



**The Cytotoxic Effect of Hua Khao Yen on Human Breast Cancer Cell Lines:
The Cytotoxic Mechanism Studies at Both Cellular and Molecular Levels**

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ชื่อวิทยานิพนธ์	ฤทธิ์การต้านเซลล์มะเร็งเต้านมของหัวข้าวเย็น : กลไกการออกฤทธิ์ในระดับเซลล์และอณูชีววิทยา
ผู้เขียน	นางสาวจิราพร แซ่คู
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บทคัดย่อ

หัวข้าวเย็นเป็นสมุนไพรที่นิยมใช้ในการรักษาโรคต่างๆของหมอพื้นบ้านรวมถึงการรักษา มะเร็ง จากการศึกษากิจกรรมต้านมะเร็งของสารสกัดชั้นน้ำและเอทานอลของหัวข้าวเย็นทั้ง 5 ชนิด โดยวิธี Sulphorhodamine (SRB) พบว่า สารสกัดชั้นน้ำของ *Dioscorea membranacea* มีฤทธิ์ต้านมะเร็งเต้านมทั้งสองชนิด (MCF-7 และ MDA-MB 468) โดยมีค่า IC_{50} เท่ากับ 5.5 และ 26.9 $\mu\text{g/ml}$ ตามลำดับ แต่ไม่มีฤทธิ์ต่อมะเร็งปากมดลูก (HeLa) สารสกัดชั้นเอทานอลของ *Dioscorea membranacea* มีฤทธิ์ต้านมะเร็งทั้ง MCF-7, MDA-MB 468 และ HeLa โดยมี IC_{50} เท่ากับ 29.2, 5.5 และ 15.0 $\mu\text{g/ml}$ ตามลำดับ และสารสกัดชั้นเอทานอลของ *Dioscorea birmanica* มีฤทธิ์ต้านเซลล์มะเร็งทั้งสามชนิดเช่นเดียวกัน โดยมีค่า IC_{50} เท่ากับ 34.2, 16.3 และ 27.2 $\mu\text{g/ml}$ ตามลำดับ นอกจากนี้พบว่าสารสกัดชั้นเอทานอลของ *Smilax corbularia* และ *Smilax glabra* มีฤทธิ์ต้านเฉพาะมะเร็งเต้านมชนิด MDA-MB 468 จากการศึกษาฤทธิ์การเหนี่ยวนำให้มีการตายแบบ apoptosis ของสารสกัดที่มีฤทธิ์ต้านมะเร็งโดยวิธี TUNEL พบว่าสารสกัดชั้นน้ำของ *Dioscorea membranacea* และสารสกัดชั้นเอทานอลของ *Dioscorea birmanica* สามารถเหนี่ยวนำให้เกิดการตายแบบ apoptosis ในเซลล์มะเร็งปากมดลูกและมะเร็งเต้านมชนิด MCF-7 ในลักษณะของ dose-dependent manner การศึกษาครั้งนี้ยังพบอีกว่า Dioscorealide B ซึ่งเป็นสารออกฤทธิ์ที่แยกได้จากหัวข้าวเย็นชนิด *Dioscorea membranacea* มีฤทธิ์ต้านเซลล์มะเร็งเต้านมชนิด MCF-7 มากกว่าชนิด MDA-MB 468 (IC_{50} เท่ากับ 2.82 และ 9.93 μM) จากการใช้วิธี Annexin V พบว่าสาร Dioscorealide B (ความเข้มข้น 3, 6 and 12 μM) สามารถเหนี่ยวนำให้เกิดการตายแบบ apoptosis ในเซลล์มะเร็งเต้านมชนิด MCF-7 ได้ 7-12 เท่าเมื่อเทียบกับกลุ่ม control และจากการใช้ caspase-Glo® assay พบว่า Dioscorealide B มีการกระตุ้น caspase-8, -9 และ 7 แสดงให้เห็นว่าการเหนี่ยวนำให้เกิด apoptosis ของสาร Dioscorealide B มีกลไกผ่านทั้งทาง extrinsic และ intrinsic pathway นอกจากนี้การศึกษากการแสดงออกของยีนที่เกี่ยวข้องโดยวิธี RT-PCR และ western blot analysis พบว่ากลไกการเกิด apoptosis ของสาร Dioscorealide B เกิดจากการกระตุ้นให้มีการ

แสดงออกของ p53 และ Bax และลดการแสดงออกของโปรตีน Bcl-2 ในระดับ mRNA อีกทั้งยังเพิ่มการแสดงออกของโปรตีน Bax และ Bak และลดการแสดงออกของโปรตีน Bcl-2 ส่งผลให้มีการกระตุ้น caspase-8, -9 และ -7 และทำให้เซลล์มะเร็งเต้านมเกิดการตายแบบ apoptosis ในที่สุด ข้อมูลจากการศึกษาครั้งนี้แสดงให้เห็นถึงฤทธิ์การต้านมะเร็งเต้านมและกลไกของสาร Dioscorealide B ซึ่งเป็นข้อมูลพื้นฐานในการพัฒนาสาร Dioscorealide B ในการรักษามะเร็งต่อไป

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ABSTRACT

Hua-Khao-Yen is a Thai medicinal plant commonly used by folk doctors to diseases especially cancer. The ethanolic- and water extracts from five species of Thai medicinal plants known as Hua Khao Yen were tested for their cytotoxic activity against HeLa, cervical cancer cell line and MCF-7 and MDA-MB 468 which represented as estrogen dependent and -independent breast carcinoma. Using Sulphorhodamine B (SRB) assay, the data showed that the water extract of *Dioscorea membranacea* had high cytotoxic activity in MCF-7 and MDA-MB 468 (IC_{50} = 5.5 and 26.9 μ g/ml, respectively) but it had no effect on HeLa. The ethanolic extract of *Dioscorea membranacea* showed cytotoxic activity against HeLa, MCF-7 and MDA-MB 468 cells (IC_{50} = 29.2, 5.5 and 15.0 μ g/ml, respectively) as well as *Dioscorea birmanica* (IC_{50} = 34.2, 16.3 and 27.2 μ g/ml, respectively). The ethanolic extract of *Smilax corbularia* and *Smilax glabra* had the cytotoxic effect only on MDA-MB 468. The water and ethanolic extracts of the other species exhibited no cytotoxic activity against HeLa and MCF-7. TUNEL assay revealed that the water extract and ethanolic of *Dioscorea membranacea* and the ethanolic extract of *Dioscorea birmanica* exhibited the induction of apoptosis in HeLa and MCF-7 cells in a dose-dependent manner. Dioscorealide B is a bioactive compound isolated from the rhizome of *Dioscorea membranacea*. In the present study, Dioscorealide B had stronger cytotoxic effect on MCF-7 than MDA-MB 468 (IC_{50} = 2.82 and 9.93 μ M, respectively). To determine whether this active compound induces apoptosis in MCF-7, Annexin V assay was performed and the results showed that the number of apoptotic cells were increased to 7-12 folds compared with control after treatment with various concentrations of Dioscorealide B (3, 6 and 12 μ M) for 24 hours. Using caspase-Glo[®] assay, the data revealed that Dioscorealide B also induced the activation of caspase-8, -9

and -7 suggesting that the mechanism of this compound might be involved in both intrinsic and extrinsic apoptotic pathway. In addition, the results from RT-PCR and western blot analysis demonstrated that Dioscorealide B-induced apoptosis was associated with reduction of antiapoptotic Bcl-2 and induction of p53 and proapoptotic Bax and Bak expression leading to the activation of caspase-9 and -7, respectively. Together, the results of this study provide evidence that Dioscorealide B possesses anticarcinogenic effect against human breast cancer cells and thus provide the molecular basis for the future development of Dioscorealide B as a novel and pharmacologically safe chemotherapeutic agent for breast cancer treatment.

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LIST OF ABBREVIATIONS AND SYMBOLS

A	=	Ampere
Ab	=	Antibody
AIF	=	apoptosis inducing factor
Apaf-1	=	apoptosis protease-activating factor-1
APS	=	ammonium persulfate
ATP	=	adenosine triphosphate
Bcl-2	=	B-cell lymphoma-2
bp	=	base pairs
CAD	=	caspase-activated deoxyribonuclease
CARD	=	caspase recruitment domain
cDNA	=	copy DNA
CO ₂	=	carbon dioxide
dATP	=	deoxy Adenosine triphosphate
dCTP	=	deoxy cytosine triphosphate
DD	=	death domain
DED	=	death effector domain
dGTP	=	deoxy guanosine triphosphate
DMEM	=	Dulbecco's modified Eagle medium
DMSO	=	dimethyl sulfoxide
DNA	=	deoxyribonucleic acid
dNTP	=	deoxy nucleotide triphosphate
DTT	=	D,L-dithiotreitol
dTTP	=	deoxy thymidine triphosphate
ECL	=	Enhanced Chemiluminescence
EDTA	=	ethylenediaminetetraacetic acid disodium salt
FasL	=	Fas ligand
FBS	=	fetal bovine serum
FITC	=	fluorescein isothiocyanate
g	=	relative centrifugal force
GAPDH	=	glyceraldehyde-3-phosphate dehydrogenase

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

h	=	hour(s)
HRP	=	horse reddish peroxidase
IgG	=	immunoglobulin G
kb	=	kilo base
kD/kDa	=	kilodalton
KCl	=	potassium chloride
m	=	meter
MEM	=	Eagle's minimum essential medium
mg	=	milligram
min	=	minute(s)
MgCl ₂	=	magnesium chloride
ml	=	milliliter
mm	=	millimeter
mM	=	millimolar
mol	=	mole
mRNA	=	messenger RNA
MW	=	molecular weight
nM	=	nanomolar
nm	=	nanometer
NaHCO ₃	=	sodium hydrogen carbonate
NaOH	=	sodium hydroxide
PARP	=	poly(ADP-ribose) polymerase
PBS	=	phosphate-buffered saline
PCR	=	polymerase chain reaction
PI	=	propidium iodide
RT	=	room temperature
RT-PCR	=	reverse transcription polymerase chain reaction
SD	=	standard deviation
SDS	=	sodium dodecyl sulfate
PAGE	=	Polyacrilamide Gel Electrophoresis

LIST OF ABBREVIATIONS AND SYMBOLS (CONTINUED)

TBE	=	buffer containing Tris, boric acid and EDTA
TBS-T	=	Tris-buffered saline with Tween 20
TE	=	PBS containing 0.05% trypsin and 0.02% EDTA
TEMED	=	N,N,N',N'-tetramethylethylenediamine
TNF	=	tumor necrosis factor
Tris	=	Tris(hydroxymethyl)aminomethane
U	=	unit(s)
UV	=	ultraviolet
V	=	Volt
v/v	=	volume per volume
w/v	=	weight per volume
μg	=	microgram
μl	=	microliter

CHAPTER 1

INTRODUCTION

1.1 Breast cancer

Cancers are a group of diseases in which cells display uncontrolled growth, invasion, and sometimes metastasis or spread to other locations in the body via lymph or blood (Hanahan, 2000). Cancer is caused by both external factors such as chemicals, radiation, tobacco and infectious organisms and internal factors such as inherited mutations, hormones, and immune condition. Most types of cancer cells eventually form a lump or mass called a tumor and are named after the part of the body where the tumor originates (American Cancer Society, 2007).

The term breast cancer refers to a malignant tumor that has developed from cells in the breast. The female breast is made up mainly of *lobules* (milk-producing glands), *ducts* (tiny tubes that carry the milk from the lobules to the nipple), and *stroma* (fatty tissue and connective tissue surrounding the ducts and lobules, blood vessels, and lymphatic vessels) as seen in Figure 1. Most breast cancers begin in the cells that line the ducts (*ductal* cancers). Some begin in the cells that line the lobules (*lobular* cancers), while a small number start in other tissues (National Cancer Institute, 2005).

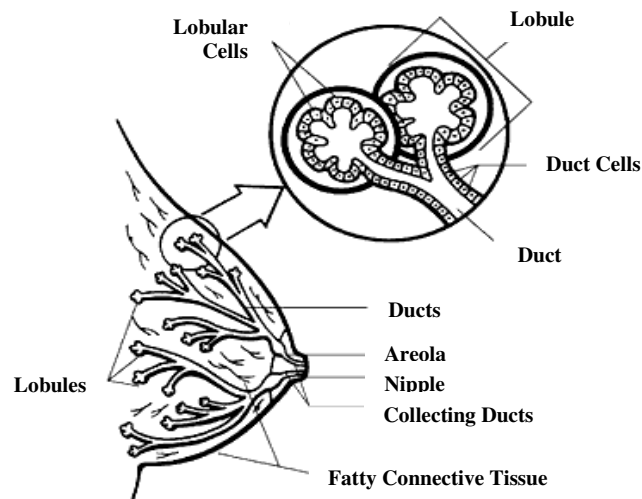


Figure 1 Normal structure of the breasts

1.1.1 Stages of Breast Cancer

Each cancer stage is based on the size of the tumor, whether the cancer is invasive or non-invasive, whether lymph nodes are involved, and whether the cancer has spread beyond the breast. The stage is the most important, as it takes into consideration size, local involvement, lymph node status and whether metastatic disease is present. The higher the stage at diagnosis, the worse the prognosis. The stage is raised by the invasiveness of disease to lymph nodes, chest wall, skin or beyond, and the aggressiveness of the cancer cells (Halpern et al, 2006; National Breast and Ovarian Cancer Centre, 2008).

Stage 0

Stage 0 is used to describe non-invasive breast cancers, such as DCIS and LCIS. In stage 0, there is no evidence of cancer cells or non-cancerous abnormal cells breaking out of the part of the breast in which they started, or of getting through to or invading neighboring normal tissue.

Stage I

Stage I describes invasive breast cancer (cancer cells are breaking through to or invading neighboring normal tissue) in which:

- the tumor measures up to 2 centimeters, AND
- no lymph nodes are involved

Stage II

Stage II is divided into subcategories known as IIA and IIB.

Stage IIA describes invasive breast cancer in which:

- no tumor can be found in the breast, but cancer cells are found in the axillary lymph nodes (the lymph nodes under the arm), OR
- the tumor measures 2 centimeters or less and has spread to the axillary lymph nodes, OR
- the tumor is larger than 2 centimeters but not larger than 5 centimeters and has not spread to the axillary lymph nodes.

Stage IIB describes invasive breast cancer in which:

- the tumor is larger than 2 but not larger than 5 centimeters and has spread to the axillary lymph nodes, OR
- the tumor is larger than 5 centimeters but has not spread to the axillary lymph nodes.

Stage III

Stage III is divided into subcategories known as IIIA, IIIB, and IIIC.

Stage IIIA describes invasive breast cancer in which either:

- no tumor is found in the breast. Cancer is found in axillary lymph nodes that are clumped together or sticking to other structures, or cancer may have spread to lymph nodes near the breastbone, OR
- the tumor is 5 centimeters or smaller and has spread to axillary lymph nodes that are clumped together or sticking to other structures, OR
- the tumor is larger than 5 centimeters and has spread to axillary lymph nodes that are clumped together or sticking to other structures

Stage IIIB describes invasive breast cancer in which:

- the tumor may be any size and has spread to the chest wall and/or skin of the breast AND
- may have spread to axillary lymph nodes that are clumped together or sticking to other structures, or cancer may have spread to lymph nodes near the breastbone
- Inflammatory breast cancer is considered at least Stage IIIB.

Stage IIIC describes invasive breast cancer in which:

- there may be no sign of cancer in the breast or, if there is a tumor, it may be any size and may have spread to the chest wall and/or the skin of the breast, AND
- the cancer has spread to lymph nodes above or below the collarbone, AND
- the cancer may have spread to axillary lymph nodes or to lymph nodes near the breastbone.

Stage IV

Stage IV describes invasive breast cancer in which:

- the cancer has spread to other organs of the body, usually the lungs, liver, bone, or brain

“Metastatic at presentation” means that the breast cancer has spread beyond the breast and nearby lymph nodes, even though this is the first diagnosis of breast cancer. The reason for this is that the primary breast cancer was not found when it was only inside the breast. Metastatic cancer is considered Stage IV.

1.1.2 Risk factor for breast cancer

Research has shown that women with certain risk factors are more likely than others to develop breast carcinoma. A risk factor is something that may increase the chance of developing a disease. Many of the known breast cancer risk factors, such as age, family history, age at first full-term pregnancy, early menarche, late menopause, and breast density, are not easily modifiable. However, other factors associated with increased breast cancer risk such as postmenopausal obesity, use of post-menopausal hormones, alcohol consumption, and physical inactivity are modifiable.

1.1.2.1 Increasing age

Age is the most important risk factor for breast cancer. An individual woman’s breast cancer risk may be higher or lower depending on personal risk factors, experiences, and other factors not yet fully understood. The chance of getting breast cancer goes up as a woman gets older. Most cases of breast cancer occur in women over 60. This disease is not common before menopause (Osteen et al, 2001; Chlebowski et al, 2005).

1.1.2.2 Family history of breast cancer/genetic predisposition

Women with a family history of breast cancer, especially in a first-degree relative (mother, sister, or daughter), have an increased risk of developing breast cancer. The risk is higher if more than one first-degree relative has developed breast cancer and

increases the younger the relative was at the time of diagnosis. (American Cancer Society, 2007).

About 5% to 10% of breast cancer cases are thought to be hereditary, resulting directly from gene mutations inherited from a parent. The most common inherited mutations are those of the BRCA1 and BRCA2 genes. In normal cells, these genes help to prevent cancer by making proteins that help keep the cells from growing abnormally. Women with an inherited BRCA1 or BRCA2 mutation have up to an 80% chance of developing breast cancer during their lifetime. In addition, women with these inherited mutations have an increased risk for developing ovarian cancer (Venkitaraman, 2002).

Other gene changes, e.g. ATM, CHEK2 and p53, might also lead to inherited breast cancers. These genes do not impart the same level of breast cancer risk as the BRCA genes, and do not frequently cause inherited breast cancer. Certain families with a high rate of breast cancer have been found to have mutations of ATM gene which normally helps repair damaged DNA. The CHEK2 gene increases breast cancer risk about two fold when it is mutated. In women who carry the CHEK2 mutation and have a strong family history of breast cancer, the risk is greatly increased. In addition, mutations of the p53 tumor suppressor gene can increase the risk of developing breast cancer, as well as several other cancers such as leukemia, brain tumors, and sarcomas (Ford et al, 1998; Antoniou et al, 2003; Yin, 2003).

