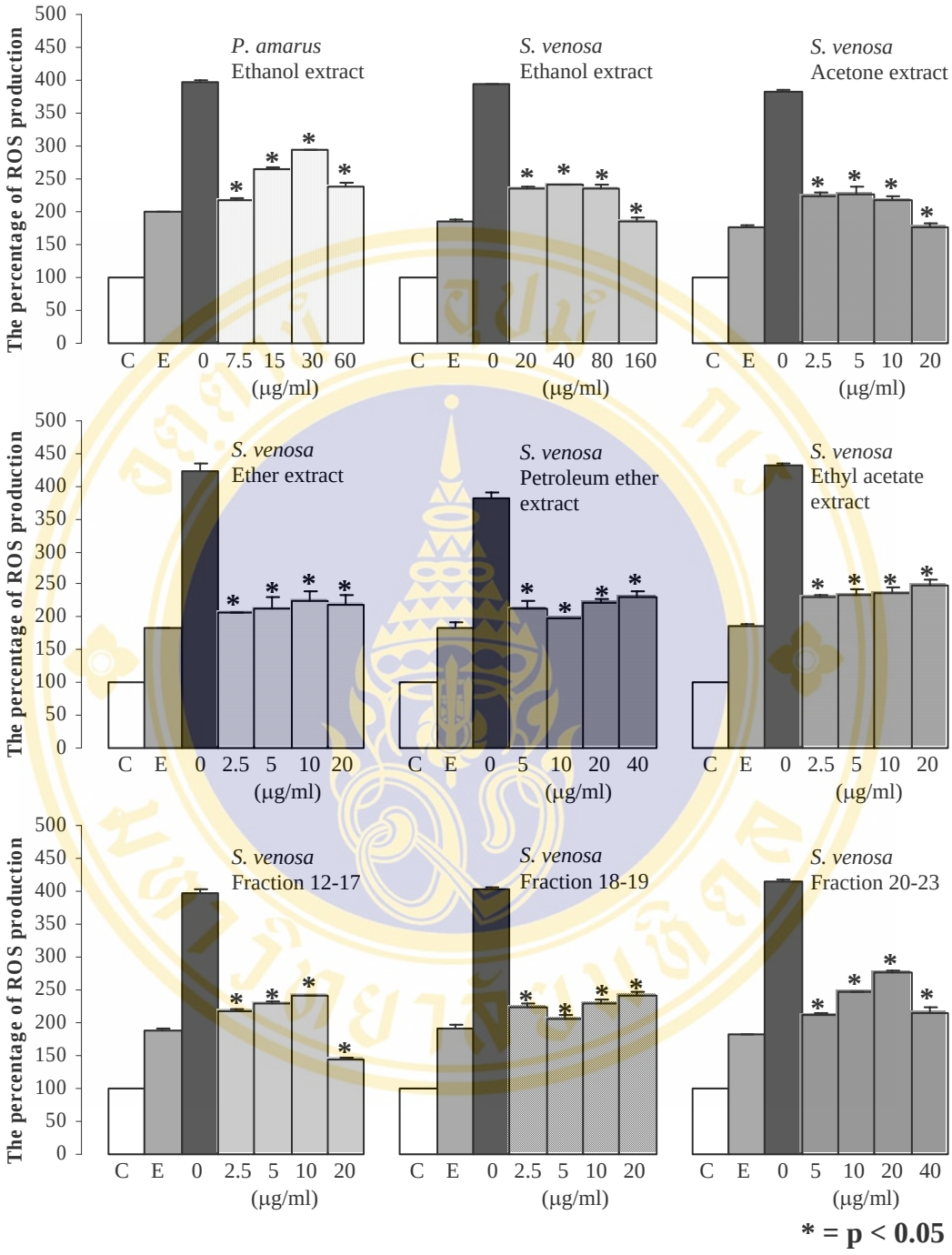


Figure 41 Inhibition of reactive oxygen species (ROS) production of **SKBR3** cells after treated with medicinal plant extracts.

C: Control; untreated SKBR3 cells.

E: SKBR3 treated with 4 mM of H_2O_2 and 100 μg/ml of Vitamin E.

O: SKBR3 treated with 4 mM of H_2O_2.

8.2 Determination of nitric oxide by Griess reaction

Nitric oxide ($^{\bullet}NO$) is an endogenous mediator of numerous physiological processes. Numerous techniques for detection of nitric oxide have been reported, including spectrophotometric, fluorescent, chemiluminescent and chromatographic assay. The simplest and most frequently applied method employs colorimetric detection with Griess reagents or Griess reaction. Griess reaction can quantify nitrites and nitrates after their reduction.

The Griess reaction provides to determine the cellular production of nitric oxide ($^{\bullet}NO$) by measuring its stable product nitrite in the cell culture supernatant. Cells synthesize nitric oxide by utilizing different isoforms of the enzyme nitric oxide synthase (NOS) which convert L-arginine to L-citrulline and $^{\bullet}NO$. In the presence of oxygen $^{\bullet}NO$ decompose to nitrite (NO_2^-) and nitrate (NO_3^-). Griess reagent (sulfanilamide/N-(1-naphthyl) ethylenediamine dihydrochloride) was added to cell culture supernatant converts nitrite into a purple azo dye which can be quantified photometrically and thus used as a parameter for the NO synthesis of cultured cells.

Nitric oxide was measured indirectly as nitrite by the Griess reagent. The inducer was sodium nitroprusside as a donor of nitric oxide. The results of Griess reaction of medicinal plant extracts against SKOV3 and SKBR3 were shown in following Figures 42 and 43, respectively.

The summary of all our experiments were presented in Table 15 for SKOV3 and Table 16 for SKBR3.

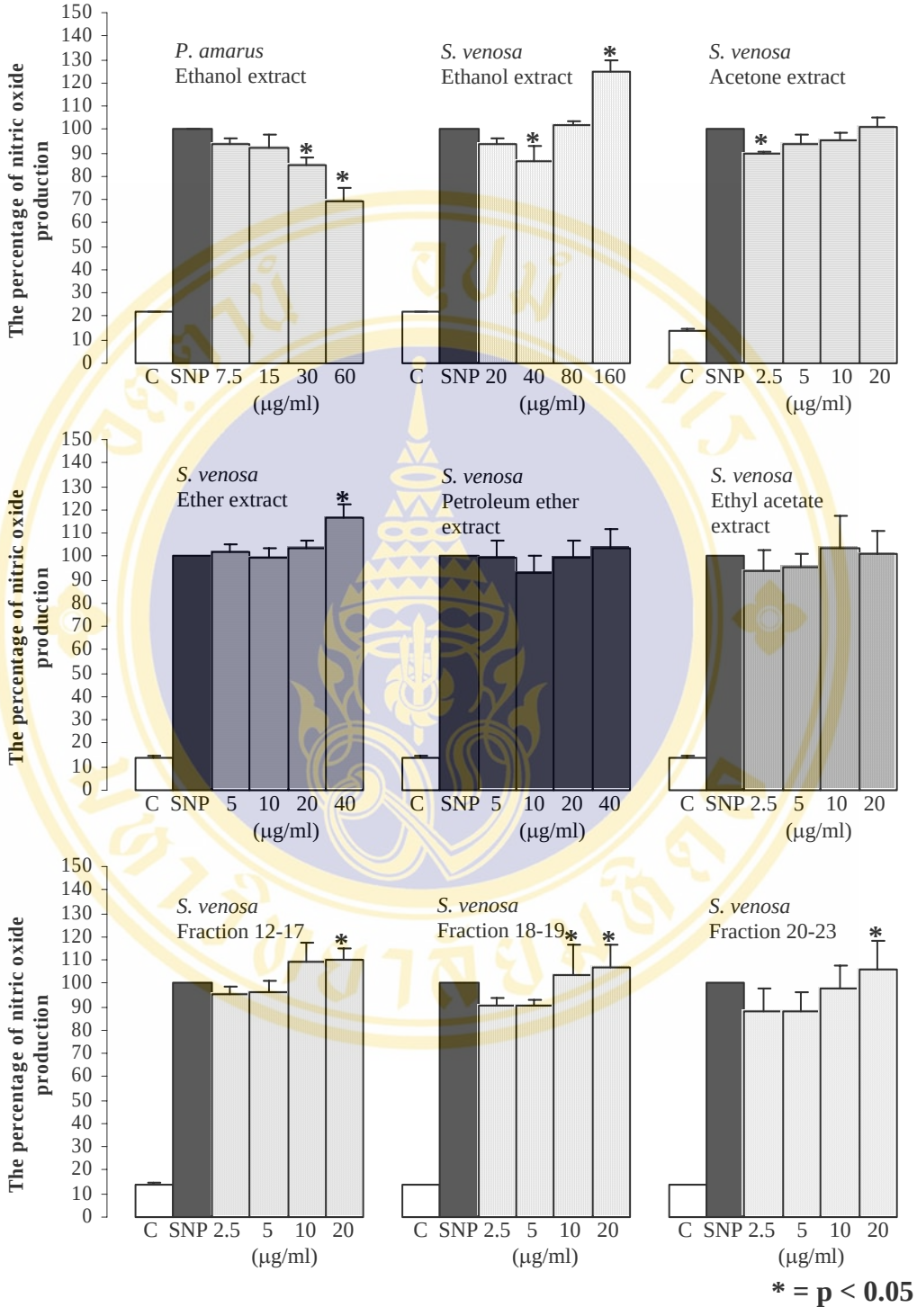


Figure 42 Effect of nitric oxide production of **SKOV3** cells against various concentrations of medicinal plant extracts.

C: Control; untreated SKOV3 cells.

SNP: SKOV3 treated with 10 µM SNP.