1.1.2.3 Hormonal factors

Reproductive hormones are thought to influence breast cancer risk through effects on cell proliferation and DNA damage, as well as promotion of cancer growth. Early menarche (<12 years), older age at menopause (>55 years), older age at first full-term pregnancy (>30 years), and fewer number of pregnancies may increase a woman's risk of breast cancer by affecting the levels of reproductive hormones produced by the body (Hulka et al, 2001). In addition, use of oral contraceptives may slightly increase the risk of breast cancer; however, women who have stopped using oral contraceptives for 10 years or more have the same risk as women who have never used the pill (Shantakumar et al, 2007). Recent use of combination hormone replacement therapy (HRT), which combines estrogen and progestin, has been shown to increase breast cancer risk, with higher risk associated

with longer use (Rossouw et al, 2002). Estrogen alone can be prescribed for women without a uterus. This is commonly known as estrogen replacement therapy (ERT or ET) and does not appear to increase the risk of developing breast cancer (Zhang et al, 2007).

1.1.2.4 Clinical factors

High breast tissue density (a mammographic indicator of the amount of glandular tissue relative to fatty tissue in the breast) has been shown to be a strong independent risk factor for the development of breast cancer. In several studies, women with the highest levels of breast density were found to have a 4- to 6-fold increased risk of breast cancer, compared with women with the least dense breasts (Barlow et al, 2006; Boyd et al, 2007). Some types of benign breast conditions are more closely linked to breast cancer risk than others (Hartmann et al, 2005).

1.1.2.5 Other factors

Obesity increases risk of postmenopausal (but not premenopausal) breast cancer, as does weight gain during adulthood (Eliassen, 2006). In addition, women who are physically inactive throughout life and drink alcohol have an increased risk of breast cancer (Boffetta et al, 2006; Hamajima et al, 2008).

1.2 Incidence of Breast cancer

Cancer is the third leading cause of death worldwide (12% all of deaths), only preceded by cardiovascular disease (30% all of deaths), and by infectious and parasitic disease (19% all of deaths) (Mathers et al, 2001). Between 2000 and 2020, the total number of cases of cancer in the developing world is predicted to increase by 73% and to increase by 29% in the developed world (Parkin et al, 2001). Cancer was the major cause of death in America, Europe, Asia and Thailand (Statistical annex, 2002) but in Hong Kong it was the leading cause of death in 1996–2001 (Stanley, 2001).

Interestingly, cancer has been the leading cause of death in Thailand for several years and the rate of people dying is still increasing every year. Statistics from the National Statistical Office of Thailand indicated that 49,628 Thai people died from cancer in 2003 (National statistical office, 2003).

Breast cancer is the most common cancer in women worldwide, comprising 16% of all female cancers. It is estimated that 519 000 women died in 2004 due to breast cancer, and although breast cancer is thought to be a disease of the developed world, a majority (69%) of all breast cancer deaths occurs in developing countries (WHO, 2008). According to the American Cancer Society, 192,370 new cases of invasive breast cancer will be diagnosed in women in the U.S. and about 40,170 breast cancer deaths are expected in 2009 (American Cancer Society, 2009). Moreover, the statistics from the National Cancer Institute of Thailand showed that breast cancer was the second leading cause of death of people in Thailand after cervical cancer during the period 1998–2000. The estimated incidence rate is 20.5 per 100 000 women and 0.2 per 100,000 men. The incidence has been increasing in the past decade. The highest incidence rate of female breast cancer is in Bangkok (ASR = 24.3) and for male breast cancer is in Songkhla (ASR = 0.4). The lowest incidence for females is in Nakhon Phanom (ASR = 10.1) and for males is in Lampang and Rayong (ASR = 0). Breast cancer is the most common cancer in female in Bangkok, the second in Songkhla after cervical cancer, and the third in Chiangmai and Lampang after cervical and lung cancer. In 2008, the hospital-based cancer registry of National Cancer Institute of Thailand revealed that breast cancer is the most common cancer in female, comprising 43% of patients and Public Health statistics showed that breast cancer is the first leading cause of cancer death in female and the death rates have been increasing (Bureau of Health Policy and Strategy, 2008).

These statistics accentuate the urgent need for improvements in detection, diagnosis, and treatment of breast cancer. Current progress in diagnosis and therapy has improved the survival of women in estrogen-dependent breast cancer. However, the treatment options available for estrogen-independent tumors are far from satisfactory, and consequently carry a poorer prognosis (Tze-chen et al, 2005). The breast cancer might result from interactions between genetic elements, various possible environmental factors, and also difference in ethnicity (Brinton et al, 2002; Hsiao et al, 2004). This disease is currently controlled through surgery and/or radiotherapy, and is frequently supported by adjuvant chemo- or hormonotherapies. Unfortunately, these classical treatments are

impeded by unwanted side effects and, most importantly, the development of tumor resistance (Boilk, 1996). Therefore, undoubtedly there is an urgent need for novel and effective therapies against breast cancer. Thus the medicinal use of naturally occurring substances or natural agents becomes necessary for an alternative for the patients suffering from cancer.

1.3 Plant extracts in cancer treatment

The objective of cancer chemotherapy is to kill cancer cells with as little damage as possible to normal cells (Halliwell and Gutteridge, 1988) so the discovery of anticancer agents must be related to novel molecular targets which should be defined and developed for specific typical cancer cells but are less toxic to normal cells or have a unique mechanism of action for a specific type of cancer (Pezzuto, 1997).

Over the centuries, human beings have used some plant constituents, for example, to prepare poisonous spearheads for warfare and hunting. Furthermore, plant-derived substances have traditionally played important roles in the treatment of human diseases. Plants have formed the basis for traditional medicine systems that have been used for thousands of years in countries with ancient civilizations such as China (Chang and But, 1986), India (Kapoor, 1990) and Thailand (Subchareon, 1998). Nowadays, about 80% of the world population residing in third world countries still depends almost entirely on plant products for their primary health care. The remaining 20% of individuals living in the first world use, in more than 25% of cases, pharmaceuticals which have been directly derived from plant products (Dennis et al, 2000). World Health Organization estimated that approximately 80% of the world's inhabitants rely mainly on traditional medicines for their primary health care and at least 119 chemical substances derived from 90 plant species can be considered as important drugs currently in use in one or more countries (Farnsworth et al, 1985). 74% of these 119 compounds were discovered as a result of chemical studies directed at the isolation of the active substances from plants used in traditional medicine (Cragg et al, 1997). Therefore, the usage of ethnopharmacology, or traditional use, is channeled for discovery of new biologically-active molecules (Houghton, 1995).

The basis of traditional medicine is in its use for a number of years and therefore its clinical existence comes as a presumption. However, for bringing more objectivity and also to confirm traditional claims, systematic clinical trials are necessary. In ayurvedic medicine research, clinical experiences, observations or available data becomes a starting point. In conventional drug research, it comes at the end. Thus, the drug discovery based on ayurveda follows a 'reverse pharmacology' path which is defined as the sciences of integrating documented clinical experiences and experiential observations into leads by transdisciplinary exploratory studies and further developing these into drug candidates or formulations through robust preclinical and clinical research (Vaidya et al, 2006).

The drug discovery process is becoming more and more complex and capital-intensive, and such companies remain 'target rich' but 'lead poor', with lead discovery as a greater bottleneck. In such a situation, industrialization of drug discovery process is underway. Although high throughput screening (HTS) and combinatorial chemical synthesis are explored with great hope, general experience tells us that in most companies the investments in these technologies have not reaped rewards in new lead discovery as expected (Bhushan et al, 2004). Despite technological advances, genomics and bioinformatics predictions, actually the number of new molecular (chemical and biological) entities has dropped during the year 2002 to less than 20/year compared to over 50/year in 1996 (King, 2002). It is estimated that to develop one successful drug, about 12-15 years and US\$ 900 million are required. The pharmaceutical industry is currently spending over US\$ 45 billion every year with about 20-25 new potential drugs and the average cost of a successful drug that enters the market is estimated to be about US\$ 5 billion per drug (Goodfellow, 2003). These call for systematic and critical review of methods and mindset involved in drug discovery today and indicates the need to rediscover the drug discovery process afresh (Chauthaiwale, 2003).

The critical retrospection of the whole drug discovery process indicates that it is becoming more complex, with drugs failing at the end of the pipeline even in Phase III or Phase IV, making it more expensive and time consuming. New drug discovery must overcome such problems and become more dynamic, focused and predictive, where safety and efficacy issues are addressed along side the developmental costs. Development of new chemical/ molecular entity into therapeutic drugs takes several years and is capital-intensive. The risks are also high and the success rate not good. Powerful new technologies such as HTS and combinatorial chemistry are revolutionizing drug discovery. But natural

products still offer unmatched structural variety, especially as new environmental niches are explored, and their usefulness can be further extended by engineering the proteins that produce them and using them to probe biological pathways (Verdine, 1996). Rediscovery of the connection between plants and health is responsible for launching a new generation of botanical therapeutics that include plant-derived pharmaceuticals, multicomponent botanical drugs, dietary supplements, functional food and plant-produced recombinant proteins. Many of these products will soon complement conventional pharmaceuticals in the treatment, prevention and diagnosis of diseases, while at the same time adding value to agriculture. Such complementation can be accelerated by developing better tools for the efficient exploration of diverse and mutually interacting arrays of phytochemicals and for the manipulation of the ability of the plant to synthesize natural products and complex proteins (Raskin et al, 1996).

The accepted modality for cancer treatment involves surgery, radiation and drugs, singly or in combination. Unfortunately, these classical treatments are impeded by unwanted side effects and, most importantly, the development of tumor resistance. For example, intensive treatment with radiotherapy or chemotherapy is commonly associated with the range of adverse side effects from nausea to bone marrow failure. Patients who obtain intensive treatment may experience a decline in quality of life (Boilk, 1996).

Cancer chemotherapeutic agents can often provide temporary relief from symptoms, prolongation of life and occasionally, treatments. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damage to normal cells. This ideal situation is achievable by inducing apoptosis in cancer cells. The life span of both normal and cancer cells is significantly affected by the rate of apoptosis. Therefore, modulating apoptosis may be useful in the management and therapy or prevention of cancer. Although modification or synthesis of known drugs continues as an important aspect of research, many synthetic works has contributed relatively small improvements over prototype drugs. There is a continued need for new templates to use in the design of potential chemotherapeutic agents. Notably, natural products are providing such templates. Recent studies on anticancer agents of plant origin have yielded an impressive array of novel structures. Moreover, epidemiological studies suggest that consumption of diets containing vegetables and fruits, major sources of phytochemicals and micronutrients, may decrease the risk of developing cancer (Boik, 1996; Taraphdar et al, 2001; Kuo et al, 2005).

Approximately five decades of systemic drug discovery and development have established a respectable armamentarium of useful chemotherapeutic agents (Chabner, 1991), as well as a number of important successes in the treatment and management of human cancer (Jessup et al, 1996). Nevertheless, the need for more effective anti-neoplastic agents remains. The most common tumors of the adult are resistant to available antineoplastic drugs, and the majority of these agents have only limited anti-solid tumor activity (Yarbro, 1992). Table 1 illustrates the latter statement, showing that only 5 of the 25 listed commonly used anti-cancer drugs elicit preferential anti-solid tumor activity. Furthermore, these agents have little impact on survival rates. This problem has recently been addressed in a contribution from medical oncologists from five continents, all arriving at the same conclusion on the inadequacy of current chemotherapeutic agents for the treatment of advanced solid malignancies (Sikora et al, 1999).

Plant-derived compounds were of great significance to cancer therapy (Table 2). It was, for instance, only upon the addition of the *Vinca* alkaloid vincristine or oncovin (isolated from *Catharanthus roseus*, Apocynaceae (Johnson et al, 1963)) to mechlorethamine, prednisone, and procarbazine (the MOPP regimen) that the first cures in a human cancer (Hodgkin's disease) were achieved (DeVita et al, 1970). The combination of the epipodophyllotoxin etoposide (derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *P. emodi*, Berberidaceae (Stähelin, 1973), bleomycin, and cisplatin is currently a highly active and curative regimen in testicular cancer (Williams et al, 1987). Etoposide is furthermore one of the most active agents against small cell lung carcinoma (Chabner, 1991; Williams, 1987; Harvey, 1999). The more recent development of the structurally and mechanistically novel taxanes (extracted from the bark of the Taxaceae *Taxus brevifolia*, *T. canadensis*, or *T. baccata* (Wani et al, 1971) and the camptothecins (derived from the bark and wood of the Nyssaceae *Camptotheca acuminata* (Wani et al, 1966) in the 1990s represented a landmark in cancer research because of their significant anti-solid tumor efficacy. Paclitaxel is in many countries approved for the treatment of ovarian and breast carcinoma and also has important activity against non-small cell lung cancer (McGuire, 1996). Irinotecan and topotecan are semi-synthetics from the lead compound camptothecin which are approved for the treatment of advanced colorectal cancer (Bertino, 1997), and as second-line chemotherapy in ovarian carcinoma, respectively. These agents are also active against several other solid malignancies such as carcinoma of the lung, cervix, and ovary (Creemers et al, 1996).

Some products from plants are known to induce apoptosis in malignant cells but not in normal cells. It has become increasingly evident that apoptosis is an important mode of action for many anti-cancer agents, including alkylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) and cisplatin, ionizing radiation, topoisomerase inhibitor etoposide, cytokine tumour necrosis factor (TNF), taxol, and N-substituted benzamides such as 3-chloroprocainamide and metoclopramide. Apoptotic induction has been a new target for innovative mechanism based drug discovery. Therefore, it is considered important to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them. Evidence has emerged from various studies that suggest that products derived from plants are useful in the treatment and the prevention of cancer. Chemopreventive agents comprise a diverse group of compounds with different mechanisms of action, but, their ultimate ability to induce apoptosis may represent a unifying concept for the mechanism of chemoprevention (Table 3). Understanding the modes of action of these compounds should provide useful information for their possible application in cancer prevention and perhaps also in cancer therapy (Taraphdar et al, 2001).

Table 1 Relative antisolid tumor activity of commonly used antineoplastic drugs (Dennis et al, 2000)

Exclusively used against hematological malignancies	Used against hematological and solid malignancies	Exclusively used against solid malignancies
L - asparaginase Busulfan Chlorambucil Cytarabine Daunorubicin Mechlorethamine 6 - Mercaptopurine Procarbazine 6 - Thioguanine	Bleomycin Cyclophosphamide Dacarbazine Dactinomycin Doxorubicin Etoposide Ifosfamide Melphalan Methotrexate Vinblastine	Carboplatin Cisplatin 5 - Fluorouracil Mitomycin C Tamoxifen

Table 2 Some cytotoxic drugs developed from plant sources (Dennis et al, 2000)

Drug	Mechanism of action	Plant source
Vinblastine, vincristine	Inhibition of tubulin polymerization	<i>Catharanthus roseus</i> (Apocynaceae)
Etoposide, teniposide	Inhibition of topoisomerase II	<i>Podophyllum peltatum</i> , <i>P. emodi</i> (Berberidaceae)
Paclitaxel, docetaxel	Promotion of tubulin stabilization	<i>Taxus brevifolia</i> (Taxaceae)
Irinotecan, topotecan, 9-aminocamptothecin, 9-nitrocamptothecin	Inhibition of topoisomerase I	<i>Camptotheca acuminata</i>

Table 3 Apoptosis induced by natural compounds (Taraphdar et al, 2001)

Phenolics	Tumour cell lines	Parameters
<p>Phenolic acids and phenols</p> <p>Gallic acid, caffeic acid, tannic acid, curcumin, capsaicin, resveratrol, yakuchinones, [6]-gingerol, [6]-paradol</p>	<p>Myelogenous leukaemia (HL-60, ML-1, U-937, THP-1)</p>	<p>Cytotoxicity, DNA fragmentation, morphological characteristics</p>
<p>Polyphenolic flavonoids</p> <p>Genistein, genistin, daidzein, biochanin A, tangeretin, baicalein, quercetin, myricetin, apigenin, kaempferol</p>	<p>Promyelocytic leukaemia (HL-60), prostate cancer (PCa, LNCaP, DU-145, PC-3), lung cancer (H-460), bladder cancer (HT-1376, UM-UC-3, RT-4, J-82, TCCSUP), hepatocellular carcinoma (HCC), colon cancer (Caco-2, HT-29)</p>	<p>Cytotoxicity, cell-cycle arrest, morphological characteristics</p>
<p>Tea polyphenols</p> <p>(-)-Epigallocatechin gallate, (-)-Epicatechingallate, (-)-Epigallocatechin, (-)-Epicatechin</p>	<p>Lung cancer (PC-9), lymphoid leukaemia (MOLT-4B), stomach cancer (KATO III)</p>	<p>Cytotoxicity, DNA synthesis, DNA fragmentation</p>

1.4 Apoptosis

In the human body, somatic cells are born by mitosis and almost all will die by apoptosis, a physiological process of cellular suicide. Cell numbers are regulated by a balance between proliferation and programmed cell death (apoptosis); for instance, in haemopoiesis, the stem cells in the bone marrow self-renew and also produce progeny, which then become committed to differentiate and finally they undergo apoptosis. Both the cell proliferation and apoptosis are active processes that are regulated by mitogenic growth factors and negative growth factors, as well as by survival factors. Cancers can occur when this balance is disturbed, either by an increase in cell proliferation or a decrease in cell death. Consequently, the goal of cancer therapy is to promote the death of cancer cells with fewer side effects on normal cells (Boik, 1996; Thomas, 1996).

1.4.1 The significance of apoptosis

Since the mid-nineteenth century, many observations have indicated that cell death plays a considerable role during physiological processes of multicellular organisms, particularly during embryogenesis and metamorphosis (Gluecksmann, 1951; Lockshin, 2001). The term programmed cell death was introduced in 1964, proposing that cell death during development is not of an accidental nature but follows a sequence of controlled steps leading to locally and temporally defined self-destruction (Lockshin, 1964). Eventually, the term apoptosis had been coined in order to describe the morphological processes leading to controlled cellular self-destruction and was first introduced in a publication by Kerr, Wyllie and Currie (Kerr, 1972). Apoptosis is of Greek origin, having the meaning "falling off or dropping off", in an analogy to leaves falling off trees or petals dropping off flowers.

Apoptosis is a normal component of the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by

contrast, is a process in which cells play an active role in their own death (which is why apoptosis is often referred to as cell suicide).

The development and maintenance of multicellular biological systems depends on a sophisticated interplay between the cells forming the organism; it sometimes even seems to involve an altruistic behaviour of individual cells in favour of the organism as a whole. During development many cells are produced in excess which eventually undergo programmed cell death and thereby contribute to sculpturing many organs and tissues (Meier, 2000). A particularly instructive example for the implication of programmed cell death in animal development is the formation of free and independent digits by massive cell death in the interdigital mesenchymal tissue (Zuzarte-Luis, 2002). Other examples are the development of the brain, during which half of the neurons that are initially created will die in later stages when the adult brain is formed (Hutchins, 1998) and the development of the reproductive organs (Meier, 2000). Also, cells of an adult organism constantly undergo physiological cell death that must be balanced with proliferation in order to maintain homeostasis in terms of constant cell numbers. The majority of the developing lymphocytes die either during genetic rearrangement events in the formation of the antigen receptor, during negative selection or in the periphery, thereby tightly controlling the pool of highly efficient and functional but not self-reactive immune cells and at the same time keeping lymphocyte numbers relatively constant (Rathmell, 2002). Taken together, apoptotic processes are of widespread biological significance, being involved in, for example, development, differentiation, proliferation/ homeostasis, regulation and function of the immune system and in the removal of defect and therefore harmful cells. Therefore, dysfunction or dysregulation of the apoptotic program is involved in a variety of pathological conditions. Defects in apoptosis can cause cancer, autoimmune diseases and spreading of viral infections, whereas neurodegenerative diseases such as Alzheimer's disease and acquired immuno-deficiency syndrome (AIDS) are caused or enhanced by excessive apoptosis (Fadeel, 1999).

In the human body about 100,000 cells are produced every second by mitosis and a similar number die by apoptosis (Vaux and Korsmeyer, 1999, Cell) !

Development and Morphogenesis:

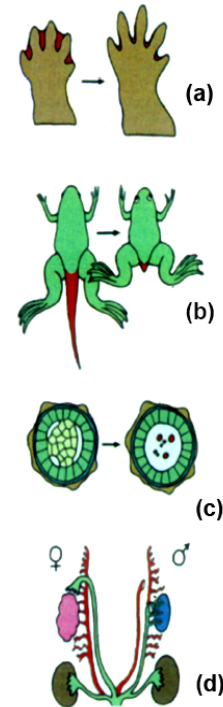
- 131 of the 1,090 somatic cells die during *C.elegans* development
- during limb formation separate digits evolve by death of interdigital mesenchymal tissue (a)
- ablation of cells no longer needed such as the amphibian tadpole tail during metamorphosis (b)
- demise of cells allows sculpturing of hollow structures (c)
- formation of reproductive organs (d)
(Müllerian duct → uterus, deleted in males; Wolffian duct → male organs, deleted in females)
- massive cell death occurs during early development of the nervous system (> 50 percent of all neurons die)

Homeostasis:

- a paradigm for the involvement of apoptosis in homeostasis is the immune system: several millions of B and T cells are generated every day and the majority (> 95 percent) of those die during maturation (death by neglect, negative selection) or by AICD of peripheral immune cells)

Deletion of damaged and dangerous cells:

- Cells with severely damaged DNA that cannot be repaired appropriately usually are removed by apoptosis
- Inappropriate mitogenic signalling that is in conflict with the environmental or cellular status of the cell usually results in cell cycle arrest or apoptosis
- Autoreactive cells of the immune system are deleted by apoptosis
- Elimination of infected cells



(a)

(b)

(c)

(d)

Figure 2 Examples of physiological cell death. Modified from Vaux and Korsmeyer, 1999

1.4.2 Morphological features of apoptosis

Apoptosis is characterized by a series of distinct morphological and biochemical alterations to the cell such as DNA fragmentation, cell shrinkage, chromatin condensation and plasma membrane blebbing (Wyllie, 1980). Upon receiving specific signals instructing the cells to undergo apoptosis, a number of distinctive changes occur in the cell. A family of proteins known as caspases is typically activated in the early stages of apoptosis. These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus (Shi, 2002).

Apoptotic cells display distinctive morphology during the apoptotic process, and this can be seen in Figure 3. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton (A). The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a “horse-shoe” like appearance (B). Cells continue to shrink (C), packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death. In order to promote their phagocytosis by macrophages, apoptotic cells often undergo plasma membrane changes that trigger the macrophage response. One such change is the translocation of phosphatidylserine from the inside of the cell to the outer surface. The end stages of apoptosis are often characterised by the appearance of membrane blebs (D) or blisters. Small vesicles called apoptotic bodies are also sometimes observed (D, arrow).

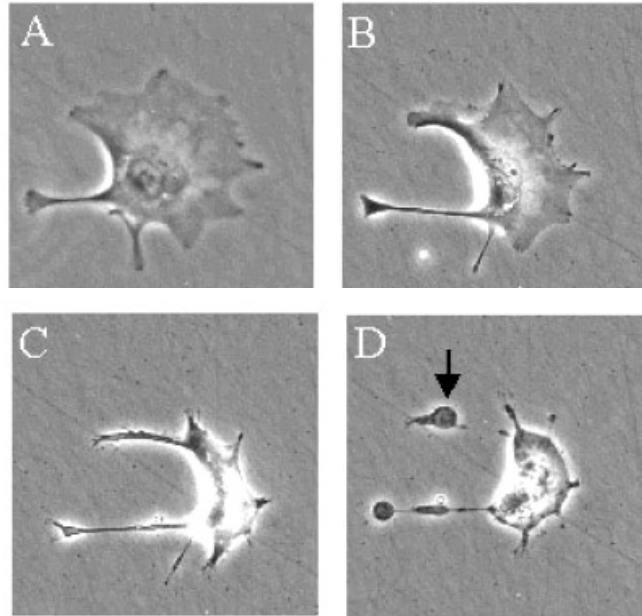


Figure 3 Morphology of an apoptotic trophoblast cell as captured by time-lapse microscopy (images taken over a 6 hour period). (<http://www.sgul.ac.uk/depts/immunology/~dash/apoptosis/intro.html>)

Necrosis and apoptosis are the patterns of cell death. Necrosis can be induced in massive cell injury often accompanied by inflammation (Figure 4). In contrast to the accidental death of cells that results from an acute injury, apoptosis or programmed cell death is an active process with specific morphological changes, which are characterized by chromatin condensation, nuclear DNA fragmentation, cell shrinkage, plasma membrane blebbing, and membrane enclosed cell fragment (apoptosis body) formation (Zhang, et al, 2003). Apoptosis is a distinct intrinsic cell death program that occurs in various physiological and pathological situations (Hengartner, 2000). Killing of tumor cells by cytotoxic therapies (e.g. chemotherapy, γ -irradiation, immunotherapy or suicidal gene therapy) is predominantly mediated by triggering apoptosis in cancer cells (Herr and Debatin, 2001). Nowadays, the central theme of cancer research is to understand how the tumor cells resist apoptosis in response to triggers which typically induce cell cycle arrest or cell death in their untransformed counterparts and evidence has suggested that the rate of cell death, the other side of the balance, is just as important (McGill and Fisher, 1997).

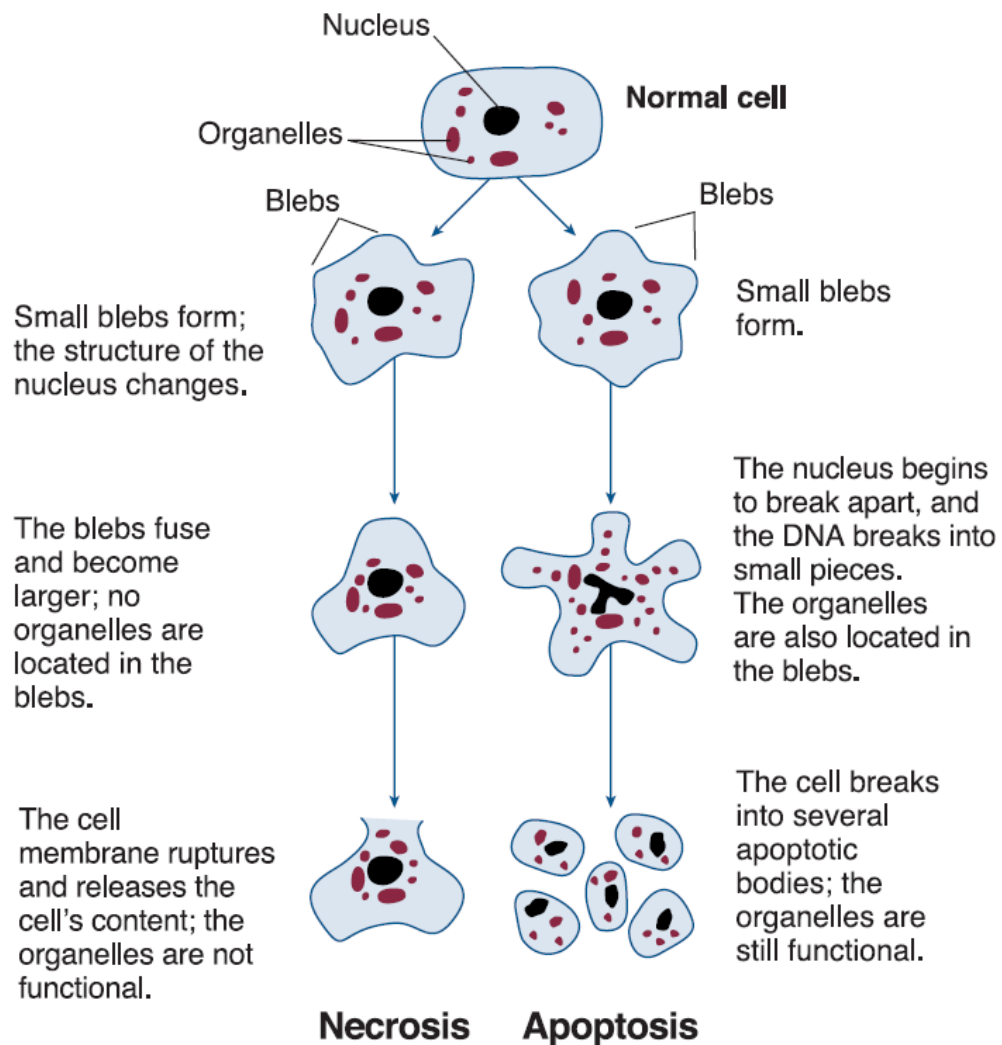


Figure 4 Structural changes of cells undergoing necrosis or apoptosis. Apoptosis includes cellular shrinking, chromatin condensation and margination at the nuclear periphery with the eventual formation of membrane-bound apoptotic bodies that contain organelles, cytosol and nuclear fragments and are phagocytosed without triggering inflammatory processes. The necrotic cell swells, becomes leaky and finally is disrupted and releases its contents into the surrounding tissue resulting in inflammation. Modified from Goodlett and Horn, 2001.

1.4.3 Molecular mechanisms of apoptosis signalling pathways

Cell death with apoptotic morphology can be triggered by several stimuli, including intracellular stress and receptor-mediated signaling. For example, the ligation of cell surface receptors, DNA damage as a cause of defects in DNA repair mechanisms, treatment with cytotoxic drugs or irradiation, a lack of survival signals, contradictory cell cycle signalling or developmental death signals. These signals feed into an evolutionarily conserved intracellular machinery of execution (Green, 2000), the mechanisms of which have been mainly traced to the activity of the caspase family of cysteine proteases (Zhvivotovsky et al, 1997; Guimaraes and Linden, 2004).

Much of the understanding of cell death has come from genetic studies in the nematode *Caenorhabditis elegans* by which several genes have been identified that function in the apoptotic killing and elimination of 131 of the initially 1090 somatic cells that are generated during hermaphrodite development (Hengartner, 1999; Philchenkov, 2004). The proximal cause of apoptosis in *C. elegans* is the activation of the cysteine protease *ced-3*, which is mediated by its oligomerization at the activator protein *ced-4*. Activity of the *ced-3/ced-4* complex is regulated by the apoptosis inhibitor *ced-9* and the apoptosis inducer *egl-1* (Figure 5). Genes homologous to *ced-3*, *ced-4*, and *ced-9* were also found in mammals. Subsequent studies in mammals and in the fly, *D. melanogaster*, have identified counterparts for these *C. elegans* genes, demonstrating that the core components of the cell death machinery are conserved through evolution (Richardson, 2002). Accordingly, *ced-3* is the single *C. elegans* member of a family of cysteine proteases, the caspases, whereas *ced-4* corresponds to the mammalian apoptotic protease activating factor 1, Apaf-1, which is the core of a caspase-activating signalling complex, the apoptosome. *Egl-1* and *ced-9* are members of the Bcl-2 family of pro- or antiapoptotic proteins, respectively, which play an important role in the mediation and regulation of apoptotic signalling pathways (Yuan et al, 1993; Wolf et al, 1999).

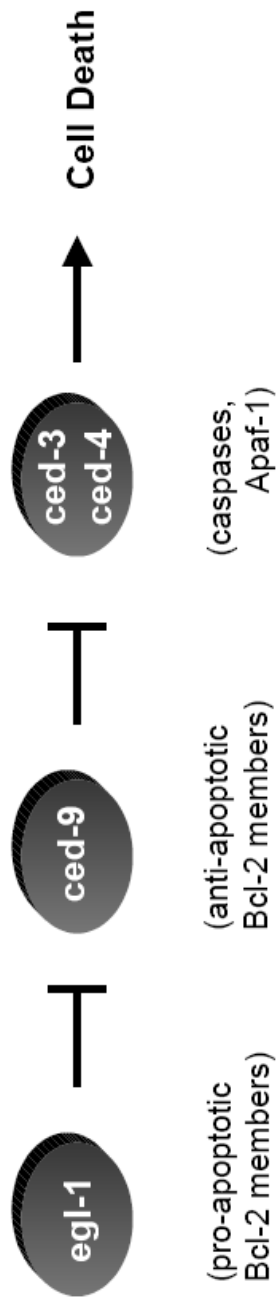


Figure 5 *C. elegans* as a model system contains basic components of the cell death machinery. Apoptosis regulation in *C. elegans* relies on a simple basic network of factors for which corresponding analogous components can also be found in higher organisms as given within brackets. Thus *egl-1* is the worm representative for mammalian proapoptotic BH3-only proteins, *ced-9* belongs to the antiapoptotic Bcl-2 family, *ced-3* is the only worm caspase, and *ced-4* is homologous to mammalian Apaf-1 (according to (Cecconi, 1999)).

1.4.3.1 Caspases

Caspases are a family of cysteine proteases that cleave target proteins at specific aspartate residues (Alnemri et al, 1996; Nicholson et al, 1995). The caspases, which are homologous to *C. elegans* ced-3, play a central role in the apoptotic signalling networks which are activated in most cases of apoptotic cell death (Yuan et al, 1993; Bratton, 2000). Actually, strictly defined, cell death can only be classified to follow a classical apoptotic mode if execution of cell death is dependent on caspase activity (Leist, 2001).

The term caspases is derived from cysteine-dependent aspartate-specific proteases: their catalytical activity depends on a critical cysteine-residue within a highly conserved active site pentapeptide QACRG, and the caspases specifically cleave their substrates after Asp residues. Caspases recognized at least four contiguous amino acids, named P4-P3-P2-P1, and cleaving after the C-terminal residue (P1), usually an Asp. Although the P1 residue was thought to be exclusively Asp, recent studies indicate that some caspases can also cleave after Glu (Hawkins et al, 2000; Srinivasula et al, 2001). Interestingly, the preferred P3 position is invariantly Glu for all mammalian caspases examined (Thornberry et al, 1997). Thus the preferred specificity of cleavage for caspases can be described as X-Glu-X-Asp. Heretofore, 7 different caspases have been identified in *Drosophila*, and 14 different members of the caspase-family have been described in mammals, with caspase-11 and caspase-12 only identified in the mouse (Denault, 2002). According to a unified nomenclature, the caspases are referred to in the order of their publication: caspase-1 is ICE (Interleukin-1 β -Converting Enzyme), the first mammalian caspase described to be a homologue of Ced-3 (Creagh, 2001). Caspase-1 as well as caspases-4, -5, -11, and -12 appear to be mainly involved in the proteolytic maturation of pro-inflammatory cytokines such as pro-IL-1 β and pro-IL-18 and their contribution to the execution of apoptosis remains questionable (Denault, 2002). Indeed, mice deficient of caspase-1 or caspase-11 develop normally and cells from those knockout mice remain sensitive to various death stimuli (Li, 1995). In contrast, gene knockout experiments targeting caspase-3 and caspase-9 resulted in perinatal mortality as a result of severe defects in brain development (Kuida, 1998), whereas caspase-8 deficient embryos died after day. This and the observation that cell lines derived from those knockout experiments are resistant to distinct apoptosis stimuli underlines the importance of caspases as

proapoptotic mediators. Indeed, caspase-3, caspase-9, caspase-8, and additionally caspases-2, -6, -7, and -10 have been recognized to play an important role in the apoptotic signalling machinery (Earnshaw, 1999).

In the cell, caspases are synthesized as inactive zymogens, the so called procaspases, which at their N-terminus carry a prodomain followed by a large and a small subunit which sometimes are separated by a linker peptide (Figure 6). Upon maturation, the procaspases are proteolytically processed between the large and small subunit, resulting in a small and a large subunit. Also, the prodomain is frequently but not necessarily removed during the activation process. A heterotetramer consisting of each two small and two large subunits then forms an active caspase.