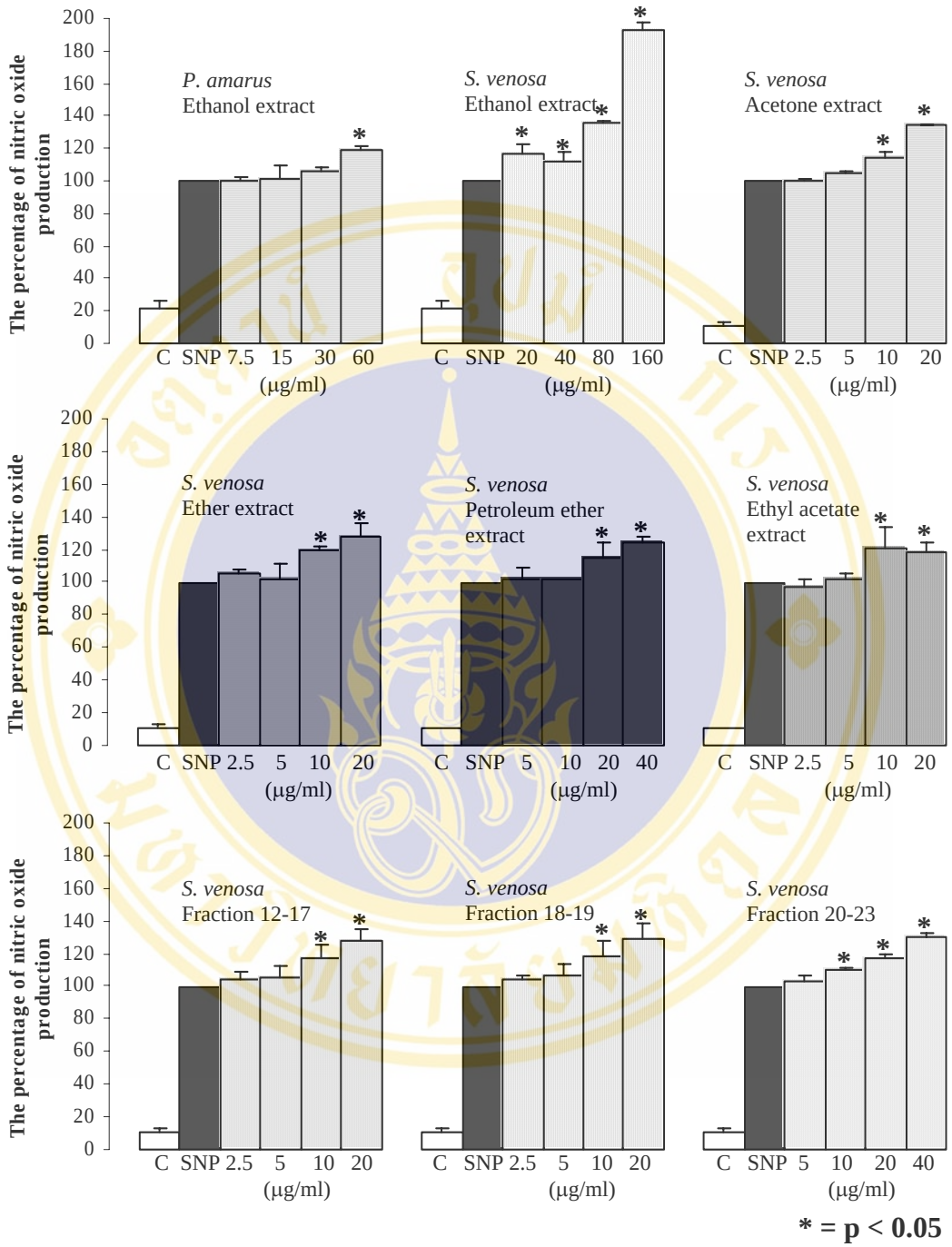


Figure 43 Effect of nitric oxide production of **SKBR3** cells against various concentrations of medicinal plant extracts.

C: Control; untreated SKBR3 cells.

SNP: SKBR3 treated with 10 μM SNP.

Table 15 The summary of both extracts and OVS1 MAb from all experiments against SKOV3 were demonstrated in this table.

Methods / Samples	Anti-proliferative assay	Apoptotic assay								Cell cycle arrest (Flow cytometry)			Antioxidative assay							
	MTT assay (µg/ml)	Morphological changes		DNA fragments		DNA comet	Annexin V-FITC						Reactive oxygen species (ROS) inhibition				Nitric oxide (NO) inhibition			
		Ho33342	Propidium iodide	2x ED_{50}	4x ED_{50}	2x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	1x ED_{50}	2x ED_{50}	4x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	4x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	4x ED_{50}
		$2xED_{50}$	$2xED_{50}$																	
OVS1 MAb	6.71	+++	+++	++ [a]	++ [a]	ND	+++ *p*	++	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. amarus 80% Ethanol	13.62	+++	+++	++	++	+++	+++ *p*	+++ *p*	++	–	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
S. venoas 80% Ethanol	35.11	+++	+++	+	+	++	+++ *p*	+++ *p*	+++ *p*	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	+++ *p*	–	+++ *p*
Acetone	3.91	ND	ND	+++	+++	+++	+++ *p*	+++ *p*	+++ *p*	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–
Ether	6.67	ND	ND	+	+	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	+++ *p*
Petroleum ether	7.03	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	–
Ethyl acetate	4.38	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	–
Fraction 12-17	3.01	ND	ND	++	++	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	+++ *p*
Fraction 18-19	1.95	ND	ND	+	+	++	+++ *p*	+++ *p*	+++ *p*	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Fraction 20-23	3.13	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	+++ *p*

+ = Positive results from +++ to +, – = Negative results, ND = not done, *p* = Statistical significance $p<0.05$, a = previous result (23).

Table 16 The summary of both extracts and OVS1 MAb from all experiments against SKBR3 were demonstrated in this table.

Methods / Samples	Anti-proliferative assay	Apoptotic assay								Cell cycle arrest (Flow cytometry)					Antioxidative assay							
	MTT assay (μg/ml)	Morphological changes		DNA fragments		DNA comet	Annexin V-FITC								Reactive oxygen species (ROS) inhibition				Nitric oxide assay (NO) inhibition			
		Ho33342	Propidium iodide	2x ED_{50}	4x ED_{50}	2x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	0.5x ED_{50}	2x ED_{50}	4x ED_{50}	T+ 2x ED_{50}	T+ 4x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	4x ED_{50}	0.5x ED_{50}	1x ED_{50}	2x ED_{50}	4x ED_{50}
		$2xED_{50}$	$2xED_{50}$																			
OVS1 MAb	7.91	+++	+++	ND	ND	ND	+++ *p*	+++ *p*	++ *p*	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
P. amarus 80% Ethanol	10.44	+++	+++	+++	+++	+++	+++ *p*	++ *p*	+ *p*	–	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	–	+++ *p*
S. venosa 80% Ethanol	39.67	+++	+++	++	++	+	++	+++	++	–	+++ *p*	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*
Acetone	5.24	ND	ND	+++	+++	++	+++ *p*	+++ *p*	+++ *p*	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Ether	4.07	ND	ND	+	+	ND	ND	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Petroleum ether	6.42	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Ethyl acetate	4.90	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Fraction 12-17	4.19	ND	ND	++	++	ND	ND	ND	ND	ND	ND	ND	ND	ND	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Fraction 18-19	2.80	ND	ND	+	++	+	++	+++	+++ *p*	–	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	+++ *p*	–	–	+++ *p*	+++ *p*
Fraction 20-23	7.03	ND	ND	+++	+++	ND	ND	ND	ND	ND	ND	ND	ND	ND	+++ p	+++ p	+++ p	+++ p	–	+++ p	+++ p	+++ p

+ = Positive results from +++ to +, – = Negative results, ND = not done, *p* = Statistical significant $p<0.05$, T+ = 0.2 μg/ml Taxol was prior added.

CHAPTER V
DISCUSSION

This study was a preliminary *in vitro* experiment to evaluate the chemotherapeutic and chemoprevention potentials of medicinal plant extracts from *Phyllanthus amarus*; 80% Ethanol extract, and *Stephania venosa*; 80% Ethanol, Acetone, Ether, Petroleum ether, Ethyl acetate, Silica gel Fraction: 12-17, 18-19 and 20-23, and OVS1 MAb. The study was performed and evaluated the activities of test substances on human ovarian cancer (SKOV3) and human breast cancer (SKBR3).

The chemotherapeutic activities consisted of two parts. The first part was the antiproliferative assay, which measured cell viability by MTT test, and apoptotic identification, which were: the determination on morphological changes, DNA damage demonstrated by DNA ladder and comet assay, and phosphatidylserine externalization. The second part was cell cycle analysis by flow cytometry which performed to detect DNA content and analysed the possible steps in cell cycle that were affected or arrested by extracts on both cancer cells at various concentrations of plants extracts and OVS1 MAb in a dose- and time-dependent manner.

The chemopreventive activities were studies on the antioxidative assay which supposed to be one of the major activities accompanied to disease-prevention. The determinations of reactive oxygen species and reactive nitrogen species, i.e., nitric oxide assay were selected for identifying the properties of the medicinal plants.

In previous reports, the aqueous extract of *P. amarus* exhibited potent anticarcinogenic activity (16). Anticancer activity of *P. amarus* may be related with the inhibition of metabolic activation of carcinogen as well as the inhibition of cell cycle regulators and DNA repair (99,116,117,127). In this study, we used 80% Ethanol extract from *P. amarus* for testing in all experiments.

The rhizomes from *S. venosa* were used as an antitumor and antiproliferation of breast cell culture (19,131,139). In this study, we used eight extracts from this plant; 80% Ethanol, Acetone, Ether, Petroleum ether and Ethyl acetate extracts which were extracted according to the solubility property in various solvents and were also further

fractionated by silica gel column chromatography; Fraction 12-17, 18-19 and 20-23 which were used for the tests.

OVS1 MAb was purified from supernatant of OVS1 hybridoma and had been reported to recognize specifically with ovarian and breast cancer cells. The OVS1 MAb was produced in Thailand by our research group and would be studied the direct effect on cells the same as other medicinal plants.

Antiproliferative activities

MTT test indicated the ED_{50} of *P. amarus* at 13.62 and 10.44 µg/ml against SKOV3 and SKBR3 cell lines, respectively (Table 13 and 14, Figure 15 and 17). The extracts from *S. venosa* showed the ED_{50} of 80% Ethanol, Acetone, Ether, Petroleum ether, Ethyl acetate, Fraction 12-17, 18-19 and 20-23 extracts on SKOV3 at 35.11, 3.91, 6.67, 7.03, 4.38, 3.01, 1.95 and 3.13 µg/ml, respectively (Table 13, Figure 15-16). SKBR3 presented the ED_{50} after treated with these extracts at 39.67, 5.24, 4.07, 6.42, 4.90, 4.19, 2.80 and 7.03 µg/ml, respectively (Table 14, Figure 17-18). These ED_{50} were classified as strong inhibition on the proliferation against these cells and the results expressed in a dose-dependent manner. The ED_{50} from *S. venosa* extracts were demonstrated that the extracts after fractionation would give stronger activity and showed more antiproliferative activity than before fractionated. Fraction 18-19 extract was the most powerful test sample on SKOV3 and SKBR3. MTT assay demonstrated that SKOV3 was more sensitive than SKBR3 when treated with various extracts. The ED_{50} of these extracts depended on the different quantities of various active compounds in plant extracts.