Caspases are generally divided into two categories, the initiator caspases which include caspase-2, -8, -9, and -10, and the effector caspases, which include caspase-3, -6, and -7 (Table 4). An initiator caspase is characterized by an extended N-terminal prodomain (>90 amino acids) important for its function, whereas an effector caspase contains 20-30 residues in its prodomain sequence. All caspases are produced in cells as catalytically inactive zymogens and must undergo proteolytic activation during apoptosis. The activation of an effector caspase (such as caspase-3 or -7) is performed by an initiator caspase (such as caspase-9) through cleavage at specific internal Asp residues that separate the large and small subunits. The initiator caspases, however, are autoactivated. As the activation of an initiator caspase in cells inevitably triggers a cascade of downstream caspase activation, it is tightly regulated and often requires the assembly of a multi-component complex under apoptotic conditions. For example, the activation of procaspase-9 is facilitated by Apaf-1 and cytochrome c (Cyt. c) (Li et al, 1997), which form a ~1.4 MDa complex dubbed an apoptosome in the presence of dATP or ATP (Rodriguez and Lazebnik, 1999; Zou et al, 1999). Once activated, the effector caspases are responsible for the proteolytic cleavage of a broad spectrum of cellular targets, leading ultimately to cell death. The known cellular substrates include structural components (such as actin and nuclear lamin), regulatory proteins (such as DNA-dependent protein kinase), inhibitors of deoxyribonuclease (such as DFF45 or ICAD), and other proapoptotic proteins and caspases. Cleavage of the DFF45 (DNA fragmentation factor 45) leads to removal of its inhibition of DFF40 or CAD (caspase-activated deoxyribonuclease), which degrades the chromosomes into nucleosomal fragments during apoptosis (Enari et al, 1998).

There are two major mechanisms that initiate the caspase cascade: the extrinsic, involving caspase-8; and the intrinsic pathway, involving caspase-9 as the apical caspase which leads to the proteolytic activation of effectors caspase including caspase-3 and -7 that then cleaves the cellular substrate, resulting in cell death as shown in Figure 7 (Kuo et al, 2005).

In extrinsic apoptosis pathways , procaspase-8 is recruited by its DEDs to the death inducing signalling complex (DISC), a membrane receptor complex formed following to the ligation of a member of the tumor necrosis factor receptor (TNFR) family (Sartorius, 2001). When bound to the DISC, several procaspase-8 molecules are in close proximity to each other and therefore are assumed to activate each other by autoproteolysis (Denault, 2002).

Intrinsic apoptosis pathways involve procaspase-9 which is activated downstream of mitochondrial proapoptotic events at the so called apoptosome, a cytosolic death signalling protein complex that is formed upon release of cytochrome c from the mitochondria (Salvesen, 2002b). In this case it is the dimerization of procaspase-9 molecules at the Apaf-1 scaffold that is responsible for caspase-9 activation (Denault, 2002). Once the initiator caspases have been activated, they can proteolytically activate the effector procaspases-3, -6, and -7 which subsequently cleave a specific set of protein substrates, including procaspases themselves, resulting in the mediation and amplification of the death signal and eventually in the execution of cell death with all the morphological and biochemical features usually observed (Earnshaw, 1999).

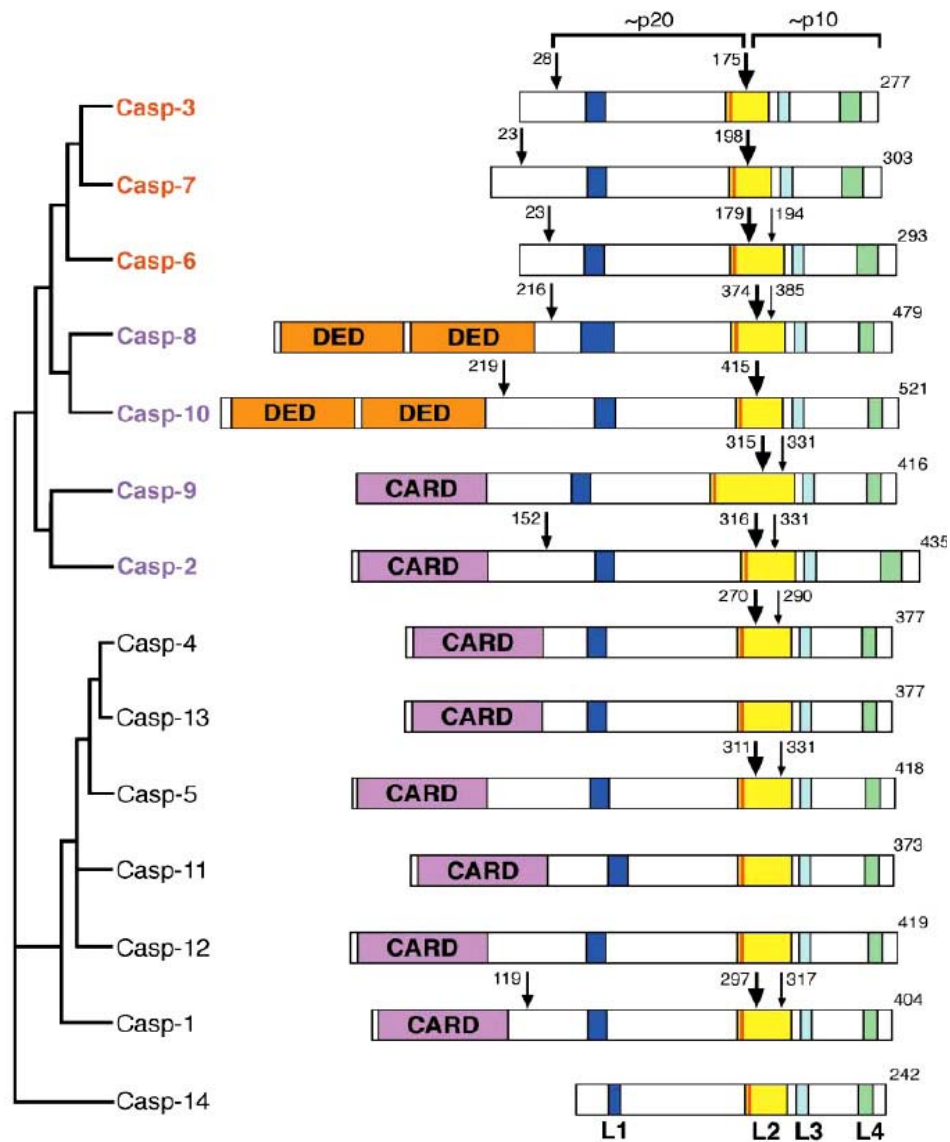


Figure 6 Schematic Diagram of the Mammalian Caspases. Except caspase-11 (mouse), -12 (mouse), and -13 (bovine), all listed caspases are of human origin. Their phylogenetic relationship (left) appears to correlate with their function in apoptosis or inflammation. The initiator and effector caspases are labeled in purple and red, respectively. (Shi, 2002)

Table 4 Structural and functional characteristics of cysteine endopeptidases of the caspase family (Philchenkov, 2004)

Enzyme	Map position of gene	Size of enzyme precursor (kDa)	Prodomain type	Active subunits (kDa)	Adaptor proteins	Caspase-activating complex	Caspase proteolytic specificities
Apoptotic initiator caspases							
Caspase-2	7q34-q35	51	Long, with CARD region	19/12	PIDD, RAIDD, PIDDosome? PACAP, DEF-CAP		VDVAD ^b
Caspase-8	2q33-q34	55	Long, with two DED-regions	18/11	FADD, DEDAF, ASC	DISC	(L/V/D)E(T/V/I)D ^c
Caspase-9	1p36.1-p36.3	45	Long, with CARD region	17/10	Apaf-1, Nod-1, Apoptosome PACAP		(L/V/I)EHD
Caspase-10	2q33-q34	55	Long, with two DED-regions	17/12	FADD, DEDAF	DISC	(I/V/L)EXD
Caspase-12 ^d	#9 ^e	50	Long, with CARD region	20/10	TRAF-2		Unknown
Apoptotic effector caspases							
Caspase-3	4q35	32	Short	17/12	NA ^f		DE(V/I)D
Caspase-6	4q25	34	Short	18/11	NA		(T/V/I)E(H/V/I)D
Caspase-7	10q25.1-q25.2	35	Short	20/12	NA		DE(V/I)D
Inflammatory caspases							
Caspase-1	11q22.2-q22.3	45	Long, with CARD region	20/10	ASC, NALP1, CARDIAK, CARD-8, Ipaf, Nod-1	Inflammasome	(W/Y/F)EHD
Caspase-4	11q22.2-q22.3	43	Long, with CARD region	20/10	Unknown		(W/L)EHD
Caspase-5	11q22.2-q22.3	48	Long, with CARD region	20/10	ASC, NALP1	Inflammasome	(W/L/F)EHD
Caspase-11 ^d	#9	42	Long, with CARD region	20/10	Unknown		(V/I/P/L)EHD
Other mammalian caspases							
Caspase-14	19p13.1	30	Short	20/10	NA		
Invertebrate caspases^g							
Ced-3		56	Long, with CARD region	17/14	Ced-4, RAIDD		DEXD
Dcp-1 ^h	58E3-59F4	36	Short	22/13	NA		DEVD
Dronc ^h	67C4-67C5	50	Long, with CARD region	20/14	DARK ⁱ		VDVAD

^a Adapted from Ref. 3 with modifications.

^b The sequence of amino acid residues is presented in P₄-P₁ direction; the proteolysis occurs after the aspartic acid residue in the P₁ position.

^c In parentheses each amino acid possibility is listed.

^d Detected in murine cells.

^e The *pseudo-caspase-12* gene is localized on human chromosome 11q22.3.

^f NA - not applicable.

^g Only several caspases are given as an example.

^h The *Drosophila* caspases.

ⁱ A *Drosophila* homologue of Apaf-1

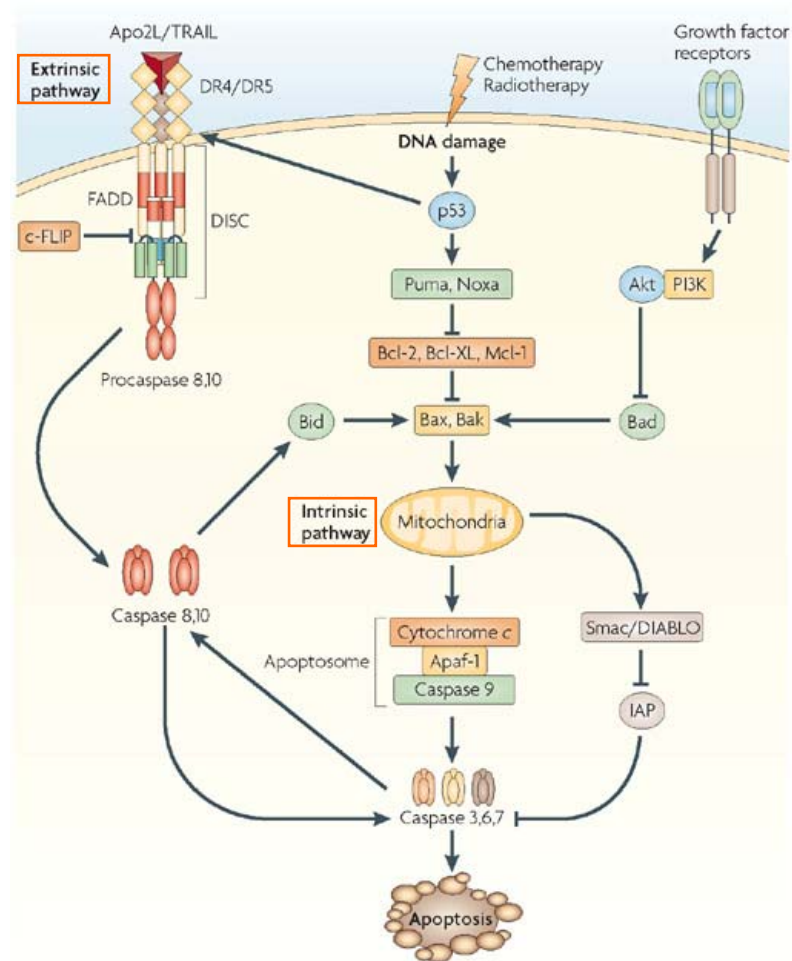


Figure 7 Schematic representation of two major apoptotic signalling pathways. Adapted from Ashkenazi, 2008.

1.4.3.2 Extrinsic apoptosis pathways

Extrinsic apoptosis signalling is mediated by the activation of so called “death receptors” which are cell surface receptors that transmit apoptotic signals after ligation with specific ligands. Death receptors belong to the tumor necrosis factor receptor (TNFR) gene superfamily, including TNFR-1, Fas/CD95, and the TRAIL receptors DR-4 and DR-5 (Ashkenazi, 2002). All members of the TNFR family consist of cysteine rich extracellular subdomains which allow them to recognize their ligands with specificity, resulting in the trimerization and activation of the respective death receptor (Naismith, 1998). Subsequent signalling is mediated by the cytoplasmic part of the death receptor which contains a conserved sequence termed the death domain (DD). Adapter molecules like FADD or TRADD themselves possess their own DDs by which they are recruited to the DDs of the activated death receptor, thereby forming the so-called death inducing signalling complex (DISC). In addition to its DD, the adaptor FADD also contains a death effector domain (DED) which through homotypic DED-DED interaction sequesters procaspase-8 to the DISC (Figure 8). As described above, the local concentration of several procaspase-8 molecules at the DISC leads to their autocatalytic activation and release of active caspase-8. Active caspase-8 then processes downstream effector caspases which subsequently cleave specific substrates resulting in cell death. Cells harboring the capacity to induce such direct and mainly caspase-dependent apoptosis pathways were classified to belong to the so called type I cells (Scaffidi, 1998).

In type II cells, the signal coming from the activated receptor does not generate a caspase signalling cascade strong enough for execution of cell death on its own. In this case, the signal needs to be amplified via mitochondria-dependent apoptotic pathways. The link between the caspase signalling cascade and the mitochondria is provided by the Bcl-2 family member Bid. Bid is cleaved by caspase-8 and in its truncated form (tBID) translocates to the mitochondria where it acts in concert with the proapoptotic Bcl-2 family members Bax and Bak to induce the release of cytochrome c and other mitochondrial proapoptotic factors into the cytosol (Luo, 1998; Yin, 2003). Cytosolic cytochrome c is binding to monomeric Apaf-1 which then, in a dATP-dependent conformational change, oligomerizes to assemble the apoptosome, a complex of wheel-like structure with a 7-fold symmetry, which triggers the activation of the initiator procaspase-9. Activated caspase-9 subsequently initiates a caspase cascade involving downstream

effector caspases such as caspase-3, caspase-7, and caspase-6, ultimately resulting in cell death (Slee, 1999).

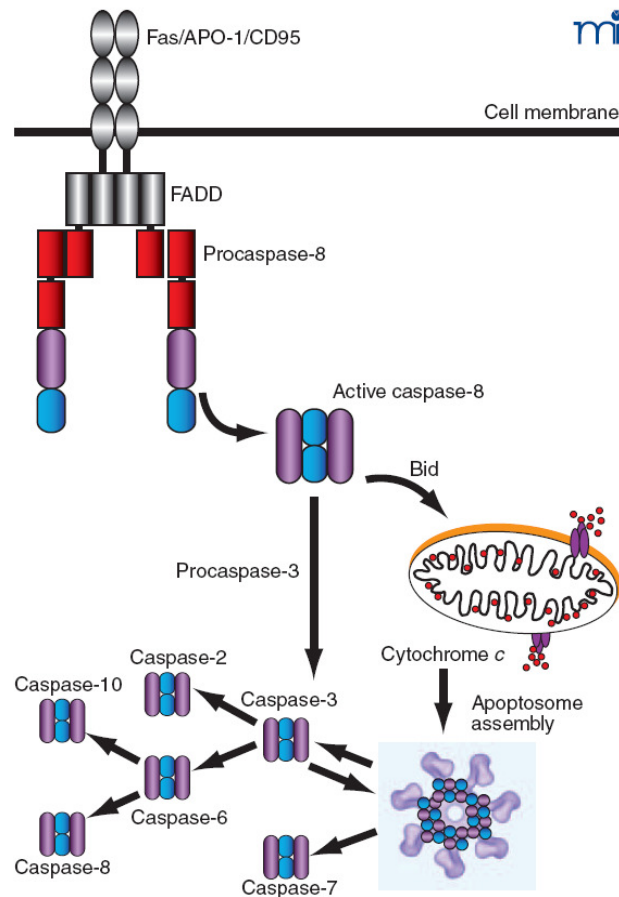


Figure 8 The extrinsic apoptosis pathway. Initiator caspases are activated through protein-protein interactions, whereas effector caspases are activated through cleavage by initiator caspases. Two major pathways to caspase activation are illustrated. Binding of Fas ligand (FasL) to Fas induces oligomerization of caspase-8 through the adapter molecule FADD. Activated caspase-8 processes and activates caspase-3, leading to further caspase activation events and substrate proteolysis. In certain cells, caspase-8 also activates the mitochondrial pathway by cleaving the Bcl-2 family member Bid, leading to the release of cytochrome c from the intermembrane space and formation of the apoptosome. FADD, Fas-associated death domain-containing molecule. Bid, Bcl-2 homology 3-interacting domain death agonist. (Hill et al, 2003)

1.4.3.3 The Bcl-2 family

In 1985, Tsujimoto et al, discovered the Bcl-2 oncogene (a founding member of the family) associated with human B-cell lymphoma/leukemia that is translocated from chromosome 18 to the immunoglobulin loci and transcriptionally activated by reciprocal translocation t(14;18). In 1988, Vaux et al, discovered the anti-apoptotic activity of this protein in a system where apoptosis was induced by deprivation of IL-3 (Vaux et al, 1988). Soon after, Bcl-2 was shown to prevent apoptosis induced by various stimuli, including serum deprivation, heat shock, and chemotherapeutic reagents (Tsujimoto, 1989), suggesting the ability of Bcl-2 to prevent apoptosis via a common pathway. Bcl-2 is also able to inhibit certain forms of necrotic cell death (Strasser et al, 1991; Kane et al, 1995), e.g. necrosis induced by hypoxia and inhibition of respiration, suggesting that apoptosis and some forms of necrosis share common steps. Such a conclusion is consistent with the inhibition of these types of necrosis by inhibitors of caspases, which are apoptosis-driving proteases (Shimizu et al, 1996).