Apoptotic determination

Study on apoptosis which is a controlled physiological process of the fundamental importance to all multicellular organisms, occurs in a morphologically and biochemically distinct manner and ultimately leads to cell suicide (145). Deregulation of apoptosis is involved in the etiology of diseases including degenerative diseases of the central nervous system, autoimmune diseases, viral infection and cancer.

The apoptosis reported in this study was performed by using the ED_{50} from MTT assay of SKOV3 for identifying on both cancer cells. Apoptosis was studied by determination of morphological changes following treatment at 30 µg/ml (two-times

of ED_{50}; $2xED_{50}$) dose of *P. amarus* extract for 48 h on SKOV3 and SKBR3. Cells expressed specific characteristic of morphological changes related with apoptotic events in Figure 19 and 20 respectively. The concentration at 80 μg/ml ($2xED_{50}$) of Ethanol extracts from *S. venosa* on SKOV3 and SKBR3 showed apoptotic-cell morphological changes (Figure 19 and 20). The morphological alteration, i.e., cell shrinkage, increased cytoplasmic density, chromatic condensation and segregation into sharply circumscribed masses, membrane blebbing and the formation of membrane-bound smooth surface called apoptotic body (145). We found that SKOV3 was more sensitive than SKBR3 after exposed with these extracts in our experiments.

The late apoptosis stage where the nucleus of cell was damaged and created DNA fragments (145). Therefore, the effects of DNA fragmentation that were determined by DNA ladder assay after treated with $2xED_{50}$ and $4xED_{50}$ of *P. amarus* extract on SKOV3 and SKBR3 (Figure 21 and 24). In the same method, we also found that all extracts from *S. venosa* against SKOV3 and SKBR3 expressed the DNA fragmentation remarkably like DNA ladder (Figure 21-23 and Figure 24-26). These results identified that all extracts could induce apoptosis on both cancer cells. Our extracts may stimulate endonuclease enzymes that cleaved DNA at internucleosomal sites to produce into about 50-300 kb fragments which were degraded into smaller pieces with lengths of oligonucleosomal multiples approximately 185 bp (145).

Moreover, the DNA damage could also be determined by comet assay (31). The intensity of the staining in the comet's tail region was presumed to be related to the DNA content and DNA damage was estimated from measurements of tail length, comet length, tail moment or tail ratio (153,154). The differential migration of DNA fragments away from the nuclear 'head' form a fluorescent 'tail', producing DNA images resembling comets (155). The non-degraded DNA in the nucleus of SKOV3 and SKBR3 cells remaining intact when subjected to electrophoresis illustrated features the same as untreated cells (Figure 27 and 29). SKOV3 and SKBR3 were exposed with 500 μM of H_2O_2 for 12 h, the highly fragmented DNA of apoptotic cells migrated away from the nuclear head as a function of the severity of cleavage showed comet shapes (Figure 27 and 29) The moderately degraded DNA of apoptotic cells after treated with extracts at $2xED_{50}$ of *P. amarus* and the extracts from *S.* venosa that were Ethanol, Acetone and Fraction 18-19 extracts against SKOV3 and SKBR3

demonstrated the comet features (Figure 27-28 and 29-30). SKOV3 exhibited highly sensitivity much more than SKBR3 when incubated with plant extracts.

During apoptosis, the phosphatidylserine (PS) externalization is one of the earliest features of apoptotic cells. In flow cytometry analysis, the concentration at $0.5xED_{50}$, ED_{50} and $2xED_{50}$ of extract from *P. amarus* and *S. venosa* that was Ethanol, Acetone and Fraction 18-19 against SKOV3 and SKBR3 expressed the phenomenon significantly in a dose-dependent manner (Figure 31 and 32).

For apoptotic experiments, we used many methods to detect apoptotic characteristic that had different reactions and various stage of events. Morphological changes could express the alteration of DNA and apoptotic body that depended on the different properties of fluorescence dye. Therefore, this assay may be a subjective determination (23,29,30). DNA fragmentation was a hallmark of the final stage of apoptosis (2,151). The previous two methods indicated only the qualitative results. Comet assay showed the potential effects of samples to the qualitative and quantitative results when using the software program to computerizing the results (31,32,154). The initial methods used in this study could demonstrate only the qualitative results of apoptosis but our later experiments were performed by flow cytometry which could be demonstrated both qualitative and quantitative results. We can select the methods which are suitable for our experiments, apoptotic stages, budget and equipments to identify apoptosis. However, the initial results could show pictures which was a confident crucial of apoptotic occurrence with less and inexpensive equipment. These techniques required cheap reagents and were also easy for manipulation. We could demonstrate clearly of morphological changes, DNA ladder, DNA comet that confirmed our plant extracts activity to induce apoptotic pathway in cancer cells.

Cell cycle analysis by flow cytometry

In flow cytometry, the discrimination of cells in G0/G1 versus S versus G2/M phases of the cell cycle is generally done by measuring cellular DNA content alone (161,162). Apoptosis was closely related to cell proliferation status while apoptosis could occur during any phase of the cell cycle. The sensitivity of proliferating cells to various death stimuli is usually cell cycle phase specific. The reagent of 5-fluorouracil can arrest cell cycle in G0/G1 phase and Paclitaxel or Taxol expresses positive effect which shows cells arrest in G2/M phase. Taxol is a standard anticancer drug for

treatment advanced stage of many cancers. We analysed SKOV3 and SKBR3 cells after treated with Taxol and observed the pattern performing effect in cell cycle (Figure 33).

The cell cycle of SKOV3 was estimated from the percentage of cell in each phase of cycle that was continuously quantitated at various times. SKOV3 cycle was determined approximately 24 h. The cell cycle of SKOV3 could be arrested in G2/M phase by Taxol after treated for 8 h (Figure 33) while treated with 5-fluorouracil cells were arrested in G0/G1 phase after 16 h incubation. We also found SKBR3 cell cycle was arrested in G2/M phase after treated with Taxol for 8 h (Figure 33) but we could not show the effect of 5-fluorouracil to arrest cell cycle on SKBR3 in our study .

The extract from *P. amarus* against SKOV3 showed cell arrest at G0/G1 significantly at 16 h (Figure 34), but only for a short time, since the G0/G1 became to normal condition afterwards. The results of extracts from *S. venosa* expressed the same pattern as *P. amarus* effect (Figure 35-36).

SKBR3 cell expressed significant arrest in G0/G1 after 1 h incubation with the extract from *P. amarus* and the extracts of Ethanol, Fraction 18-19 from *S. venosa* (Figure 37-39). SKBR3 was treated with Taxol for induced cycle to arrest at G2/M phase and washout after 16 h incubation. After Taxol treatment, the extract from *P. amarus*, the Ethanol and Fraction 18-19 extracts from *S. venosa* at $2xED_{50}$ and $4xED_{50}$ of concentrations were added in each experiment. We found that our extracts could present significantly to arrest cell cycle in G2/M phase at 16, 24 and 32 h in a time-dependent manner (Figure 37-39). Thus, we assumed that our extracts may prolong cell in G2/M phase with the same possible activity but was not powerful as Taxol.

The results on cell cycle analysis demonstrated these extracts may be efficient to arrest cell cycle in some phases of cancer cell cycle. The two cell lines have limited properties for cell cycle analysis and we have to study more in details to clarify this experiment with purified substances.

The cell cycle analysis is very useful method to study the effects and mechanisms of plant extracts in various cells. This method can estimate time spent of one cell cycle and show the initial effect on treated cell. In our study, we found that the cell cycle of SKOV3 was about 24 h but SKBR3 could not be clearly demonstration. This may be occurred from using dose to treating cell not high enough

to show the effect possibly observed. In the future, we suggested that used the high dose of plant extracts in order to see the clearly effect.

Antioxidative activities

We performed two assays on antioxidation, i.e., determination of reactive oxygen species (ROS) and nitric oxide inhibition assay. ROS are constantly generated under normal conditions as a consequence of aerobic metabolism. ROS are particularly transient species due to their highly chemical reactivity and can react with DNA, proteins, carbohydrates and lipid in a destructive manner. The DCFH-DA fluorescence probe was used to estimate for hydrogen peroxide of cytosolic ROS. The percentage of ROS production in SKOV3 and SKBR3 was significant reduced by Ethanol extract from *P. amarus* (Figure 40 and 41). SKBR3 showed more sensitivity than SKOV3 when treated with this extract at $0.5xED_{50}$, ED_{50}, $2xED_{50}$ and $4xED_{50}$ (Figure 40 and 41). It was supposed that this extract was a powerful extract to inhibit the oxidative stress on both cancer cells. All extracts from *S. venosa* at the same concentration of *P. amarus* on SKOV3 and SKBR3 presented significant ROS reduction in a dose-dependent manner (Figure 40 and 41). However, the results after incubating with these plant extracts the ROS production tended to decline remarkably. The inhibition of ROS production may be the outcome from interfering of apoptotic phenomenon after incubated with these extracts. In our study, we found that our plant extracts expressed highly antioxidative properties to combat oxidative stress on both cancer cells. It was implied that the processes of apoptosis and antioxidation of plant extracts involved each other.

A role of reactive nitrogen species (RNS) as both positive and negative regulators of cell death has been established. The anti-apoptotic activity of $^{\bullet}NO$ is evident that $^{\bullet}NO$ is a potent inhibitor of caspase activity both *in vitro* and *in vivo*. This discovery has significant implications for the inhibit regulation of apoptosis by $^{\bullet}NO$ (190). The exposure of cells to $^{\bullet}NO$ concentrations of greater than 1 μM can result in inhibition of apoptosis (190). A fine balance exists between the pro- and anti-apoptotic properties of $^{\bullet}NO$ at the level of the mitochondria.

Griess reaction, nitrite reacts with sulfanilic acid and N-(1-naphthly)-ethylenediamine dihydrochloride in the presence of phosphoric acid and produces a colored azo dye, which can be measured colorimetrically at 540 nm. This value is

proportional to the amount of nitrite in the solution. The detection limit of the Griess reagent is between 100 nM to 1 μM (189). The reduction of nitric oxide by Ethanol extract from *P. amarus* at $0.5xED_{50}$, ED_{50}, $2xED_{50}$ and $4xED_{50}$ of dose against SKOV3 and SKBR3 demonstrated significantly in a dose-dependent manner (Figure 42). The results from treatment of extracts from *S. venosa* could not be demonstrated their activities (Figure 43).

The $^{\bullet}NO$ results was assumed that reactive nitrogen species activities use different pathways to response with each extracts and in different cancer cells also showed different results. The system to detect the nitric oxide production could not be clearly demonstrated, therefore, it is necessary to improve system. This result supported the concepts on nitric oxide that a fine balance exists between the pro- and anti- apoptotic properties of nitric oxide has different implication for RNS in different cancer cells. Generally, the nitric oxide assay by Griess reaction use murine mouse macrophage cell (RAW 264.7) to detect nitric oxide but our study used SKOV3 and SKBR3 were used which may be not much sensitive on nitric oxide assay.

Monoclonal antibody

OVS1 MAb was secreted from OVS1 hybridoma cells, harvested from culture supernatant and further purified on protein A column. The hybridoma is still a stable cell line secreting OVS1 MAb. The purity of OVS1 MAb was performed on SDS-PAGE (Table 10 and 11, Figure 12 and 13). Proteins were measured by Micro BCA protein assay kit (PIERCE). The concentrations of OVS1 MAb after purification were 2.14, 1.43, 1.46, 1.07 and 0.93 mg/ml in Lot No. 1, 2, 3, 4 and 5 respectively (Table 9, Figure 11). OVS1 MAb expressed the antiproliferative property when treated on SKOV3 and SKBR3 at ED_{50} 6.71 and 7.91 μg/ml, respectively (Table 12, Figure 14).

We also found that OVS1 MAb can induce apoptotic process on both cancer cells. The determination of morphological changes, after treated with 10 μg/ml of OVS1 MAb for 48 h, were shown significant characteristic of morphological alterations as apoptotic characteristic (Figure 19 and 20). We also detected the induction of apoptotic property by determination of phosphatidylserine by Annexin V-FITC assay. The results showed that, SKOV3 and SKBR3 treated with ED_{50}, $2xED_{50}$ and $4xED_{50}$ of OVS1 MAb, were presented significant in a dose-dependent manner of the phophatidylserine determination (Figure 31 and 32).

Future approach

The study of extracts from *P. amarus* and *S. venosa* support the potential of these medicinal plants as new drugs for cancer chemotherapy and chemoprevention. The intensive research has to perform to clarify the concrete activities of extracts and purified constituents, especially in mechanisms of action, roles in different pathways of apoptosis and cell cycle arrest.

Monoclonal antibodies showed high affinity and specific on the target antigen, so it can use widely for diagnosis and treatment of disease. MAb alone can be used to eliminate tumor cells and also are served as a vehicle to transport the cytotoxic agents (drug, toxin or compounds from plant extracts) specifically to target tumor cells expressing the tumor associated antigen (6,150). The conjugation of MAb with cytotoxic agent produced a substance called an immunotoxin which can penetrate into tumor and not bind significant to non-tumor cells. Therefore, immunotoxin can decrease and limit the side effect of therapy. In our previous study of combined OVS1 MAb with Taxol to treat cells showed enhancing or synergistic effect on antiproliferation and apoptotic effect when compared with OVS1 MAb or Taxol alone (12,23).

From the present result (Table 15 and 16) suggested that we should continue to pursue studying, the potential active compounds in plant extracts from *P. amarus* and *S. venosa* that affect on cancer. Since the plant extracts showed promising results on antiproliferation and inducing apoptotic process as anticancer agent, we can combine or conjugate these compounds to OVS1 MAb as a new delivery system for targeting on tumor cells. Further study on chemotherapeutic and chemopreventive mechanisms of compounds from *P. amarus* and *S. venosa* that have potential regulation for cancer patient would be beneficial to increase the quality life and may be the discovery of a new natural product to fight with cancer.

CHAPTER VI
CONCLUSION

Cancer is the third common leading cause of death in Thailand and death from cancer still increasing each year. Thai medicinal plants have been widely used as folk medicine. Many studies on compound from plants have been found many interesting biological and pharmacological activities. The research and development of plant-derived drugs are one of the most attention as a novel drug. Phytotherapy is considered as an alternative remedy to combat cancer and less side-effect substances.

Cancer-chemotherapeutic activities of *P. amarus, S. venosa* extracts and OVS1 MAb were demonstrated significant effect on antiproliferation against SKOV3 and SKBR3 cancer cells in a dose-dependent manner by MTT assay. All extracts and OVS1 MAb could clearly express apoptosis in cancer cells by; determination on morphological changes with Ho33342 and propidium iodide staining, DNA fragmentation, DNA damage by comet assay and phosphatidylserine translocation by Annexin V-FITC assay. All extracts and OVS1 MAb could induce apoptosis phenomenon in both cancer cells, thus, they could be applied as an anticancer drug in the future.

The effect of cell cycle analysis by flow cytometry from our study suggested that the extracts from *P. amarus* and *S. venosa* may arrest SKBR3 cells at G2/M phase with time- and dose-dependent manner. Some cell lines have limited properties for cell cycle analysis and we have to study more in details to clarify this experiment and try with the purified substances.

Roles of extracts on chemoprevention as an antioxidation which could prevent oxidative damage considered to be an important factor on carcinogenesis. All extracts in this study, exhibited prominent reduction in reactive oxygen species (ROS), especially Ethyl acetate extract from *S. venosa*. The obtained result of the Nitric oxide (NO) assay by Griess reaction indicated that the extracts had little effect on both cancer cells and does not lead to any significant inhibition in the tested system.

All extracts showed properties as a chemotherapy agent by inducing the apoptotic pathway of cancer cells. The partially purified substance from *S. venosa*, i.e., Fraction 18-19 showed strong property on antiproliferation assay and had remarkable apoptosis. This extract might arrest cancer cell at G2/M phase and also showed antioxidative property.

This study provided information on *P. amarus* and *S. venosa* which presented significant potential in cancer chemotherapy and chemoprevention. We should continue to pursue the study by isolation of the active compound from these plants that can reveal the previous identified activities. Since the plant extracts showed promising results on the antiproliferation and inducing apoptotic process as anticancer agent, we can combine or conjugate these compounds after intensive study to OVS1 MAb for targeting on tumor cells, as a new delivery system. Further study on chemotherapeutic or chemopreventive mechanisms of compounds from *P. amarus* and *S. venosa* may be the discovery of a new natural product to fight cancer or increase the quality life for cancer patients.

REFERENCES

1. Saengruchi S, editor. Tumor registry Siriraj cancer center statistical report 2003. Siriraj cancer center: Supavanichkarnpim; 2004.
2. Cooper GM. The cell a molecular approach. 2nd ed. Washington DC: ASM Press; 2000.
3. Wattenberg L, Lipkin M, Boone CW, Kelloff GJ. Cancer chemoprevention. Tokyo: CRD Press; 1992.
4. Gray D, Sprent J. Immunological memory. Berlin: Springer-Verlag; 1990.
5. Boyer CM, Lidor Y, Lottich SC, Bast RC. Antigenic cell surface marker in human solid tumors. Antibody immunoconjugates radiopharmaceuticals. 1988;1: 105-62.
6. Johnstone A, Thorpe R. Immunochemistry in practice. 1st ed. London: Blackwell Scientific Publication; 1982.
7. Roitt I, Brostoff J, Male D. Immunology. 6th ed. Edinburgh: Mosby; 2001.
8. Neungton N, Moongkarndi P, Neungton S, Laohathai K, Srisawat C, Wachirutmanggur L, et al. Establishment of OVS1 and OVS2 monoclonal antibodies recognizing human ovarian mucinous cystadenocarcinoma. Asian Pacific J Allergy Immunol 1995;13:47-53.
9. Isarangkul W. Ovarian epithelial tumors in Thai women: A histological anglysis of 291 cases. Genecol Oncol 1984;17:326-39.
10. Vatanasapt V, Martin H, Sriplung H, Chindavijak K, Sontipong S, et al. Cancer in Thailand 1988-1991. Lyon: 1993. IARC Technical report No. 16:49.
11. Neungton N, Moongkarndi P, Laohathai K, Neungton S, Juntrachotiwit P. Immuno-histological characterization of OVS1 and OVS2 monoclonal antibodies recognizing human ovarian mucinous cystadenocarcinoma. Asian Pacific J Allergy Immunol 1992;10:129-34.

12. Kaslungka S. Cytotoxicity and apoptosis of ovarian cancer and breast cancer cell lines induced by OVS1 monoclonal antibody and paclitaxel [M.Sc. Thesis in Microbiology]. Bangkok: Faculty of Graduate Studies, Mahidol University; 1999.
13. Kosem N. Antiproliferation and antioxidative effects on ovarian and breast cancer by OVS1 monoclonal antibody combinding with mongosteen and turmeric [M.Sc. Thesis in Microbiology]. Bangkok: Faculty of Graduate Studies, Mahidol University; 2003.
14. Balick MJ, Elisabetsky E, Laird SA. Medicinal resources of the tropical forest: biodiversity and its importance to human health. New York: Columbia University Press; 1996.
15. Lamar AS, Fiore M, Cundari E, Ricordy R, Cozzi R, Salvia DR. *Phyllanthus orbicularis* aqueous extract: cytotoxic, genotoxic and antimutagenic effects in the CHO cell line. Toxicol App Pharm 1999;161:231-9.
16. Rajeshkumar NV, Joy KL, Kuttan G, Ramsewak RS, Nair GM, Kuttan R. Antitumour and anticarcinogenic activity of *Phyllanthus amarus* extract. J Ethnopharmacol 2002;81:17-22.
17. Jeena JK, Joy KL, Kuttan R. Effect on *Emblica officinalis, Phyllanthus amarus* and *Picrorrhiza kurroa* on N-nitrosodiethylamine induced hepatocarcino genesis. Cancer Lett 1999;136:11-6.
18. Banerji J, Chatterjee A, Patra A, Bose P, Das R, Das B, et al. Kamaline, an unusual proaporphine and aporphine alkaloids from *Stephania venosa*. Phytochem 1994;36(4):1053-6.
19. Charles B, Bruneton J, Pharadai K, Tantisewie B, Guinaudeau H, Shamma M. Some unusual proaporphine and aporphine alkaloids form *Stephania venosa*. J Nat Prod 1987;50(6):113-7.
20. กิติศักดิ์ ลิขิตวิทยาวุฒิ, จิระพันธ์ กรึงไกร. สารต้านทานมาลาเรียจากหัวสบู่เลือด. จุฬาวิจัย 2542.
21. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 1983;65:55-63.