The Bcl-2 protein is a membrane protein that is localized to the outer mitochondria membrane, endoplasmic reticulum membrane, and nuclear envelope, whereby its NH₂-terminal is facing the cytosol. As many other members of its family, Bcl-2 has a hydrophobic domain at COOH-terminal (TM domain) that allows the insertion of the protein into the cytosolic face of the intracellular membranes; this intracellular localization is important for their function. The Bcl-2 family members are major regulators of the apoptotic process, whereas caspases (cysteine proteases) are the major executioners. The cell death-regulating activity of the Bcl-2 family members appears to depend on their ability to modulate mitochondrial function.

The Bcl-2 family is divided into three subfamilies by function and structure (Figure 9):

1. Anti-apoptotic members (Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 etc.) that show structural conservation at the four domains, BH1, BH2, BH3, and BH4 (BH stands for Bcl-2-homology);
2. Multi-domain pro-apoptotic members (Bax, Bak etc.) that share sequence homology with Bcl-2 at BH1, BH2, and BH3; and

3. Pro-apoptotic members called BH3-only proteins (Bid, Bik, Bad, Hrk (DP5), Bim, Bmf, Puma, Noxa and many others are known) that share homology only at the BH3 domain.

One of the striking features of Bcl-2 family proteins is their ability to form homodimers and heterodimers (Oltvai et al, 1993). Heterodimerization between antiapoptotic and pro-apoptotic members of this family is considered to inhibit the biological activity of their partners (Oltvai et al, 1993; Yang et al, 1995), and is mediated by the insertion of the BH3 region of a proapoptotic protein into a hydrophobic pocket composed of BH1, BH2 and BH3 from an anti-apoptotic protein (Sattler et al, 1997). In addition to BH1, BH2 and BH3, BH4 is required for anti-apoptotic activity (Huang et al, 1998), while BH3 is essential for pro-apoptotic activity. Oligopeptides corresponding to the BH3 region of Bax and Bak are able to induce apoptotic cell death (Chittenden et al, 1995). Replacement of the BH3 region of Bcl-2 by the BH3 region of Bax converts Bcl-2 from a death antagonist to a death agonist (Hunter & Parslow 1996), suggesting that there is a critical sequence difference which distinguishes the BH3 domain of the anti-apoptotic and pro-apoptotic members of the family. In addition to the regulation of apoptosis by heterodimerization of anti-apoptotic and pro-apoptotic members of the Bcl-2 family, some of these proteins have been suggested to regulate apoptosis independently of each other (Knudson and Korsmeyer, 1997). This notion might be consistent with the recent findings that some Bcl-2 family members can form ion channels in synthetic lipid membranes. Thus, the life or death of a cell may be determined by Bcl-2 family proteins in two ways, either through heterodimerization between anti-apoptotic and proapoptotic members, or through the independent functions of these proteins. In either case, the ratio between anti-apoptotic and pro-apoptotic members of the Bcl-2 family may determine the susceptibility of a cell to apoptosis.

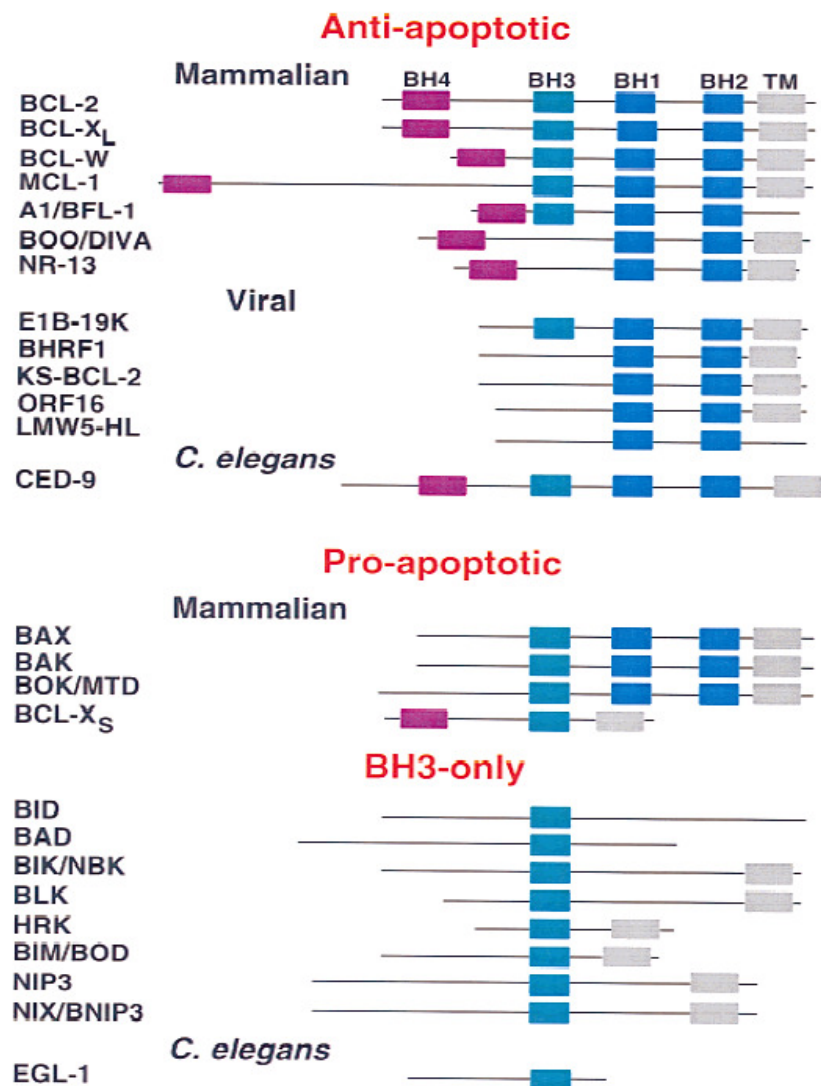


Figure 9 Summary of anti-apoptotic and pro-apoptotic BCL-2 members. BCL-2 homology regions (BH1-4) are denoted as is the carboxy-terminal hydrophobic (TM) domain. (Gross et al, 1999)

1.4.3.4 Intrinsic apoptosis pathways

The intrinsic apoptotic pathway hinges on the balance of activity between pro- and anti-apoptotic members of the Bcl-2 superfamily of proteins which act to regulate the permeability of the mitochondrial membrane (Coults et al, 2003). The anti-apoptotic Bcl-2 proteins Bcl2 and Bcl-X_L act to prevent permeabilization of the mitochondrial outer membrane by inhibiting the action of the pro-apoptotic multi-domain Bcl-2 proteins BAX (a cytosolic protein) and BAK (found in the mitochondrial membrane) (Reed, 1998). Over expression of Bcl-2 and Bcl-X_L is known to be associated with a number of human malignancies (Coults et al, 2003; Bush et al, 2003; Igney et al, 2002). Other pro-apoptotic Bcl-2 family members, including the BH3-only proteins PUMA and NOXA, act as cytosolic sensors of cell damage or stress (Karst and Li, 2007).

As shown in Figure 10, the intrinsic (mitochondrial) pathway is initiated in response to cellular signals resulting from DNA damage, a defective cell cycle, detachment from the extracellular matrix, hypoxia, loss of cell survival factors, or other types of severe cell stress. This triggers activation of specific members of the pro-apoptotic Bcl-2 protein family involved in the promotion of apoptosis, Puma and Noxa, which in turn activate the multi-domain proapoptotic proteins Bax or Bak. These two proteins move to the mitochondrial membrane and disrupt the function of the antiapoptotic Bcl-2 proteins thereby allowing permeabilization of the mitochondrial membrane. Cytochrome c and the pro-apoptotic protein SMAC/DIABLO are then able to leak from the inter-membrane space of the mitochondria into the cytosol. Cytochrome c binds the adaptor apoptotic protease activating factor-1 (APAF1), forming a large multi-protein structure known as the apoptosome. The apoptosome then recruits and activates caspase 9, which, in turn, activates the downstream effector caspases, including caspase-3, -6, and -7, leading to apoptosis (Henry-Mowatt et al, 2004).

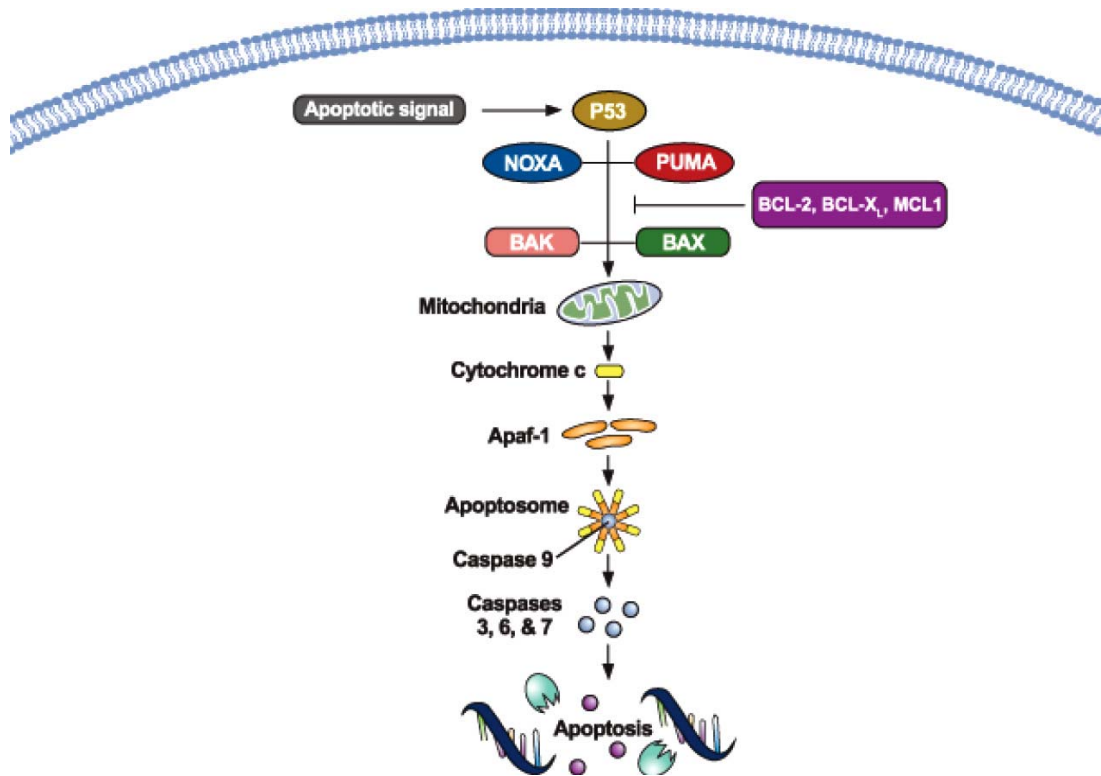


Figure 10 Elements of the intrinsic apoptotic pathway. Adapted from Ashkenazi, 2002.

1.5 General data of plants called ‘ Hua Khao Yen’

Hua Khao Yen are Thai medicinal plants which are found in many preparations from Thai traditional medicine textbooks (The Association of Traditional Medicine, 1952 and 1978; Pongboonrod, 1976; The palm leaf text studies program, 1982) and is an ingredient in as many as 2449 formulae (Division of Medical Research, 1986). These formulae are used to treat dermatopathy, lymphopathy, venereal diseases, leprosy, and cancer as well as inflammatory conditions associated with diseases such as rheumatism, infectious diseases and other pain-causing conditions. ‘Hua Khao Yen’ are found as ingredients in almost every traditional drug formula for cancer (Vimolkhunakorn, 1979).

In addition, interviews of the selected traditional doctors in Southern Thailand revealed that they used Hua Khao Yen as ingredients in their remedies for cancer which accounted for about sixty percent of the list of herbal drugs used for cancer treatment (Itharat et al, 1998). Hua Khao Yen were the best-selling medicinal plants in traditional drugstores in Thailand. From an intensive interview with 23 experienced Thai traditional doctors and excursions into the forest with them to collect Hua Khao Yen, it was found that Hua Khao Yen were the rhizomes of 5 species, i.e. *Dioscorea membranacea* Pierre (Dioscoreaceae), *D. birmanica* Prain ex Burkill, *Smilax corbularia* Kunth (Smilacaceae), *S. glabra* Roxb. and *Pygmeopremna herbacea* Prain et Burkill (Verbenaceae) as shown in Figure 11 (Itharat et al, 1999) .



Dioscorea membranacea



Dioscorea birmanica



Smilax corbularia



Smilax glabra



Pygmeopremna herbacea

Figure 11 Five species of Hua Khao Yen i.e. *Dioscorea membranacea* Pierre, *Dioscorea birmanica* Prain ex Burkill, *Smilax corbularia* Kunth, *Smilax glabra* Roxb. and *Pygmeopremna herbacea* Prain et Burkill

1.6 Review of Literature

1.6.1 *Dioscorea membranacea* Pierre

Dioscorea membranacea Pierre is a member of Dioscoreaceae; its Thai vernacular names are Phak lum phua, Phak khanong ma, Khao-yen tai and Khrua that (Supatanakul et al, 1985). It is distributed from Thailand westwards to north Burma and eastwards into Cambodia; southwards passing beyond the Isthmus of Kra into Malaysia. It grows on limestone at its southern limit (Burkill, 1951). Its rhizome has been used to treat cancer for a long time by folk doctors (Itharat, 2002; Subchareon, 1998).

A description of *Dioscorea membranacea* Pierre is shown in Figure 12. The rhizome is wide running, perhaps even up to 2 m. It is dark brown with white flesh. The stem is slightly ridged and unarmed. Leaves are deeply trifid above a short acuminate 9 nerved cordate base, two primary nerves reaching the forerunner tip along with the midrib and the second pair reaching the tips of the lateral lobes. The petioles are 1/2-2/3 of the length of the blade. Male flowers have small subsessile cymes with up to 4 flowers, sepals 1 mm long, and long-ovate. Stamens, alike the filaments insert just below the sepals 0.3 mm long. The anther is small and introse. Female flowers are on downwardly directed spike-like racemes. Outer sepals are obovate, inner ones are lanceolate, and the inner are a little shorter than the outer. Style is short. Capsules are about 1 -2 cm apart (Burkill, 1951).



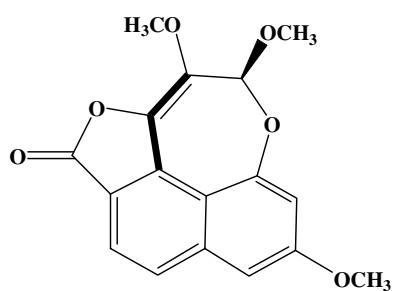
Figure 12 *Dioscorea membranacea* Pirre (original picture)

1.6.2 Chemical constituents of *Dioscorea membranacea*

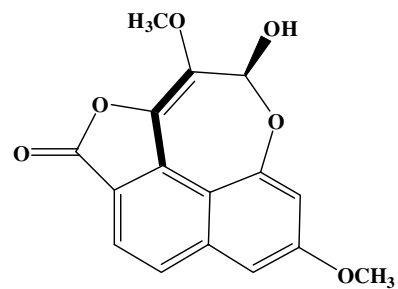
Eight compounds isolated from *Dioscorea membranacea* are shown in Table 5 and Figure 13.

Table 5 Chemical constituents found in *Dioscorea membranacea* Pierre

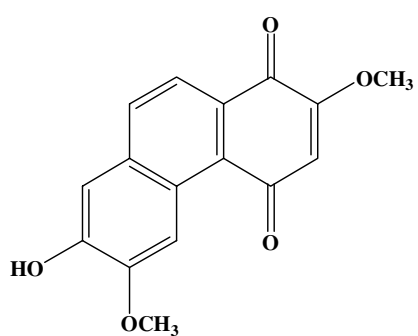
Botanical name	Part of plant used	Chemical constituents	References
<i>D. membranacea</i>	Rhizomes	Dioscorealide A	Itharat et al, 2003
		Dioscorealide B	Itharat et al, 2003
		Dioscoreanone	Itharat et al, 2003
		Stigmasterol	Itharat, 2002
		β -Sitossterol	Itharat, 2002
		Diosgenin 3-O- α -L-	Itharat, 2002
		rhamnopyranosyl (1 \rightarrow 2)- β -D	
		glucopyranoside (prosapogenin A of	
		dioscin)	
		Diosgenin 3-O- β -D-	Itharat, 2002
		glucopyranosyl (1 \rightarrow 3)- β -D	
		glucopyranoside (prosapogenin of	
		gracillin)	
		β -sitossterol 3-O- β -D-	Itharat, 2002
		glucopyranoside	