22. Moongkarndi P. Srivattana A, Boonyapraphatsara N, Puthong S, Laohathai K. Cytotoxicity assay of Hispidulin and Quercetin using colorimetric technique. Mahidol Univ J Pharm Sci 1991;18(2):25-31.
23. Moongkarndi P, Kaslungka S, Kosem N, Junnu S, Jongsomboonkusol S, Theptaranon Y, et al. Cytotoxicity and Apoptosis of ovarian and breast cancer cell lines induced by OVS1 monoclonal antibody and Paclitaxel. Asian Pacific J Allergy Immunol 2003;21:31-41.
24. Studzinski GP. Cell growth and apoptosis: a practical approach. New York: IRL Press; 1995.
25. Kaufmann SH. Apoptosis: pharmacological implications and therapeutic opportunities. San Diego: Academic Press; 1997.
26. Conhen JJ. Overview: Mechanisms of apoptosis. Immunol Today 1993;14(3):126-30.
27. Kroemer G, Petit P, Zamzami N, Vayssiere JL, Mignotte B. The biochemistry of programmed cell death. FASEB J 1995;9:1277-87.
28. Gougeon ML, Lecoeur H, editors. Evaluation of apoptosis. J Immunol Methods 2002;265:1-2.
29. Reid S, Cross R, Snow C. Combined Hoechst 33342 and merocyanine 540 staining to examine murine B cell cycle stage, viability and apoptosis. J Immunol Methods 1996;192:43-54.
30. Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. J Immunol Methods 1991;139(2):271-9.
31. Fairbairn WD, Olive LP, O'Neil LK. The comet assay: a comprehensive review. Mutat Res 1995;339:1277-87.
32. Roser S, Pool-Zobel LB, Rechkemmer G. Contribution of apoptosis to responses in the comet assay. Mut Res 2001;497:169-75.
33. Shounan Y, Feng X, O'Connell PJ. Apoptosis detection by Annexin V binding a novel method for the quantitation of cell-mediated cytotoxicity. J Immunol Methods 1998;217:61-70.

34. Vindelov LL, Christensen IJ. Some methods and application of flow cytometric DNA analysis in clinical and experimental oncology. Eur J Haematol 1989;42:69-76.
35. Gong J, Traganos F, Darzynkiewicz Z. A selective procedure for DNA extraction from apoptotic cells applicable for gel electrophoresis and flow cytometry. Anal Biochem 1994;218:314-9.
36. Shureigi I, Reddy P, Brenner DE. Chemoprevention: general perspective. Crit Rev in Oncol Hematol 2000;33:157-67.
37. Decensi A, Coata A. Recent advances in cancer chemoprevention, with emphasis on breast and colorectal cancer. Eur J Cancer 2000;36:694-709.
38. Carbone PP. Advances in the systemic treatment of cancers in the elderly. Crit Rev in Oncol Hematol 2000;35:201-18.
39. Halliwell B, Gutteridge JMC. Free radicals in biology and medicine. Oxford: Academic Press; 1999.
40. Ashmawy MB. The role of natural antioxidants and synthetic nonsteroidal antiinflammatory drugs in the chemoprevention of cancer. Saudi Pharm J 1999;7:1-13.
41. Rice-Evans CA, Diplock AT, Symons MCR. Techniques in free radical research. Amsterdam: Elservier; 1991.
42. Workshop on cell culture and free radicals. Bangkok: Faculty of Pharmacy, Mahidol University; 1999.
43. Pervin S, Singh R, Gau CL, Edamatsu H, Toamnoi F, Chaudhuri G. Potentiation of nitric oxide-induced apoptosis of MDA-MB-468 cells by Farnesyltransferase inhibitor: implication in breast cancer. Cancer Res 2001;61:4701-6.
44. Kimura H, Ogura T, Kurashima Y, Weisz A, Esumi H. Effects of nitric oxide donors on vascular endothelial growth factor gene induction. Biochem Biophys Res Commun 2002;296:976-82.
45. Jang TJ, Kim DK. Inducible nitric oxide synthase expression of tumor and stromal cells is associated with the progression of 7,12-dimethylbenz [a] anthracene-induced rat mammary tumor. Cancer Lett 2002;182:121-6.

46. World Health Organisation. The World Health Organisation report 2003. Geneva: WHO Graphics; 2004.
47. Tannock IF, Hill RP. The basic science of oncology. New York: McGraw-Hill; 1998.
48. Kraiphibul P, editor. Annual report 2003 Ramathibodi Cancer Registry. Facalty of Medicine Ramathibodi Hospital: Mahidol University; 2004.
49. Sheldon H. Boyd's introduction to the study of disease. Philadelphia: Lea and Febiger; 1992.
50. Underwood JCE. General and systematic pathology. London: Churchill Livingstone; 1992.
51. Berek JS, Thomas GM, Ozols RF. Ovarian cancer. In: Holland JF, Bast Jr RC, Morton DL, Ill EF, Kufe DW, Weichselbaum RR, editors. Cancer medicine. 4th ed. Philadelphia: Williams & Wilkins; 1997. p. 2289-326.
52. Cooper GM. Elements of human cancer. Boston: Janes and Bartlett; 1992.
53. Hogber T, Kagedal B. Long-term follow-up of ovarian cancer with monthly detections of serum CA125. Gynecol Oncol 1992;46(2):191-8.
54. Robertson DM, Stephenson T, Pruysers E, Burger H, McCloud P, Tsigos A, et al. Inhibins/activins as diagnostic markers, for ovarian cancer. Mol Cell Endocrinol 2002;191:97-103.
55. วรชัย รัตนธราธร. Ovarian cancer. ใน: วรชัย รัตนธราธร, บรรณาธิการ. ตำราการรักษาโรค กรุงเทพมหานคร: โฮลิสติก; 2538. หน้า 126-48.
56. Pratt WB, Ruddor RW, Ensminger WD, Maybaum J. The anticancer drugs. 2nd ed. Oxford: Oxford University Press; 1994.
57. Morton DL, Jones RC. Principles of surgical oncology. In: Holland JF, Bast Jr RC, Morton DL, Ill EF, Kufe DW, Weichselbaum RR, editors. Cancer medicine. 4th ed. Philadelphia: Williams and Wilkins; 1997. p. 651-64.
58. อาคม เชียรศิลป์. ความรู้เกี่ยวกับเคมีบำบัด. กรุงเทพมหานคร: ฟาร์มิตาเลีย คาร์โล เออร์มา; 2531.
59. Weichselbaum RR, Chen G, Hallahan DE. Biological and physical basis of radiation oncology. In: Holland JF, Bast Jr RC, Morton DL, Ill EF, Kufe DW, Weichselbaum RR, editors. Cancer medicine. 4th ed. Philadelphia: Williams and Wilkins; 1997. p. 697-726.

60. Reed JC. Apoptosis. San Diego: Academic Press; 2000.
61. Fisher B, Osborne CK, Margolese RG, Bloomer WD. Neoplasms of the breast. In: Holland JF, Bast Jr RC, Morton DL, Ill EF, Kufe DW, Weichselbaum RR, editors. Cancer medicine. 4th ed. Philadelphia: Williams and Wilkins; 1997. p. 2349-429.
62. MacMahao B, Cole P, Brown J. Etiology of human breast cancer. J Natl Cancer Inst 1973;50:21.
63. Miller AB. An overview of hormone-associated cancer. Cancer Res 1973;38:3985.
64. Kelsey JL. A review of the epidemiology of human breast cancer. Epidemiol rev 1979;1:74.
65. Haagensen CD. Diseases of the breast. 3rd ed. Philadelphia: WB Saunders; 1979.
66. Catteau A, Morris JR. BRCA1 methylation: a significant role in tumor development? Cancer Biol 2002;12:359-71.
67. Forbes JF. The incidence of breast cancer: the global burden, public health consideration. Semin Oncol 1997;24:SI20-35.
68. Hunter DJ, Willett WC. Diet, body size and breast cancer. Epideminol Rev 1993;15:110-32.
69. Snyderwine EG. Some perspective on the nutritional aspects of breast cancer research. Cancer 1994;74:1070-7.
70. Gaubatz JW. Cooked food mutagens and breast cancer. Nutrition 1996;12:449-550.
71. Wakabayashi K, Nagao M, Esumi H, Sugimura T. Food derived mutagens and carcinogens. Cancer Res 1992;52: 2092s-8s.
72. Nagao M, Ushijima T, Wakabayashi K, Ochiai M, Kushida H, Sugimura T. Dietary carcinogens and mammary carcinogenesis. Cancer 1994;74:1063-9.
73. DeStefeni E, Ronco A, Mendilaharsu M, Guidobono M, Deneo-Pellegrini H. Meat intake, heterocyclic amines and risk of breast cancer: a case-control study in Uruguay. Cancer Epideminol Biomarkers Prev 1997;6:573-81.

74. Zheng W, Gustafson DR, Sinha R, Cerhan JR, Moore D, Hong CP, et al. Well-done meat intake and the risk of breast cancer. J natl Cancer Inst 1998;90:1724-9.

75. วรชัย รัตนธราธร. Breast cancer: state of the art adjuvant chemotherapy for breast cancer. ใน: วรชัย รัตนธราธร, บรรณาธิการ. ตำราการรักษาโรคมะเร็ง. กรุงเทพมหานคร: โฮลิสติก; 2538. หน้า 74-95.

76. World Health Oragnisation. Chemotherapy of solid tumours. Geneva: The Organisation; 1977.

77. Grever MR, Grieshaber CK. Toxicology by organ system. In: Holland JF, Bast Jr RC Morton DL, I11 EF, Kufe DW, Weichselbaum RR, editors. Cancer medicine. 4th ed. Philadelphia: Williams and Wilkins; 1997. p. 891-904.

78. Dhinakar S, Kompala PT, editors. Cell serapation science and technology. Washington DC: Chemical society. 1991. p. 301.

79. Hurrell JGR, Monoclonal hybridoma monoclonal antibodies: techniques and applications. Baca Raton: CRC Press; 1982.

80. Lyle L. Production and applications of monoclonal antibodies. MD & DI 1982;83(June):41-4.

81. Enrlich P. Collected studies on immunology. 11th ed. New York: John Wiley & Sons; 1906. p. 442-7.

82. Foon KA, Todd RF. Immunolgic classification of leukemia and lymphoma. Blood 1986;68:1-31.

83. Bagshawe KD. Tumor markers. Where do we go from here? Br J Cancer 1983;48:167-75.

84. Fritsche HA. Some new and prospective tumor markers; there nature and clinical stutus. Lab Management 1986;25-34.

85. Crowther ME, Britton KE, Granowska M, Shephered JH. Monoclonal antibodies and their usefulness in epithelial ovarian cancer. Br J Obstet Gynecol 1989;96:516-21.

86. Scott AM, Cebon J. Clinical promise of tumour immunology. Lancet 1997;349:19SII-22SII.

87. Glennie MJ, Johnson PWM. Clinical trials of antibody therapy. Rev Immunol Today 2000;21(8):403-10.

88. Biddle WC, Foon KA. Monoclonal antibodies in therapy, alone or conjugated with drugs. In: Mitchell MS, editor. Biological approach to cancer treatment and biomodulation. 1st ed. America: McGraw-Hill; 1993. p. 209-51.
89. Haskell CM. Cancer treatment. Philadelphia: W.B. Saunders; 1985.
90. Janeway CA, Travers P, Hunt S, Walport M. Immunobiology: the immune system in health and disease. 3rd ed. London: Garland Publishing; 1997.
91. Dhir H, Roy AK, Sharma A. Relative efficiency of *Phyllanthus emblica* fruit extract and ascorbic acid in modifying lead and aluminium induced sister chromatid exchanges in mouse bone marrow. Environ Mol Mutag 1993;21:229-236.
92. Cozzi R, Ricordy R, Aglitti T, Gatta V, Perticone P, Salvia R. Ascorbic acid and β-carotene as modulators of oxidative damage. Carcinogenesis 1997;18(1):223-8.
93. Weiss RF, Fintelman V. Herbal medicine. 2nd ed. Stuttgart: Thieme; 2000.
94. Saralamp P, Chuakul W, Temsiririrkkul R, Clayton T. Medicinal Plants in Thailand. Bangkok: Amarin Printing & Publishing; 1996.
95. Gupta ML, Gupta TK, Bhargava FP. A study of antifertility effects of some indigenous drugs. J Res Indian Med 1994;6:112-7.
96. Ihantola-Vormisto AJ, Summannen H, Kankaaranta H, Vuorela ZM, Asmawi C, Moilanen E. Anti-inflammatory activity of extracts from leaves of *Phyllanthus emblica.* Planta Med 1997;63(6):518-24.
97. Srividya N, Periwal S. Diuretic, hypotensive and hypoglycaemic effect of *Phyllanthus amarus*. Indian J Exp Biol 1995;33:861-4.
98. Unander DW, Webster GL, Blumberg BS. Usage and bioassays in *Phyllanthus* (Euphorbiaceae) Part IV clustering of antiviral uses and other effects. J Ethnoparmacol 1995;45:1-18.
99. Calixto JB, Santos ARS, Yunes RAC. A review of the plants of the genus *Phyllanthus* their chemistry, pharmacology and therapeutic potential. Med Res Rev 1998;18:225-58.
100. Thyagarajan SP. Effect of *Phyllanthus amarus* with chronic carriers of hepatitis B virus. Lancet 1988;764-6.

101. Unander DW, Venkateswaran PS, Millman I, Bryan H, Blumberg BS. *Phyllanthus* species: sources of new antiviral compounds. In: Janick J, Simon JE, editors. Advances in new Crops. Timber Press; 1990. p. 518-21.

102. Ott M, Thyagarajan SP, Gupta S. *Phyllanthus amarus* suppresses hepatitis B virus by interrupting interactions between HBV enhancer I and cellular transcription factors. Eur J Clin Inv 1997;27(11):908-15.

103. Liu KCSC, Lin MT, Lee SS, Chiou JF, Ren S, Lien EJ. Antiviral tannins from two *Phyllanthus* species. Planta Med 1999;65:43-6.

104. Chang CW, Lin MT, Lee S, Liu KCSC, Hsu FL, Lin JY. Differential inhibition of reverse transcriptase and DNA cellular polymerase-α activities by lignins isolated from Chinese herbs, *Phyllanthus myrtifolius* Moon, and tannins from *Lonicera japonica* Thunb and *Castanopsis hystrix*. Antiviral Res 1995;27:367-74.

105. Hnatyszyn O, Broussalis A, Herrera G, Muschietti L, Coussio J, Martino V, et al. Argentine plant extracts active against polymerase and ribonuclease H activities of HIV-1 reverse transcriptase, Phytother Res 1999;13:206-9.

106. Vinayagamoothy T. Antibacterial activity of medicinal plants of Sri Lanka, Ceylon. J Sci Biol Soc 1982;191:50-9.

107. Omulokoli E, Khan B, Chabra SC. Antiplasmodial activity of four Kenyan medicinal plants. J Ethnopharmacol 1997;56(2):133-7.

108. Filho CV, Santos AR, Campos RO, Miguel OG, Yunes RA, Ferrari FI, et al. Chemical and pharmacological studies of *Phyllanthus carolinensis* in mice. J Pharm Pharmacol 1996;48(12):1231-6.

109. Petit GR, Schaufelberger DE, Nieman RA, Dufresne C, Saenz-Renauld JA. Antineoplastic agents 177 isolation and structure of phyllanthostatin. J Nat Prod 1990;53:1406-13.

110. Blumberg BS, Millman I, Venkateswaran PS, Thyagarajan SP. Hepatitis B virus and hepatocellular carcinoma-treatment of HBV carriers with *Phyllanthus amarus*. Cancer Detect Prev 1990;14:195-201.

111. Roy AK, Dhir H, Sharman A. Modification of metal induced micronuclei formation in mouse bone marrow erythrocytes by *Phyllanthus* fruit extract and ascorbic acid. Toxicol Lett 1992;62:9-17.

112. Gowrishanker B, Vivekanandan OS. *In vivo* studies of a crude extract of *Phyllanthus amarus* L. in modifying the genotoxicity induced in *Vicia faba* L. by tannery effluents. Mutat Res 1994;322:185-92.

113. Unander DW, Venkateswaran PS, Milliman I, Blumberg BS. Inhibition of hepatitis B virus-dependent DNA polymerase by species of *Phyllanthus*; genetic and environmental effects on this activity. Brenesia 1991;34:27-40.

114. Ferrer M, Sanchez-Lamar A, Fuentes JL, Barbe J, Llagostera M. Studies of the antimutagenesis of *Phyllanthus orbicularis*: mechanisms involved against aromatic amines. Mut Res 2001;498:99-105.

115. Botany and Weed Science Division [Online]. Bangkok: Available from: http://www.doa.go.th/botany/pham.html [Acessed 1 Mar 2005].

116. Satyavati GV, Raina MK, Sharma M. Medical plants of India. Delhi: Cambridge Prining: 1976.

117. รุ่งระวี เติมศิริฤกษ์กุล, วงศ์สถิต ฉั่วกุล, สมภพ ประธานธุรารักษ์, พร้อมจิต ศรลัมพ์, วิชิต เปานิล, นพมาศ สุนทรเจริญนนท์, สมุนไพรยาไทยที่ควรรู้. กรุงเทพมหานคร: อมรินทร์พริ้นติ้ง แอนด์ พับลิชชิ่ง; 2542.

118. Farnsworth N, Bunyapraphatsara N. Thai medicinal plants: recommended for primary health care system. Bangkok: Prachachon; 1992.

119. นันทวัน บุญยะประภัศร, อรนุช โชคชัยเจริญพร. สมุนไพรไม้พื้นบ้าน. กรุงเทพมหานคร: สำนักพิมพ์ประชาชน; 2542.

120. Foo LY, Wong H. Phyllanthusiin D, an unusual hydrolysable tannin from *Phyllanthus amarus*. Phytochemistry 1992;31:711-3.

121. Foo LY. Amariin, a di-hydrohexahydroxydiphenoyl hydrolysable tannin from *Phyllanthus amarus*. Phytochemistry 1993;33:487-91.

122. Somanabandhu A, Nitayangkura S, Mahidol C, Ruchirawat S, Likhitwitayawuid K, Shieh HL, et al. 1H-and 14C-NMR assignments of phyllanthin and hypophyllanthin: lignans that enhance cytotoxicity with cultured multidrug-resistant cells. J Nat Prod 1993;56:233-9.

123. Macrae WD, Hudson JB, Towers GHN. Studies on the pharmacological activity of Amazonian Euphorbiaceae. J Ethnopharmacol 1988;222:143-72.

124. Thyagarajan SP, Thirunulakantan K, Subramanian S, Sundaravelu T. *in vitro* inactivation of HBsAg by *Ecliptalba Hassk* and *Phyllanthus niruri* Linn. Ind J Med Res 1982;76:124-30.

125. Lee DC, Ott M. Thyagarajan SP, Shafritz DA, Burk RD, Gupta S. *Phyllanthus amarus* down-regulates hepatitis B virus mRNA transcription and replication. Eur J Clin Invest 1996;26(12):1069.

126. Dhir H, Roy AK, Sharma A, Talukder G. Modification of clastogenicity of lead and aluminium in mouse bone marrow cells by dietary ingestion of *Phyllanthus emblica* fruit extract. Mutat Res 1990;241:305-12.

127. Sripanidkulchai B, Tattawasart U, Laupatarakasem P, Vinitketkumneun U, Sripanidkulchai K, Furihata C, et al. Antimutagenic an anticarciongenic effects of *Phyllanthus amarus*. Phytomedicine 2002;9:26-32.

128. Chopra RN, Neyer SL, Chopra IC. Glossary of Indian Medicinal Plants CSIR. India: 1956. p. 191

129. Development Alternatives Group [Online]. New Delhi: Available from: http://www.devalt.org/newsletter/jul03/of_4.htm [Accessed 1 Mar 2005].

130. Available form: http://www.saboten.co.jp/ sub1/meisai3/15517.htm. [Accessed 1 Mar 2005].

131. Forman LL. Menispermaceae. In: Flora of Thailand. The forest Herbarium. 5th ed. Royal Forest Department; Bangkok:1991. p. 300-65.

132. Perry LM, Metzger J. Medicinal plants of East and Southeast Asia. Cambridge, Messachusetts: MIT Press; 1980.

133. Tomita M. Die alkaloide der Menispermaceae Pfflanzen, Fortschritte der chemie Organischer Naturstoffe vol. 9, Berlin: Springer-Verlag; 1952.

134. Shamma M. The isoquinoline alkaloids chemistry and pharmacology. New York: Academic Press; 1972.

135. Kunitomo J, Murakami Y, Oshikata M, Shingu t, Akasu M, Lu S, et al. The alkaloids of *Stephania sasakii*: structure of five new alkaloids. Phytochemistry 1980;19:2735-9.

136. Buckingham J, editor. Dictionary of natural products. London: Chapman & Hall; 1994.

137. Guinaudeau H, Shamma M, Tanitsewie B, Pharadai K. Aporphine alkaloids oxygenated at C-7. J Nat Prod 1982;45:355-7.

138. Pharadai K, Pharadai T, Tantisewie B, Guinaudeau H, Freyer AJ, Shamma M. (-)-0-Acetylsukhodianine and oxostephanosine: two new aporphinoids from *Stephania venosa*. J Nat Prod 1985;48:658-9.

139. Keawpradub N, Itharat A, Tantikarnkul A, Rugleng S, Inruspong R. Cytotoxic alkaloids from the tuber of *Stephania venosa*. Songklanakarin J Sci Technol 2001;23(2):225-34.

140. Cordell GA. Introduction to alkaloids a biogenetic approach. New York: John wiley & sons; 1981.

141. Simon S, Rios JL, Villar A. Pharmacological activities of Protoberberine alksloids. Plants Med Phytother 1989;23:202-5.