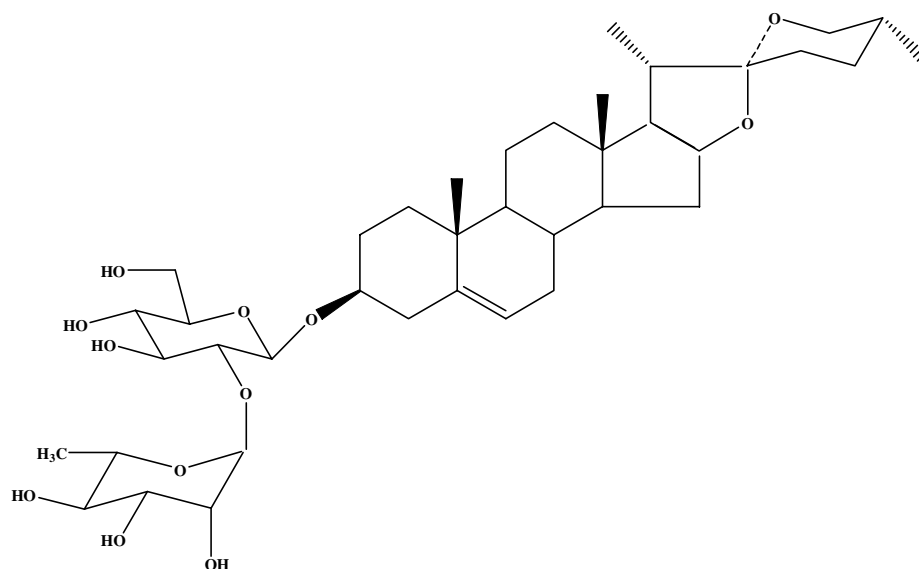
Dioscorealide A

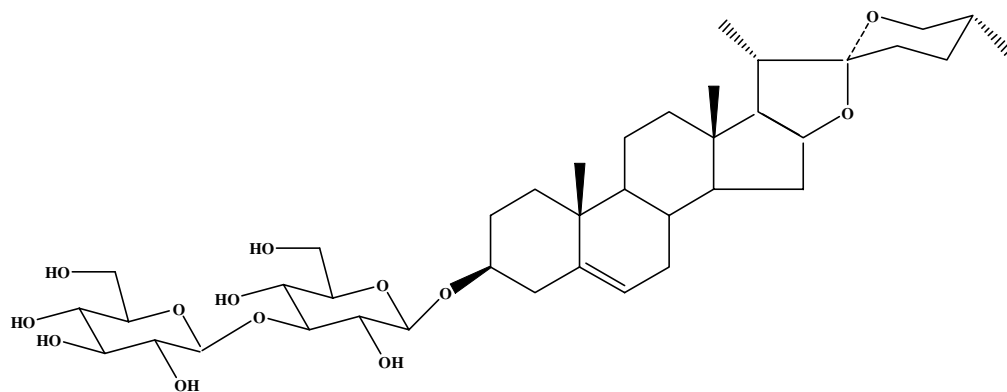


Dioscorealide B

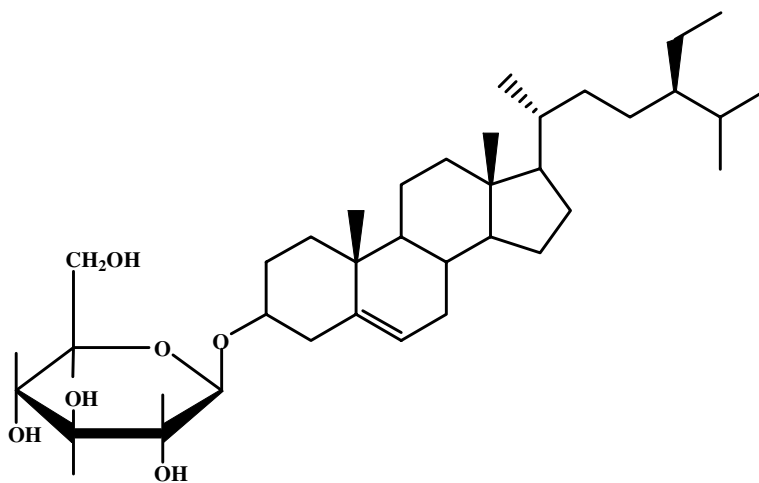


Dioscoreanone

Diosgenin 3-O- α -L-rhamnopyranosyl (1 \rightarrow 2)- β -D-glucopyranoside**Figure 13** Structures of chemical constituents found in *D. membranacea*. (Itharat, 2002)

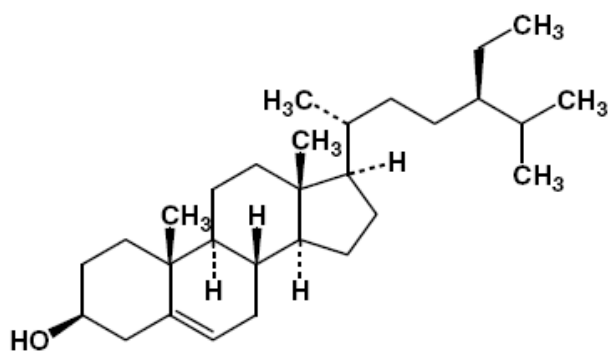


Diosgenin 3-*O*- β -*D*-glucopyranosyl (1 \rightarrow 3)- β -*D*-glucopyranoside

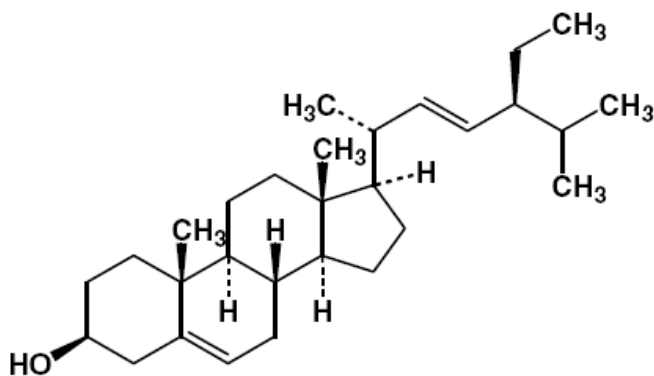


β -Sitosterol 3-*O*- β -*D*-glucopyranoside

Figure 13 (continued)



β -Sitosterol



Stigmasterol

Figure 13 (continued)

1.6.3 Biological activity of *Dioscorea membranacea*

Previous studies revealed antimicrobial, antioxidant, anti-HIV-1 protease, anti-HIV-1 integrase and nitric oxide inhibitory activities of *Dioscorea membranacea*. In addition, the extract of *D. membranacea* showed cytotoxic effect on several cancer cell lines. The biological activity of other *Dioscorea* species is shown in Table 6.

The study on anti-HIV-1 protease and HIV-1 integrase activities of Hua Khao Yen, including *Dioscorea birmanica*, *D. membranacea*, *Smilax corbularia*, *S. glabra* and *Pygmaeopremna herbacea*, was conducted to test for their inhibitory effects against HIV-1 protease (HIV-PR) and HIV-1 integrase (HIV-1 IN). The data indicated that only ethanolic extract of *D. membranacea* had appreciable activity ($IC_{50} = 48 \mu\text{g/ml}$) against HIV-1 PR; however, both the water and ethanolic extract of *D. membranacea* had no anti-HIV-1 IN activity ($IC_{50} > 100 \mu\text{g/ml}$) (Tewtrakul et al,2006).

Besides, five species of Hua Khao Yen were examined for their inhibitory activities against lipopolysaccharide (LPS) induced nitric oxide (NO) production in RAW264.7 cell lines. The results showed that an ethanolic extract of *D. membranacea* had the most potent inhibitory activity, with an IC_{50} value of $23.6 \mu\text{g/ml}$ (Tewtrakul et al,2006).

Remarkably, the study of Itharat (2002) revealed antioxidant, antimicrobial and anticancer activities of the extract from the rhizome of *Dioscorea membranacea*. Using DPPH and lipid peroxidation of liposome assay, the ethanolic extract of this plant showed antioxidant activity with EC_{50} value of $16.5 \mu\text{g/ml}$ and $8.1 \mu\text{g/ml}$, respectively. The results of antibacterial screening demonstrated that the ethanolic extract of *D. membranacea* showed antibacterial effect against *Staphylococcus aureus* ($MIC < 1.25 \text{ mg/ml}$), *Bacillus subtilis* ($MIC < 1.25 \text{ mg/ml}$), *Escherichia coli* ($MIC = 2.5 \text{ mg/ml}$) and *Epidermotophytum floccosum* ($MIC < 1.25 \text{ mg/ml}$). Moreover, anti-cancer activity of five species of Hua Khao Yen were determined by sulphoramine B (SRB) assay and the results indicated that the ethanolic extract of *Dioscorea membranacea* had a significantly cytotoxic effect on lung (COR-L23), colon (LS-174T) and breast cancer (MCF-7) cells (IC_{50} at exposure time 72 h = 6.2, 16.7, 12.0 $\mu\text{g/ml}$, respectively). Aqueous and ethanolic extract of *D. membranacea* rhizomes were the most cytotoxic against cancer cell lines in which the water extract showed a specifically cytotoxic effect against breast cancer

lines (MCF-7), although the ethanolic extract showed a greater effect against lung (COR-L23) and colon cancer cell lines (LS-174T). Furthermore, the water and ethanolic extract of this plant showed a slight activity in normal cells (SVK-14) (Itharat, 2002).

Table 6 Biological activities of *Dioscorea* species

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea alata</i>	Rhizome	Immunomodulatory	Dioscorin was able to stimulate nitric oxide production in RAW264.7 cells and enhanced the phagocytosis against <i>E.coli</i> . Dioscorin also induced IL-6, TNF- α , and IL-1 β production in RAW 264.7 cells and human monocytes and stimulated proliferation of splenic cells from BALB/c mice.	Liu et al, 2007
	Rhizome	Hypotensive	Powdered-yam-products (PYP) reduce the blood pressure in spontaneously hypertensive rats (SHR)	Liu et al, 2009
<i>D. batatas</i>	Tuber	Antioxidant	Dioscorin showed scavenging activity against DPPH (EC_{50} = 1.43 nM).	Hou et al, 2001

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea batatas</i>	Tuber	Antioxidant	<p>Yam tuber mucilage (YTM) exhibited antioxidant activities in a series of in vitro tests, including DPPH (IC_{50} = 0.86 mg/mL) and hydroxyl radical (IC_{50} = 22 μg/mL) scavenging activity assays, reducing power test, anti-lipid peroxidation and anti-human low density lipoprotein peroxidation tests (IC_{50} = 145.46 μg/mL) using butylated hydroxytoluene (BHT), reduced glutathione, or ascorbic acid for comparisons.</p>	Hou et al, 2002
	Rhizome	Immune modulatory	<p>The cytotoxic activity of mouse splenocyte against leukemia cell was increased in the presence of Yam mucopolysaccharide (YMP). The production of IFN-γ was significantly increased in the YMP treated splenocytes. YMP significantly increased the viability of peritoneal macrophages.</p>	Choi, 2004

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea birmanica</i>	Rhizome	Cytotoxic	Ethanolic extract active against COR-L23 (IC ₅₀ =7.4 µg/ml) and MCF-7 (IC ₅₀ =16.3 µg/ml).	Itharat et al, 2004
<i>D. bridgesii</i>	Dried tuber	Antioxidant	Mathanolic extract showed weak activity with DPPH assay at 50 µg/ml.	Schmada et al, 1999
<i>D. bulbifera</i>	Dried rhizome	Cytotoxic	Water extract active against CA-mammary microalveolar at 50 µg/ml.	Sato, 1989
	Bulb	Antibacterial	8-epidiosbulbin E acetate (EEA) exhibited broad-spectrum plasmid-curing activity against multidrug-resistant (MDR) bacteria.	Shriram et al, 2008
<i>D. collettii</i> var. <i>hypoglauca</i>	Dried rhizome	Cytotoxic	Prosaponin A, dioscin, gracillin isolated from ethanolic extract active against human acute myeloid leukemia K562(IC ₅₀ <3 µg/ml)	Hu et al, 1996
	Dried rhizome	Cytotoxic	Protoneodioscin, protodioscin, protoneo-gracillin, protogracillin against the cancer cell line of K562	Hu et al, 1997

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea colletii</i> var. <i>hypoglauca</i>	Dried rhizome	Cytotoxic	Methyl protogracillin showed selectivity against colon cancer cell line(KM12), central nervous system (CNS) cancer cell line (U251), two melanoma cell lines (MALME-3M and M14), two renal cancer cell lines (786-0 and UO-31) and breast cancer cell line (MDA-MB-231) with $GI_{50} = 2.0 \mu\text{M}$.	Hu et al, 2001
	Dried rhizome	Cytotoxic	Protoneodioscin active against Leukemia, CNS cancer, and prostate cancer while melanoma, ovarian cancer, and renal cancer are less sensitive. The preliminary animal studies showed that the maximum tolerant dose of protoneodioscin was 600 mg/kg to mice.	Hu et al, 2002

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea colletii</i> var. <i>hypoglauca</i>	Dried rhizome	Cytotoxic	Protodioscin showed selectively against leukemia line (MOLT-4), NSCLC line (A549/ATCC), two colon cancer cell lines (HCT-116 and SW-620), CNS cancer cell line (SNB-75), melanoma cell line (LOX IMVI), and one renal cancer cell line (786 - 0) with $GI_{50} < 2.0 \mu\text{M}$.	Hu et al, 2002
	Dried rhizome	Cytotoxic	Methyl protoneodioscin showed selectively against non-small cell lung cancer (NSCLC) line (A549/ATCC), colon cancer cell line (HCT-116), two central nervous system (CNS) cancer cell lines (SF-539 and SNB-75), melanoma cell line (M14), renal cancer cell line (CAKI-1), one prostate cancer (DU-145) and two breast cancer cell lines (HS 578T and MDA-MB-435) with $GI_{50} \leq 2.0 \mu\text{M}$.	Hu et al, 2002

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea collettii</i> var. <i>hypoglauca</i>	Dried rhizome	Cytotoxic	Methyl protoneogracillin was cytotoxic selectively against two leukemia lines (CCRF-CEM and RPMT-8226), colon cancer cell line (KM12), two central nervous system (CNS) cancer cell lines (SF-539 and U251), melanoma line (M14), renal cancer cell line (786-0), prostate cancer cell line (DU-145), and one breast cancer cell line (MDA-MB-435), with $GI_{50} \leq 2.0 \mu\text{M}$.	Hu et al, 2003
	Dried rhizome	Cytotoxic	Methyl protodioscin showed strong cytotoxicity against most cell lines from solid tumors with $GI_{50} \leq 10.0 \mu\text{M}$, selectively against one colon cancer cell line (HCT-15) and one breast cancer cell line (MDA-MB-435) with $GI_{50} < 2.0 \mu\text{M}$ but moderate cytotoxicity was shown against leukemia cell lines with GI_{50} 10-30 μM .	Hu et al, 2003

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea collettii</i> var. <i>hypoglauca</i>	Dried rhizome	Anticancer	Treatment of methyl protodioscin resulted in G2/M arrest and apoptosis in HepG2 cells. These effects were attributed to down-regulation of Cyclin B1 and the signaling pathways leading to up-regulation of Bax and down-regulation of BCL-2,	Wang et al, 2006
<i>D. deltoidea</i> var. <i>orbiculata</i>	Fresh rhizome	Antineoplastic	Orbiculatoside A was isolated and identified as 3-O-beta-D-glucopyranosyl-ergost-5-ene-3beta, 26-diol-26-O-beta-D-glucopyranosyl(1→3)-[beta-D-glucopyranosyl(1→2)-beta-D-glucopyranosyl(1→6)]-beta-D-glucopyranoside by NMR techniques. This compound was cytotoxic to cancer cell lines K562, HCT-15, A549, HT1080 and A2780a	Shen et al, 2002
<i>D. dregena</i>	Tuber	Antibacterial	Methanolic extract active against <i>Staphylococcus aureus</i> , <i>Pseudomonas auroginosa</i>	Kelmanson et al, 2000

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea fitshauensis</i>	Rhizome	Anticancer	Prosapogenin B of dioscin (P.B) exerts anti-proliferative effect on K562 cells through inducing apoptosis of the cells as evidence by the nuclear chromatin condensation, nuclear fragmentation, plasma membrane bleb formation, the increase of the detected DNA ladder and the percentage of apoptotic bodies in the sub-G0/G(1).	Wang et al, 2003
	Rhizome	Anticancer	Diosgenin-3-O-alpha-L-rhamnopyranosyl-(1→4)-beta-D-glucopyranoside (DRG) inhibited the proliferation of human cancer cells, A431, A2780, A549, K562, and HCT-15. DRG induce apoptosis on HCT-15 cells, which involves the reduction of the mitochondrial potential, the release of cytochrome c, and the down-regulation of the ratio of Bcl-2/Bax expression level.	Wang et al, 2004

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea hispida</i>	Dried rhizome	Cytotoxic	Methanolic extract inactive against MT-4 cell lines ($IC_{100} > 228 \mu\text{g/ml}$)	Otake et al, 1995
<i>D. japonica</i>	Dried root	Cytotoxic	Methanolic extract active against L-1210 ($IC_{50} < 20 \mu\text{g/ml}$)	Nam et al, 1995
	Rhizome	Immunomodulatory	Dioscorins induced expression of the pro-inflammatory cytokines and stimulate phagocytosis of RAW 264.7 and enhanced proliferation of $CD4^+$, $CD8^+$, and Th1 cells in spleen and $CD19^+$ cells in both spleen and thymus.	Lin et al, 2009
<i>D. panthaica</i>	Rhizome	Cytotoxic	Doscoresides C and D, pregnadienolone 3-O-beta-gracillimatriose, pregnadienolone 3-O-beta-chacotrioside and pseudoproto-dioscin showed mild cytotoxicity against A375, L929, and HeLa.	Dong et al, 2001

Table 6 (continued)

Botanical name	Part of plant used	Activities	Results of biological activities	References
<i>Dioscorea panthaica</i>	Rhizome	Cytotoxic	Dioscin, progenin II, 3- O-[bis-alpha- L- rhamnopyranosyl-(1→2 and 1→4)- beta-D-glucopyranosyl]-22 R, 25 R-spirost-5-ene-3 beta,20 alpha-diol and 3- O-[bis-alpha-L-rhamnopyranosyl-(1→2 and 1→4)- beta- D-glucopyranosyl]-25 R-furost-5-ene-3 beta, 22 alpha, 26-triol showed significant cytotoxicity against A375, L929, and HeLa.	Dong et al, 2004
<i>D. preussii</i>	leaves	Cytotoxic	Methanolic extract exhibited cytotoxic activities toward human monocytes	Lamidi et al, 2005
<i>D. villosa</i>	Root	Cytotoxic	Extract of <i>D. villosa</i> showed cytotoxic effect on neuroblastoma (LC ₅₀ = 31 µg/ml)	Mazzio et al, 2008
	Rhizome	Renotoxic	Ethyl acetate, methanol and 50% aqueous methanol extracts of <i>Dioscorea villosa</i> showed significant toxicity to normal renal mammalian fibroblasts (NRK49F) and tubular epithelial cells (NRK52E).	Wojcikowski et al, 2008

1.6.4 Biological activity of Dioscorealide B

Dioscorealide B is one of the isolated compounds from the ethanolic extract of *Dioscorea membranacea*. Previous studies demonstrated that Dioscorealide B possessed anti-allergic and nitric oxide inhibitory activities and served as a selective cytotoxic agent.