142. Bhakuni DS, Jain S. Protoberberine alkaloids in alkaloids. New York: Academic Press; 1988.

143. Ingkaninan K, Yuyaem T, Thongnoi W. Anti-cholinesterase activity of *Stephania*. Naresuan Univ J 2001;9(1):35-40.

144. Kerr JFR, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide ranging implications in tissue kinetics. Br J Cancer 1972;26:239-57.

145. Jessie ALS, Panchal N, Li D, Gan Y. Apoptosis: a new pharmacodynamic endpoint. Pharm Res 1997; 14(12): 1659-71.

146. Sluyser M. Apoptosis in normal development and cancer. London: Taylor and Francis; 1996.

147. Bender MedSystems Inc [Online]. California: Available form: http:// www.bendermedsystems.com/ 112.html [Accessed 1 Mar 2005].

148. University of Otago [Online].Dunedin: 2003. Available form: http:// www-micro.msb.le.ac.uk/ 3035/kalmakoff/baculo/... [Accessed 1 Mar 2005].

149. Centro de Estudios Científicos [Online]. Valdivia: 2005. Available form: http:// www.md.ucl.ac.be/.../ RPR20011/sld046.htm [Accessed 1 Mar 2005].

150. Fei R, Shaoyang L. Combination antigene therapy targeting c-myc and c-erbB_2 in the ovarian cancer COC_1 cell line. Gynecol Oncol 2002;85:40-4.

151. Gooch JL, Yee D. Strain-specific differences information of apoptotic DNA ladders in MCF-7 breast cancer cells. Cancer Lett 1999;144(1):31-7.

152. McKelvey VM. The single cell gel electrophoresis assay (COMET assay): A European Review. Mutat Res 1993;288:47-63.

153. Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA damages in individual mammalian cells. Biochem Biophys Res Commun 1984;123:291-8.

154. Olive PL, Banath JP, Durand RE. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells measured using the "comet" assay. Radiat Res 1990;122:69-72.

155. Olive PL, Vikse CM, Banath JP. Use of the comet assay to identify cell sensitive to tirapazamine in multicell spheroids and tumors in mice. Cancer Res 1996;56:4460-3.

156. Olive PL, Banath JP. Multicell spheroid response to drugs predicted with the comet assay. Cancer Res 1997;57:5528-33.

157. Available form: http://www.lfw.pennnet.com/Articles/ Article_Display.cfm?... [Accessed 1 Mar 2005].

158. University of Bern [Online]. Bern: 2004. Available form: http://www.pathology.wustl.edu/ html/facilitiesfacsfc.html [Accessed 1 Mar 2005].

159. Available form: http:// www.pathology.wustl.edu/ html/facilitiesfacsfc.html [Accessed 1 Mar 2005].

160. Vermes I, Haanen C, Nakken HS, Reutelingsperger C. A novel assay for apoptosis: flow cytometric detection of phosphatidyl serine expression on early apoptotic cells using fluorescein labelled Annxin V. J Immumol Methods 1995;184:39-51.

161. Ormerod MG. Investigating the relationship between the cell cycle and apoptosis using flow cytometry. J Immunol Methods 2002;265:73-80.

162. Darzynkiewicz Z, Bruno S, Del Bino G, Gorczyca W, Hotz MA, Lassota P, et al. Features of apoptotic cells measured by flow cytometry. Cytometry 1992;13:795-808.

163. Darzynkiewicz Z, Juan G, Li X, Gorczyca W, Murrakami T, Traganos F. Cytometry in cell necrobiology: analysis of apoptosis and accidental cell death (necrosis). Cytometry 1997;27:1-20.

164. Ormerod MG. The study of apoptotic cells by flow cytometry. Leukemia 1998;12:1013-25.

165. Ormerod MG. Using flow cytometry to follow the apoptotic cascade. Redox Report 2001;6:275-87.

166. Ormerod MG. Analysis of DNA-general methods. In :Ormerod MG, editor. Flow cytometry: A practical approach. 3rd ed. Oxford: IRL Press at Oxford University Press; 2000. p. 83-97.

167. Ormerod MG. Flow cytomertry in the study of apoptosis. In Ormerod MG, editor. Flow cytometry: A practical approach. 3rd ed. Oxford: IRL Press at Oxford University Press; 2000. p. 235-47.

168. ปริ่มเฉนียน มุ่งการดี. ความรู้ทั่วไปและหลักการใช้เคมีบำบัด. ใน: ภาควิชาเภสัชวิทยาคลีนิก. ยาต้านจุลชีพ ยาเคมีบำบัด และยาที่มีผลต่อภูมิคุ้มกัน. กรุงเทพ: สำนักพิมพ์ มหาวิทยาลัยสุโขทัยธรรมาธิราช; 2545. หน้า 410-451.

169. Available form: http:// www.bioteach.ubc.ca/ CellBiology/TheCellCycle/ [Accessed 1 Mar 2005].

170. McCord JM. The evolution of free radicals and oxidative stress. Am J Med 2000;108:652-9.

171. Forstermann U, Gath I, Schwarz P, Closs EI, Kleinert H. Isoforms of nitric oxide synthase. Biochem Pharmacol 1995;50(9):1321-32.

172. Koppenol WH, Moreno JJ, Pryor WA, Ischiropoulos H, Beckman JS. Peroxynitrite, a cloaked oxidant formed by nitric oxide and superoxide. Chem Res Toxicol 1992;5:834-9.

173. Castro L, Freeman BA. Reactive oxygen species in human health and disease. Nutrition 2001;17:161-5.

174. Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997;272:20963-6.

175. Baik SC, Youn HS, Chung MH, Lee WK, Cho MJ, Ko GH, et al. Increased oxidative DNA damage in *Helicobacter pylori* infected human gastric mucosa. Cancer Res 1996;56:1279-82.

176. Ballinger SW, Bouder TG, Davis GS, Judice SA, Nicklas JA, Albertini RJ. Mitochondrial genome damage associated with cigarette smoking. Cancer Res 1996;56:5692-7.

177. Chen Q, Marsh J, Ames B, Mossman B. Detection of 8-oxo-2′-deoxyguanosine, a marker of oxidative DNA damage, in culture medium from human mesothelial cells exposed to crocidolite asbestos. Carcinogenesis 1996;17:2525-7.

178. Hiraku Y, Kawanishi S. Oxidative DNA damage and apoptosis induced by benzene metabolites. Cancer Res 1996;56:5172-8.

179. Stadtman ER, Oliver CN. Metal-catalyzed oxidation of proteins: physiological consequences. J Biol Chem 1991;266:2005-8.

180. Castro L, Rodriguez M, Radi R. Aconitase is readily inactivated by peroxynitrite, but not by its precursor, nitric oxide. J Biol Chem 1994;269:29409-15.

181. Souza JM, Radi R. Glyceraldehyde-3-phosphate dehydrogenase inactivation by peroxynitrite. Arch Biochem Biophys 1998;360:187-94.

182. Ara J, Przedborski S, Naini AB, Jackson-Lewis V, Trifiletti RR, Horwitz J, et al. Inactivation of tyrosine hydroxylase by nitration following exposure to peroxynitrite and 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Proc Natl Acad Sci USA 1998;95:7659-63.

183. Knight JA. Free radicals, antioxidants, aging, and disease. Washington DC: AACC; 1999.

184. Wink DA, Mitchell JB. Chemical biology of nitric oxide: insights into regulatory, cytotoxic, and cytoprotective mechanisms of nitric oxide. Free Radical Biol Med 1998;25:434-56.

185. Stamler JS. Redox signaling: nitrosylation and related target interactions of nitric oxide. Cell 1994;78:931-6.

186. Mayer B, John M, Bohme E. Purification of a Ca^{2+}/calmodulin-dependent nitric oxide synthase from porcine cerebellum. Cofactor-role of tetrahydrobiopterin. FEBS Lett 1990;277:215-9.

187. Pollock JS, Forstermann U, Mitchell JA, Warner TD, Schmidt HH, Nakane M, et al. Purification and characterization of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proc Natl Acad USA 1991;88:10480-4.

188. Forstermann U, Closs EI, Pollock JS, Nakane M, Schwarz P, Gath I, et al. Nitric oxide synthase isozymes. Characterization, purification, molecular cloning and fuctions. Hypertension 1994;23:1121-31.

189. Nathan C, Xie QW. Nitric oxide synthases: roles, tolls and controls. Cell 1994;78:915-8.

190. Taylor BS, Alarcon LH, Billiar TR. Inducible nitric oxide synthase in the liver: regulation and function. Biochemistry (Mosc) 1998;63:766-81.

191. Sandau K, Pfeilschifter J, Brune B. The balance between nitric oxide and superoxide determines apoptotic and necrotic cell death of rat mesangial cells. J Immunol 1997;158:4938-46.

192. Steller H. Mechanisms and genes of cellular suicide. Science 1995;267:1445-9.

193. Bernhard B, Knethen AV, Sandau KB. Nitric oxide and its role in apoptosis. Eur J Pharm 1998;351;261-72.

194. Basu TK, Temple NJ, Garg ML. Antioxidants in human health and disease. New York: CABI; 1999.

295. Brune B, Knethen AV, Sandau KB. Review nitric oxide and its role in apoptosis. Eur J Pharmacol 1998;351:261-272.

196. PIERCE Chemical Company. Protein chemistry: products. n.p.; 1998.

197. นภาธร บานชื่น. ELISA: ทฤษฎีและปฏิบัติ. กรุงเทพมหานคร: สำนักพิมพ์หมอชาวบ้าน; 2536.

198. Moongkarndi P. Monoclonal antibodies against the fifth component of human complement. Heidelberg: Ruprecht-Karls Universitat; 1984.

199. Bio-Rad Laboratories. Life science research products 2000-01. n.p.; 2000.

200. Bollag DM, Rozycki MD, Edelstein SJ. Protein methods. 2nd ed. Toronto: Wiley-Liss; 1996.

201. Hames BD, Rickwood D. Gel electrophoresis of protein: a practical approach. New York: IRL Press; 1990.

202. Darling DC, Morgan SJ. Animal cells culture and media. Brisbane: John Wiley & Sons; 1994.

203. Ruffa MJ, Ferraro G, Wagner ML, Calcagno ML, Campos RH, Cavallaro L. Cytotoxic effect of Argentine medicinal plant extracts on human hepatocellular carcinoma cell line. J Ethnopharmacol 2002;79:335-9.

204. American Type Culture Collection [Online]. ATCC Bioproduct; 2005. Available from: http://www.atcc.org/Products/MttCell.cfm [Accessed 1 Mar 2005].