Eight isolated compounds from *D. membranacea* shown in Table 3 were investigated for their anti-allergic activities using RBL-2H3 cells. The results revealed that Dioscorealide B had the highest activity with an IC_{50} value of 5.7 μ M, followed by Dioscoreanone ($IC_{50} = 7.7 \mu$ M), Dioscorealide A ($IC_{50} = 27.9 \mu$ M), and Diosgenin ($IC_{50} = 29.9 \mu$ M), respectively, whereas other compounds exhibited moderate and weak activities. It was found that Dioscoreanone exhibited the most potency against TNF- α release ($IC_{50} = 8.1 \mu$ M), followed by Dioscorealide B ($IC_{50} = 22.0 \mu$ M) and Dioscorealide A ($IC_{50} = 33.1 \mu$ M), respectively. For an inhibition on IL-4 release, Dioscoreanone showed the highest inhibitory activity with an IC_{50} value of 6.0 μ M, followed by Dioscorealide A ($IC_{50} = 36.2 \mu$ M) and Dioscorealide B ($IC_{50} = 73.6 \mu$ M). The result indicated that Dioscorealide B showed a stronger effect on the early phase reaction than the late phase one, whereas Dioscorealide A and Dioscoreanone possessed comparable activities in both phases (Tewtrakul and Itharat, 2006). In addition, the inhibitory activity against LPS induced NO production in RAW 264.7 was examined. It was found that Dioscorealide B had a nitric oxide inhibitory activity with IC_{50} of 24.9 μ M, which was stronger than that of NO synthase inhibitor (L-NA, $IC_{50} = 61.8 \mu$ M) (Tewtrakul and Itharat, 2007).

To determine the cytotoxic effect of eight isolated compounds from *D. membranacea*, sulphoramine B (SRB) assay was performed. After 72 hours of incubation, the data revealed that Dioscorealide B selectively inhibits the proliferation of lung cancer cell (CORL-23) ($IC_{50} = 5.3 \mu$ M) and particularly breast cancer cell (MCF-7) ($IC_{50} = 1.7 \mu$ M) without being significantly cytotoxic towards non-malignant cells (SVK-14) ($IC_{50} = 145 \mu$ M) (Itharat, 2002). However, the mechanism of Dioscorealide B against cancer hasn't been elucidated. Thus, it is of great interest to examine the antiproliferative activity and mechanisms of Dioscorealide B of *D. membranacea* which might eventually be useful in the treatment of breast carcinoma.

1.7 Objectives

1. To investigate the cytotoxic effect of Hua Khao Yen extracts and its active compound on human breast cancer cells.
2. To elucidate the molecular mechanism(s) of active compound isolated from Hua Khao Yen in human breast cancer cells.

CHAPTER 2

RESEARCH METHODOLOGY

2.1 Materials

2.1.1 Plant Materials

The parts of plants that were reported to be used against cancer by folk doctors in Thailand were collected from several regions of Thailand from January to March, 2003. Authentication of plant materials was carried out at the herbarium of the Department of Forestry Bangkok, Thailand, where the herbarium vouchers have been kept to specify plant and species identified. Other plants have been kept as specimens in the herbarium of Southern Center of Thai Medicinal Plant at the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand.

2.1.2 Chemicals and reagents

All standard chemicals were obtained from Sigma-Aldrich, USA; PIERCE, USA; Amersham Biosciences, UK; QIAGEN, Germany; BIO-RAD, USA and Promega, USA.

2.1.3 Primers

Primers and conventional PCR conditions for detection of p53, p21, p27, p57, Bax, Bcl-2 and GAPDH transcripts were shown in Table 7.

Table 7 Primers and conventional PCR conditions for detection of p53, p21, p27, p57, Bax, Bcl-2 and GAPDH transcripts

Gene	Sense(S) and antisense (AS) primers	PCR condition	Size (bp)
p53	S: 5'GCTCTGTTTACTGTACCACCATCC 3' AS: 5' CTCTCGGAACATCTCGAAGCG 3'	95°C/1 min, 60°C/1 min and 72°C/1min for 35 cycles	352
p21	S: 5'CTCAGAGGAGGCGCCATG 3' AS: 5'GGGCGGATTAGGGCTTCC 3'	95°C/1 min, 60°C/1 min and 72°C/1min for 35 cycles	500
p27	S: 5' TTGCCCGAGTTCTACTACAGA 3' AS: 5' CGTTTGACGTCTTCTGAGGCC 3'	95°C/1 min, 60°C/1 min and 72°C/1min for 35 cycles	344
p57	S: 5'CGCAGATTTCTTCGCCAAGCG3' AS: 5'GGGACCAGTGACCTTCTCGG3'	95°C/1 min, 60°C/1 min and 72°C/1min for 35 cycles	334
Bax	S: 5' CACCAGCTCTGAGCAGAT 3' AS: 5' CAGCCTTGAGCACCAAGTT 3'	95°C/30 sec, 50°C/30 sec and 72°C/1min for 35 cycles	300
Bcl-2	S: 5' TGTGGCCTTCTTTGAGTTCG 3' AS: 5' TCACTTGTGGCCAGATAGG 3'	95°C/1 min, 60°C/1 min and 72°C/1min for 35 cycles	280
GAPDH	S: 5'GAAGGTGAAGGTCGGAGT 3' AS: 5'GAAGATGGTGATGGGATTTC3'	95°C/1 min, 60°C/45 sec and 72°C/1min for 35 cycles	226

2.1.4 Primary antibodies

All primary antibodies were diluted in Tris-buffered saline with Tween 20 (TBST). The dilution of the Bax-antibody (US biological, USA) was 1:500, of Bcl-2-antibody (Santa Cruz, Santa Cruz) 1:300 and of Bak antibody (Calbiochem) 1: 1000. Caspase-7 antibody (Cell Signaling) was diluted 1:1,000, p21 and p27 Antibody (DAKO) was diluted 1:100. Actin antibody (Sigma-Aldrich) was used in a dilution of 1:1,000.

2.1.5 Secondary antibodies

All secondary antibodies were purchased from Amersham Biosciences, UK. Horse reddish peroxidase (HRP) labeled Anti-mouse IgG and Anti-rabbit IgG for western blot were used in 1:10,000 - 1:50,000 dilutions in TBST.

2.2 Preparation of plant extracts

Parts of these plants were washed with water to remove the remaining sand and to reduce the microbial load. The cleaned plant materials were cut into small pieces and dried at 50 °C, powdered and extracted in a similar way to that practiced by Thai traditional doctors, e.g. water extraction and ethanolic extraction.

2.2.1 Water extracts

To produce the water extract of each plant, dried ground plant materials (250g) were boiled for 30 minutes in distilled water and filtered by filter cloth. Then the filtrate was concentrated and dried by lyophilizer.

2.2.2 Ethanol extracts

For the ethanolic extracts, dried ground plant material (250g) was macerated with 95% ethanol for 3 days, filtered and concentrated to dryness under pressure. The marc was macerated 2 times and dried by evaporator. All extracts of each plant was combined and calculated percentage of yield.

2.3 Isolation of Dioscorealide B

The rhizomes of *D. membranacea* (1 kg) were dried, powdered, and extracted with ethanol, and the ethanol concentrated under reduced pressure to obtain 31 g of ethanolic extract. Crude ethanolic extract (10 g) was then chromatographed over silica gel using chloroform (10 x 100 mL), chloroform/methanol (1:1, 10 x 100 mL), and methanol (10 x 100 mL), respectively. Each fraction was dried and evaporated to yield 2.8, 0.2, and 6.7 g, respectively, these fractions being noted as FA, FB and FC. The chloroform fraction (FA, 2 g), the most active fraction, was then re-chromatographed over silica gel using hexane, chloroform, and methanol gradient as follows; hexane/chloroform 6:4 (1000 mL), 8:2 (1000 mL), 95:5 (1000 mL), 9:1 (500 mL) and 7:3 (500 mL), respectively. The compound structure was elucidated by chemical and spectroscopic means. The spectral data of each compound was compared with those of the reported ones.

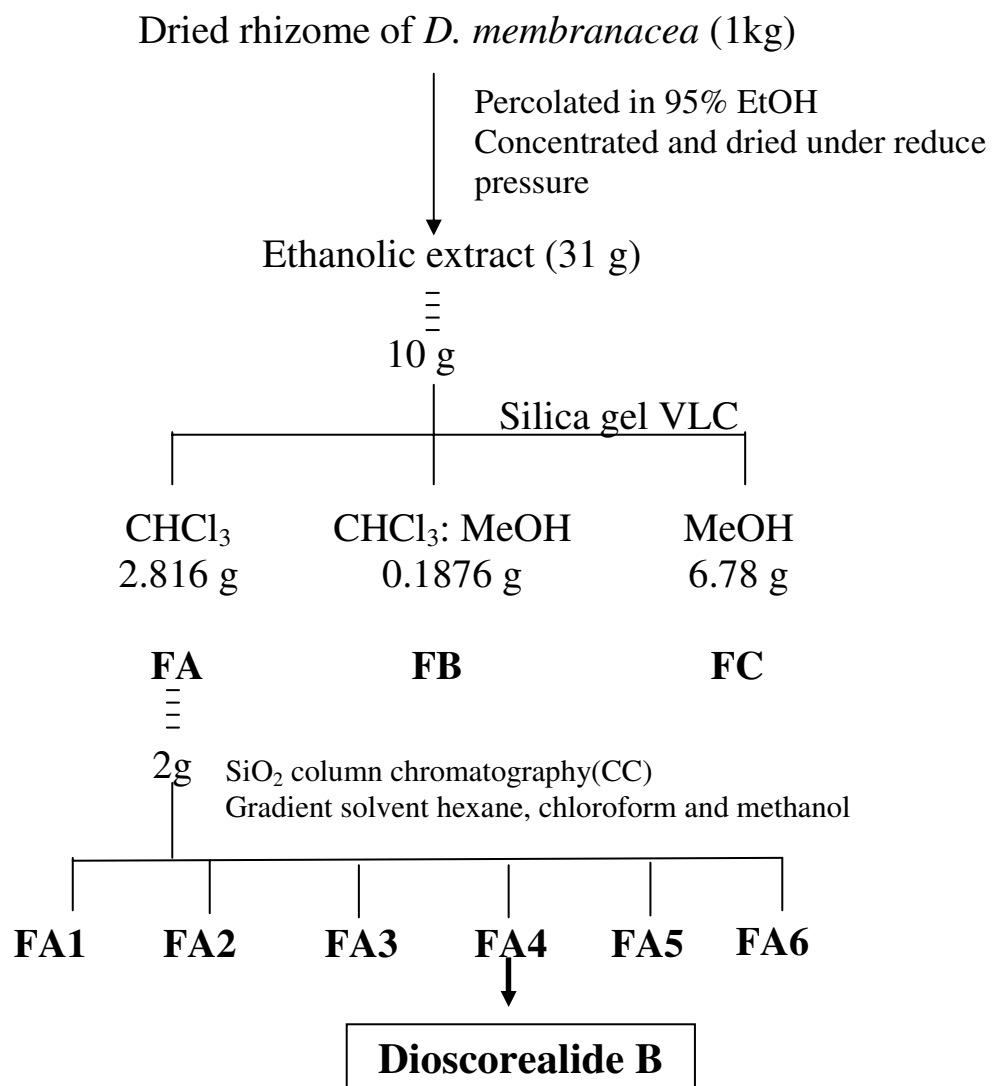


Figure 14 Isolation of Dioscorealide B from the rhizome of *D. membranacea* (Itharat, 2002)

2.4 Approaches to the study of apoptosis

2.4.1 Human cell lines and Cell culture

Cervical cancer cell line, HeLa and two human breast cancer cell lines: MCF-7 (estrogen receptor positive) and MDA-MB 468 (estrogen receptor negative) were used in this study. MCF-7 and HeLa were kindly provided by Dr. Pongpun Siripong Cancer Research Institute of Thailand, Bangkok Thailand and MDA-MB 468 was acquired from the American Type Culture Collection (HTB-132). MCF-7 and MDA-MB 468 cells were grown in DMEM (GIBCO™) containing 10% fetal bovine serum (GIBCO™) with 100,000 U/L of penicillin and 100 mg/L of streptomycin. HeLa cells were cultured in MEM (GIBCO™) containing 10% fetal bovine serum (GIBCO™) with 100,000 U/L of penicillin and 100 mg/L of streptomycin. All cells were maintained in a humidified 37°C incubator with 5% CO₂.

Cancer cells were cultured in T75 flasks and split 1:10 – 1:20 every 3–4 days. This was performed as follows: Growth medium was replaced by 2 ml PBS containing 0.05% trypsin. Afterwards, cells were incubated 2–5 min at 37°C. After detaching the cells, trypsin was inhibited by addition of serum containing growth medium. The cells were centrifuged at 1200 rpm; the cell pellet was re-suspended in fresh growth medium. Then cells were seeded in 6-well plates with a density of 5×10^5 cells/well for Annexin-V assay, RNA and protein extraction, in 24-well plates with a density of 3×10^4 /well for TUNEL assay or in 96-well plates with a density of 3×10^3 and 10^4 cells/well for sulphorhodamine B and caspase activity assay, respectively.

2.4.2 *In vitro* Assay for Cytotoxic Activity

The antiproliferative assay, SRB (sulphorhodamine B) assay, was performed according to the method of Skehan et al (1990). This colorimetric assay estimates cell number indirectly by staining total cellular protein with the dye SRB. The principle of SRB, which is a bright pink aminoxanthene dye, is that it is an anionic protein

stain containing two sulphonic groups, which bind electrostatically to basic amino acid residues of cellular protein under mildly acidic condition. The protein-bound dye is extracted from cells and solubilized for spectrophotometry by weak bases. This colorimetric assay can be used to estimate cell number indirectly only for monolayer by providing a sensitive index of total cellular protein content which is linearly related to cell density (Skehan et al, 1990). This assay was found to give good results over both high and low cell densities (Freshney, 1994).

Sulphorhodamine B (SRB) assay

According to their growth profiles, the optimal plating densities of the cell line MCF-7 and MDA-MB 468 were determined to be 3×10^3 and 5×10^3 cells/well, respectively to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analyzed by SRB assay (Skehan et al, 1990). 100 μ l/well of these cell suspensions were seeded in 96-well microtiter plates and incubated to allow cell attachment. After 24h, the cells were treated with the extracts and pure compounds. Each extract was initially dissolved in a quantity of DMSO (Sigma) for ethanolic extracts, or sterile distilled water for water extracts and vinblastine sulphate (Sigma) used as positive control. The first screening was 50 μ g/ml of each extract, which was tested against all cancer cells, and the results of a percentage of cell survival less than 50 % at an exposure time of 72 hours was considered to be active. According to National Cancer Institute guidelines (Boyd, 1997) extracts with IC_{50} values < 20 μ g /ml were considered active. The active extracts were further diluted in medium to produce the required concentrations. 100 μ l/well of each concentration was added to the plates to obtain final concentrations of 0.5, 1, 2.5, 5, 10, 50, 100 μ g/ml for the active extract, the final mixture used for treating the cell contained not more than 0.1% of the solvent, the same as in solvent control wells. The plates were incubated for selected exposure time of 72 hours. At the end of each exposure time, the medium was removed. The wells were then washed with medium, and 200 μ l of fresh medium were added to each well. The plates were incubated for a recovery period of 72 hours. On the seventh day of the culture period, cells were fixed by 100 μ l of ice-cold 40%

trichloroacetic acid (TCA, Aldrich Chemical) per well, incubated at 4°C for 1 hour in the refrigerator and washed 5 times with tap water to wash non viable cells, so viable cells were fixed as monolayer in each well. 50 µl of SRB solution (0.4% w/v in 1% acetic acid, Sigma) was added to each well and left in contact with the cells for 30 min; then the plates were washed 4 times with 1% acetic acid until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base (tris (hydroxy methyl) aminomethane, pH 10.5) (Sigma) was added to each well to solubilize the dye. The plates were shaken gently for 20 minutes on a gyratory shaker. The absorbance of each well (6 replicate) was read on a Power Wave X plate reader at 492 nm as an indication of cell number. Cell survival was measured as the percentage absorbance compared with the control (non-treated cells). The IC₅₀ values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine.

2.4.3 *In situ* apoptotic cell death detection by TUNEL assay

One of the first biochemical hallmarks indentified for apoptosis is the cleavage of DNA at internucleosomal sites. This specific cleavage leads to the formation of nucleosomal fragments of 180–200 bp lengths, readily observed as a ladder on agarose gels. (Yin et al, 2003)

Terminal deoxynucleotidyl transferase (TdT) mediated dUTP–biotin nick end labeling (TUNEL) detects the DNA fragmentation of apoptotic cells by exploiting the fact that the DNA breaks expose a large number of 3'–hydroxyl ends. Biotinylated nucleotide is incorporated at the 3'–OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, (rTdT) enzyme. Horseradish peroxidase–labeled streptavidin (Streptavidin HRP) is then bound to these biotinylated nucleotides, which are detected using the peroxidase substrate, hydrogen peroxide, and the stable chromogen, diaminobenzidine (DAB). Using this procedure, apoptotic nuclei are stained dark brown.

TUNEL assay

The following assay procedure was modified from Promega's information and the product components of Dead End™ Colorimetric TUNEL System (Cat.# G7360). At the end of each exposure, the medium of each well was moved to centrifuge tubes, one tube for one concentration. The cells were washed with 100 µl of PBS and the substances were put in the previous centrifuge tubes. Then 200 µl of 0.025% trypsin-EDTA were added to each well to make single a cell suspension and the cells were incubated for 10 minutes. The single cell suspensions were removed to the previous centrifuge tubes and then the cells were spun at 1,000 rpm for 10 minutes. Then, the supernatant solutions were removed and the cells were resuspended with PBS.