205. Sanker KP, Obana S, Nakata M, Kitajima I, Maruyama I. Anandamide induces apoptosis of PC-12 cells: involvement of superoxide and caspase-3. FABS Lett 2000;472:39-44.

206. Whiteside G, Cougnon N, Hunt SP, Munglani R. An improved method for detection of apoptosis in tissue sections and cell culture, using the TUNEL technique combined with Hoechst stain. Brian Res Brain Res Protoc 1998; 2(2):161-4.

207. Rickwood D, Hames BD. Gel electrophoresis of nucleic acid: a practical approach. Oxford: IRL Press; 1990.

208. วีณา จิรัจฉริยากูล, บรรณาธิการ. ยาและผลิตภัณฑ์ธรรมชาติ. กรุงเทพ: ภาควิชาเภสัชวินิจฉัย คณะเภสัชศาสตร์ มหาวิทยาลัยมหิดล; 2534.

209. ดำรง ทิพย์โยธา. การวิเคราะห์ข้อมูลทางสถิติด้วย SPSS for window version 10. กรุงเทพ: โรงพิมพ์แห่งจุฬาลงกรณ์มหาวิทยาลัย; 2545.

210. Abbas AK, Lichtman AH, Pober FS. Cellular and molecular immunology. 4th ed. Philadelphia: W.B. Saunders; 2000.

211. Stites DP, Stobo JD, Well JV. Basic and clinical immunology. 6th ed. California: Appleton and Lange; 1987.

APPENDIX

APPENDIX A

MEDIA AND REAGENTS

1. RPMI 1640 medium

RPMI 1640 medium (BIOCHROM AG Cat.No. T121-01) (with L-glutamine and 25 mM HEPES buffer, without $NaHCO_3$)	10.43 g
$NaHCO_3$	2 g
100 μg/ml Streptomycin - 100 units/ml Penicillin G	10 ml
Sterile water for injection to make	1000 ml

Adjust pH to 7.0 - 7.2 with 1 N HCl or 1 N NaOH before adjust volume with water and sterilize by cellulose acetate filter (pore size 0.2 μm).

2. RPMI 1640 medium for hybridoma

RPMI 1640 medium (BIOCHROM AG Cat.No. T121-01) (with L-glutamine and 25 mM HEPES buffer, without $NaHCO_3$)	10.43 g
Glucose	2 g
Pyruvic acid	0.11 g
L-glutamine	0.10 g
NaHCO3	2 g
100 μg/ml Streptomycin-100 units/ml Penicillin G	10 ml
Sterile water for injection to make	1000 ml

Adjust pH to 7.0-7.2 with 1 N HCl or 1 N NaOH before adjust volume with water and sterilize by cellulose acetate filter (pore size 0.2 μm).

3. Freezing medium

RPMI 1640 medium	67%
Fetal calf serum	25%
Dimethysulfoxide (DMSO)	8%

4. Hank's balanced salt solution

Hank's balanced salt	9.79 g
$NaHCO_2$	3.50 g
Distilled water to make	1000 ml

Adjust pH to 7.0 with 1 N HCl or 1 N NaOH and sterilize by cellulose acetate filter (pore size 0.2 μm).

5. Phosphate buffer saline (PBS)

0.2 M phosphate buffer (PB)

0.2 M NaH_2PO_4 pH 4.5	27.998 g/l
0.2 M Na_2HPO_4 pH 9.4	35.598 g/l

Add NaH_2PO_4 to Na_2HPO_4 dropwise to adjust the pH to 7.4

0.01 M PBS

0.2 M PB pH 7.4	50 ml
NaCl	8.76 g
Distilled water to make	1000 ml

Sterilize by cellulose acetate filter (pore size 0.2 μm).

6. 0.1 M Sodium citrate pH 3.0

Sodium citrate	29.41 g
Distilled water to make	1000 ml

Adjust pH to 3.0 with 1 N HCl before adjust volume with water.

7. 0.5 M Tris-HCl pH 6.8

Tris-(hydroxymethyl) aminomethane	60.57 g
Distilled water to make	1000 ml

Adjust pH to 6.8 with 1 N HCl before adjust volume with water.

8. 1.5 M Tris-HCl pH 8.8

Tris-(hydroxymethyl) aminomethane	181.71 g
Distilled water to make	1000 ml

Adjust pH to 8.8 with 1 N HCl before adjust volume with water.

9. 10% Sodium dodecyl sulfate (SDS)

Sodium dodecyl sulfate	10 g
Distilled water to make	100 ml

Mix and heat to dissolve completely at 60°C.

10. Acrylamide stock solution

Acrylamide	30 g
N,*N'*-Methylenebisacrylamide	0.8 g
Distilled water to make	100 ml

Stir in the dark and filtrate by Whatman filter paper No.1.

11. Sample buffer for SDS-PAGE 10 ml

50% Glycerol	5 ml
10% SDS	2 ml
2-Mercaptoethanol	0.5 ml
1% Bromophenol blue	1 ml
Distilled water	0.9 ml

Store for weeks in the refrigerator or for months at -20^{o}C.

12. Electrophoresis buffer for SDS-PAGE

Tris-(hydroxymethyl) aminomethane	3 g
Glycine	14.4 g
Sodium dodecyl sulfate (SDS)	1 g
Distilled water to make	1000 ml

pH should be approximately 8.3 and store at room temperature.

13. Staining solution for SDS-PAGE

Coomasic blue	0.1 g
Methanol	40 ml
Glacial acetic acid	10 ml

14. Destaining solution for SDS-PAGE

Methanol	100 ml
Glacial acetic acid	100 ml
Distilled water	800 ml

15. 1 mg/ml Hoechst 33342 (Ho33342) stock solution

Ho33342	1 mg
Distilled water	1 ml

Store in aliquots at -20°C.

16. 1 mg/ml Propidium iodide (PI) stock solution

PI	1 mg
Distilled water	1 ml

Store in aliquots at -20°C.

17. Lysis buffer to break cells for DNA ladder assay

1M Tris-HCl pH 7.6	5 ml
0.5M EDTA pH 8.0	2 ml
2M NaCl	5 ml
10% SDS	2.5 ml
Distilled water to make	50 ml

18. 20 mg/ml Proteinase K stock solution

Proteinase K	100 mg
Distilled water	5 ml

Store in aliquots at -20°C.

19. 10 mg/ml Ribonuclease (RNase) stock solution

RNase	50 mg
Solution of 10mM Tris-HCl pH 7.5-15mM NaCl	5 ml

Heat to 100°C for 15 min and cool slowly to room temperature. Store in aliquots at -20°C.

20. 10x Tris-acetate-EDTA (TAE) buffer for agarose gel electrophoresis

Tris base	48.4 g
Glacial acetic acid	11.4 ml
0.5 M EDTA pH 8.0	20.0 ml
Distilled water to make	1000 ml

Adjust pH to 7.6 with 1 N HCl or 1 N NaOH before adjust volume with water

21. Agarose gel

Agarose	0.75 g
TAE buffer	50 ml

Mix and heat to dissolve completely.

22. Gel loading buffer for agarose gel electrophoresis

Bromophenol blue	0.25%
Glycerol	50%
EDTA	100 mM

Mix and heat to dissolve completely

23. 1% Agarose gel for comet assay

Agarose	0.015 g
0.01 M PBS	1.5 ml

Mix and heat to dissolve completely

24. 0.75% Low melting agarose

Low melting agarose	0.0075 g
0.01 M PBS	1 ml

Mix and heat to dissolve completely

25. Alkaline lysis solution for comet assay

Sodium chloride	146.1 g
EDTA	37.224 g
Tris	1.2114 g
Distilled water to make	1000 ml

Adjust pH to 10 with 1 N NaOH before adjust volume with water and add 1 ml Triton X-100 and 10 ml DMSO per 100 ml, freshly.

26. Alkaline electrophoresis buffer for comet assay

Sodium hydroxide	17.9941 g
EDTA	0.3722 g
Distilled water	1000 ml

27. Neutralization buffer for comet assay

Tris	48.456 g
Distilled water to make	1000 ml

Adjust pH to 7.5 with 1 N HCl before adjust volume with water

28. 1 mg/ml Ethidium bromide stock solution

Ethidium bromide	1 mg
Distilled water	1 ml

Store in aliquots at -20°C.

29. 0.1 M Citric acid

Citric acid monohydrate	0.2101 g
Distilled water to make	10 ml

29. DNA extract buffer for cell cycle analysis

0.2 M Na_2HPO_4	9.6 ml
0.1 M Citric acid	400 µl

30. Cell dye for cell cycle analysis

Triton X-100	100 µl
10 mg/ml RNase A	2 ml
1 mg/ml PI	200 µl
PBS	10 ml

Stir and mix completly, store at 4°C in the dark.

31. 1 mM 2′,7′-Dichlorodihydro fluorescein diacetate (DCFH-DA) stock solution

DCFH-DA	0.0048 g
DMSO	10 ml

Store in aliquots at -20°C.

32. Griess reagent

Solution A

Sulfanilamide	1 g
5% Phosphoric acid	100 ml

Solution B

N-(1-naphtyl)ethylendiamine dihydrochloride	100 mg
Distilled water	1 ml

Mix Solution A to Solution B at the same volume, freshly.

APPENDIX B

CALCULATIONS

1. R_f value of protein mobilities by SDS-PAGE method

$$R_f \text{ value} = \frac{\text{Distance of protein migration}}{\text{Distance of tracking dye migration}}$$

2. The percentage of cell viability

$$\text{The \% of cell viability} = \frac{\text{O.D. of treated cells}}{\text{O.D. of untreated cells}} \times 100$$

3. The percentage of reactive oxygen species (ROS) production

$$\text{The \% of ROS production} = \frac{\text{Fluorescence intensity of treated cells}}{\text{Fluorescence intensity of untreated cells}} \times 100$$

4. The percentage of nitric oxide (NO) production

$$\text{The \% of NO production} = \frac{\text{Fluorescence intensity of treated cells}}{\text{Fluorescence intensity of untreated cells}} \times 100$$

BIOGRAPHY

NAME	Miss Suna Jongsomboonkusol
DATE OF BIRTH	30 August 1975
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	King Mongkut University of Technology Thonburi, 1994-1997 Bachelor of Science (Microbiology) Mahidol University, 2000-2005 Master of Science (Biopharmaceutical Sciences)
RESEARCH GRANT	Graduate Studies of Mahidol University Alumni Association, Mahidol University by Pfizer Foundation (Thailand)
HOME-ADDRESS	1622/14 Soi Chan31 Chan Road Khang Thungwaddon Khet Sathorn Bangkok, Thailand 10120 Tel. 0-2211-9911