50 µl of single cell suspensions were topped on the Poly-L-Lysine-coated slides, 25x75x10 mm size (MENZEL-GLASER® , Menzel GmbH&Co KG) and then spun with the Cytospin machine (Cytospin3, SHANDON) after that the slides were allowed to air-dry for 15-30 minutes. Cells were fixed by immersing slides in 10% buffered formalin, 4% paraformaldehyde solution or 10% buffered formalin in PBS in Coplin jar for 25 minutes at room temperature. Then, the slides were washed by immersion in fresh PBS for 5 minutes at room temperature and this step was repeated (after this, slides may be stored in PBS at 4°C or in 70% ethanol at -20°C). Cells were permeabilized by immersing the slides in 0.2% Triton® X-100 solution in PBS for 10-15 minutes at room temperature. Slides were rinsed by immersion in fresh PBS for 5 minutes at room temperature and then repeated. Excess liquid was removed by tapping the slides then the cells were covered with 30 µl of Equilibration Buffer and equilibrated at room temperature for 5-10 minutes. While the cells were equilibrating, the Biotinylated Nucleotide mix was thawed on ice and sufficient rTdT reaction mix prepared for all experimental and control reaction (reaction mix: 98 µl of Equilibration Buffer, 1 µl of Biotinylated Nucleotide and 1 µl of rTdT Enzyme) and kept it on ice. The equilibrated areas were blotted around with tissue paper and 30 µl of rTdT reaction mix added to the cells on slide (the cells were not allow to dry). The cells were covered with Plastic Coverslips to ensure even distribution of the reagent and incubated at 37°C for 60 minutes inside a humidified chamber to allow the end-labeling reaction to occur. Then, the Plastic Coverslips were removed and the reactions terminated by immersing the slides in 2X SSC

(1:10 with deionized water) in a Coplin jar for 15 minutes at room temperature. The slides were washed by immersion in fresh PBS for 5 minutes at room temperature (this wash was repeated twice to remove unincorporated biotinylated nucleotides). Then, the endogenous peroxidases were blocked by immersing the slides in 0.3% hydrogen peroxide for 5 minutes at room temperature and the slides washed by immersion in PBS for 5 minutes at room temperature (repeated twice). Thirty μl of the Streptavidin HRP solution 1:500 in PBS was added to each slide and incubated for 30 minutes at room temperature. The slides were washed by immersion in PBS for 5 minutes at room temperature (repeated twice). Thirty μl of DAB solution, the mixture of 50 μl of DAB Substrate 20X buffer, 50 μl of the DAB 20X Chromogen and 50 μl of Hydrogen Peroxide 20X in 950 μl of deionized water, were added to each slide and developed until there was a light brown background (10–15 minutes, the background was not allowed to become too dark). The slides were rinsed several times in deionized water and the cells dyed by immersing the slides in methyl green solution for 5 minutes then rinsing the slides several times in deionized water. The slides were dehydrated by immersing in 70%, 95%, 100% alcohol and xylene, respectively (each immersion twice times for 5 minutes). The slides were mounted in a permanent mounting medium.

2.4.4 Quantification of Apoptotic Cells by Flow Cytometry

Heretofore, scoring of the characteristic changes in nuclear morphology, such as chromatin condensation and fragmentation, is often used to identify apoptotic cells both in vitro and in vivo. A more refined detection system is the TUNEL assay (Wijsman et al, 1993), based on the incorporation of hapten-labeled nucleotides at the sites of nicked DNA. However, the technique is rather cumbersome and does not allow for discrimination between apoptotic and necrotic cells.

A novel apoptosis detection assay has been described based on the measurement of the loss of plasma membrane asymmetry (van Engeland et al, 1998). Under normal physiological conditions, a cell maintains a strictly asymmetric distribution of phospholipids in the two leaflets of the cellular membranes with phosphatidylserine (PS) facing the cytosolic side (Devaux, 1991, Connor et al, 1992, Higgens, 1994). However, during early apoptosis this membrane asymmetry is rapidly lost without concomitant loss of

membrane integrity (Koopman et al, 1994; Homburg et al, 1995; van Engeland et al, 1996, 1997, 1998). This results in the exposure of PS at the outer leaflet of the plasma membrane, which serves a physiological role in the recognition and subsequent removal of the dying cell by means of phagocytosis (Fadok et al, 1992; Savill et al, 1993). This phenomenon can be detected by hapten labeled annexin V, which shows high affinity for PS residues in the presence of millimolar concentrations of Ca^{2+} (Andree et al, 1990). By simultaneous probing of membrane integrity by means of exclusion of the nuclear dye propidium iodide (PI), apoptotic cells can be discriminated from necrotic cells. These cells become annexin V-positive because of its binding to PS at the cytoplasmic side of the plasma membrane. Although the method was initially developed for the measurement of apoptosis in blood cells, van Engeland et al (1997) showed that the method is also applicable to measure apoptosis in adherent tumor cell cultures.

Annexin-V-FITC staining

Induction of apoptosis by Dioscorealide B treatment was measured by Annexin V-FITC apoptosis detection kit (BD Pharmingen) as described by the manufacturer's instruction. MCF-7 cells were seeded in 6-well plates and treated with Dioscorealide B at 3, 6 and 12 μM . After 24 h, cells were collected, washed with cold PBS twice, and gently resuspended in 100 μl of staining solution (containing annexin V fluorescein and propidium iodide in a HEPES buffer). After incubation at room temperature for 15 min, cells were analyzed by flow cytometry. Apoptotic and necrotic cells were determined using a FACScanTM flow cytometer (BD Biosciences). Annexin V FITC fluorescence was excited at a wavelength of 488 nm and emission was detected using a 530 \pm 40 nm bandpass filter. Propidium iodide fluorescence was excited at a wavelength of 488 nm and emission was detected using a 680 \pm 30 nm bandpass filter. To exclude cell debris and doublets, cells were appropriately gated by forward versus side scatter and pulse width, and 1 x 10⁴ gated events per sample were collected. Annexin V binds to those cells that express phosphatidylserine on the outer layer of the cell membrane, and propidium iodide stains the cellular DNA of those cells with a compromised cell membrane. This allows for the discrimination of live cells (unstained with either fluorochrome) from

apoptotic cells (stained only with annexin V) and necrotic cells (stained with both annexin V and propidium iodide)

2.4.5 Measurement of Caspase-7, -8 and -9 Activity

Activation of caspases is arguably the biochemical hallmark of apoptosis. There are several ways to detect caspase activation. Caspases are a family of cysteine proteases that cleave their substrates after aspartate residues. Existing as zymogens (also called procaspases) within un-stimulated cells, caspases are activated through the cleavage of the zymogen to form the large and small subunits, which in turn form a heterotetramer complex.

In this study, Caspase-Glo[®] 3/7, -8 and -9 assays (Promega) were used to measure caspase-7, caspase-8 and caspase-9 activities. The assay provides a luminogenic caspase-3/7, -8 or -9 substrate, which contains the tetrapeptide sequence DEVD, LETD or LEHD, in a buffer system optimized for caspase activity, luciferase activity and cell lysis. Adding a single Caspase-Glo[®] Reagent in an “add-mix-measure” format results in cell lysis, followed by caspase cleavage of the substrate and generation of a luminescent signal, produced by luciferase (Figure 15)

Caspase-activity assay

10^4 cells of MCF-7 were cultured in 96-well plates and treated with Dioscorealide B (3, 6 or 12 μ M). After the periodic incubation, 100 μ l of Caspase-Glo[®] Reagent was added to each well of a white-walled 96-well plate containing 100 μ l of blank, negative control cells or treated cells in culture medium. Because of the sensitivity of this assay, care was taken not to touch pipet tips to the wells containing samples so as to avoid cross contamination. The plate was covered with a plate sealer or lid. The contents of the wells were gently mixed using a plate shaker at 300-500 rpm for 30 seconds and were then incubated at room temperature for 1 hour. After that, the luminescence of each sample was measured in a plate-reading luminometer. To inhibit the caspase-8 and -9 activity, MCF-7 cells were pretreated with 50 μ M of the caspase-8 inhibitor Z-IETD-FMK or the

caspase-9 inhibitor Z-LEHD-FMK for 3 hours prior to treatment with 3 μ M of Dioscorealide B. The assay was performed in triplicate.

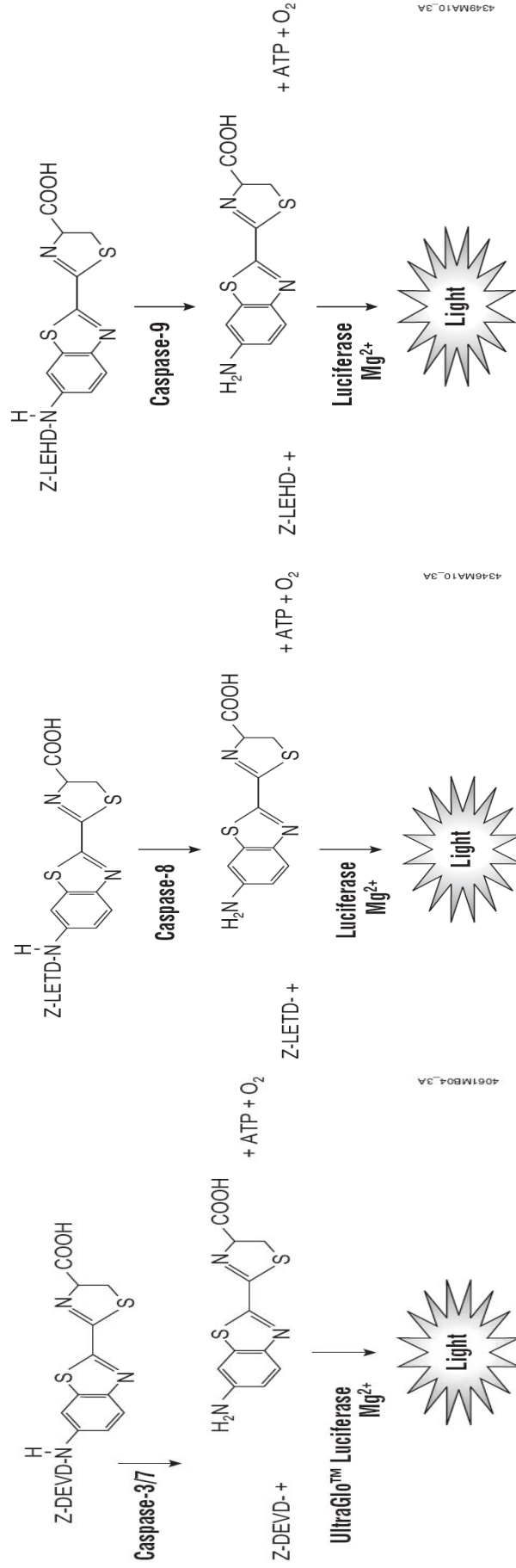


Figure 15 Caspase-3/7, -8 and -9 cleavage of the luminogenic substrate containing the DEVD, LETD and LHED sequence, respectively. Following caspase cleavage, a substrate for luciferase (amino-luciferin) is released, resulting in the luciferase reaction and the production of light.

2.4.6 RNA analysis

2.4.6.1 RNA sample preparation

For RT-PCR analysis, MCF-7 cells were harvested and then centrifuged at 1200 rpm, 4°C for 10 minutes and washed once in PBS. At least 4 wells per condition were pooled. The pellet was washed once in PBS. Briefly, samples were dissolved in TRIZOL[®] Reagent by repetitive pipetting ($5-10 \times 10^6$ cells/ml) and incubated for 5 minutes at 15 to 30°C to permit the complete dissociation of nucleoprotein complexes. Then 200 μ l of chloroform were added into each sample. Sample tubes were shaken vigorously by hand for 15 seconds and incubated at 15 to 30°C for 2 to 3 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes at 2 to 8°C. Following centrifugation, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remained exclusively in the aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA from the aqueous phase was precipitated by mixing with 500 μ l isopropyl alcohol. Samples were then incubated at 15 to 30°C for 10 minutes and centrifuged at 12,000 rpm for 10 minutes at 2 to 8°C. The supernatants were removed. After that, the RNA pellet was washed once with 75% ethanol and centrifuged at 7,500 rpm for 5 minutes at 2 to 8°C. The RNA pellet was dried. Finally, RNA was dissolved in RNase-free water and incubated for 10 minutes at 55 to 60°C. The RNA samples were stored at -70°C until they were required.

2.4.6.2 Determination of RNA amount

To determine the RNA amount in the samples, 4 μ l were diluted in 156 μ l RNase-free water. The RNA amount was determined by UV at a wavelength of 260 nm. The reference wavelength was 280 nm. OD of 1 at 260 nm represents a concentration of 40 μ g/ml RNA.

2.4.6.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR was performed using a Qiagen OneStep RT-PCR kit. 0.25 µg of total RNA was subjected to one-step RT-PCR in 25 µl reaction volume containing 2.5 µl 5x Qiagen OneStep RT-PCR buffer (Tris-Cl, KCl, (NH₄)₂SO₄, 12.5 MgCl₂, DTT; pH 8.7), 0.5 µl of 10 mmol/l deoxynucleoside triphosphate (dNTP), 0.5 µl of Qiagen OneStep RT-PCR Enzyme Mix (OmniscriptTM Reverse Transcriptase, SensiscriptTM Reverse Transcriptase and HotStarTaq[®] DNA polymerase), 0.75 µl of 10 µmol/L each primer, 0.25 µl of Rnase inhibitor and RNase-free water to 25 µl. The reverse transcription step was initiated at 50°C for 30 min, followed by PCR activation at 95°C for 15 min. The primer sequences and PCR conditions used in these experiments are shown in Table 5.

2.4.6.4 Agarose gel electrophoresis

Analysis of PCR amplification products was performed by fluorescence detection after agarose gel electrophoresis. To this end, 10 µl of each sample were mixed with bromophenol blue containing loading buffer 6x (2.4 ml EDTA solution 0.5 M, 12 ml glycerin, 5.6 ml aqua dest. and 0.04 g bromophenol blue) and were loaded onto a 1.5% agarose gel containing ethidiumbromide. The gel was prepared by dissolving 0.6 g agarose in 40 ml TBE buffer (10.8 g trizma base, 5.5 g boric acid, 0.75 g disodium EDTA), boiling everything up to 100°C and adding 40 µl of a 0.5 mg/ml ethidiumbromide solution after cooling down to about 70°C. The electrophoresis was performed for 70 minutes at 80 V. 10 µl of 100 bp DNA-ladder (NEB) were used as a size marker. PCR amplification products were detected under UV light (excitation 260 nm).

2.4.7 Protein analysis

2.4.7.1 Protein sample preparation

For protein extraction and subsequent analysis, MCF-7 cells were grown at a density of 5×10^5 cells per well in 6-well plates. MCF-7 cells were harvested and then centrifuged at 1200 rpm, 4°C for 10 minutes and washed once in PBS. At least 4 wells per condition were pooled. The pellet was washed once in PBS and lysed with 125 µl of CelLytic M (Sigma Aldrich). The cells were incubated for 15 minutes on a shaker. To pellet the cellular debris, the lysed cells were then centrifuged for 15 minutes at 12,000 rpm. Finally, the protein-containing supernatant was placed in a chilled test tube. The protein samples were stored at -70°C until they were required.

2.4.7.2 Determination of protein amount

Protein amounts in extracts were determined by Lowry's method. Protein standard BSA was diluted in distilled water to concentrations of 0.2, 0.5, 0.8, 1.2 and 1.5 mg/ml. A volume of 100 µl of either protein standard or protein samples was mixed with 4.5 ml of Lowry's reagent (Bio-Rad). The absorbance at 750 nm was determined from protein standard. The concentration of protein sample was calculated and protein amounts of the test samples were calculated from the standard curve.

2.4.7.3 Immunoblotting (Graidist et al, 2004)

The protein samples were subjected to electrophoresis on a 12 % SDS-polyacrylamide gel (Appendix A) and then transferred to a PVDF membrane. The membrane was blocked by blocking buffer (5% skim milk in TBS-T (0.1% Tween 20, 154mM NaCl, 48 mM Tris-base, pH 6.8) for 1 h and washed two times for 5 min each time with 1% skim milk in TBS-T buffer. The membrane was then incubated with primary antibodies specific to Bax (1:500), Bak (1:1000), Bcl-2 (1:300), caspase-7

(1:1000), p21 (1:100), p27 (1:100) and actin (1:1000). The blot was washed three times for 5 min each time with 1% skim milk in TBS-T buffer. The membrane was then incubated with polyclonal IgG-anti-mouse antibody for Bax, Bcl-2, Bak, p21 and p27 and with polyclonal IgG-anti-rabbit antibody for caspase-7 and actin for 1 h and, then washed three times for 10 min each time with TBS-T buffer. Bound antibodies were detected with SuperSignal[®] West Pico Chemiluminescent Substrate kit (Pierce). The excess detection reagent was drained off with 3M paper. Then the membrane was placed in a film cassette containing autoradiography film (Hyperfilm, Amersham), and exposed for 5 min. The X-ray film was developed using a developing machine

2.5 Statistical analysis

Data were expressed as means \pm SEM. Statistical comparisons of the results were made using analysis of variance (ANOVA) and a P value of less than 0.05 was considered significant.

CHAPTER 3

RESULTS

3.1 Screening of cytotoxic activity of crude extracts

The cytotoxic effect of five species of Hua Khao Yen was evaluated by the Sulphorhodamine B (SRB) assay. The results of cytotoxic activity were expressed as a concentration required for inhibiting cell growth by 50% (IC_{50} value) in Tables 6. The American NCI defined plants which give the extracts with the IC_{50} values of ≤ 20 $\mu\text{g/ml}$ and ≤ 4 $\mu\text{g/ml}$ for pure compounds as the plants with cytotoxic activity. (Boyde, 1997)

The data revealed that the water extract of *Dioscorea membranacea* had high cytotoxic activity against two type of breast cancer (MCF-7 and MDA-MB 468 were IC_{50} =5.5 and 26.9 $\mu\text{g/ml}$, respectively) but it had no effect against cervical cancer cell (HeLa). The ethanolic extract of *D. membranacea* showed cytotoxic activity against HeLa, MCF-7 and MDA-MB 468 cells (IC_{50} = 29.2, 5.5 and 15.0 $\mu\text{g/ml}$, respectively) as well as *Dioscorea birmanica* (IC_{50} = 34.2, 16.3 and 27.2 $\mu\text{g/ml}$, respectively). Besides, the result showed that the water and ethanolic extracts of the other species exhibited no cytotoxic activity against HeLa and MCF-7 and the ethanolic extract of *Smilax corbularia* and *S. glabras* had a cytotoxic effect only on MDA-MB 468.

Table 8 Cytotoxicity activity (IC_{50} $\mu\text{g/ml} \pm \text{SEM}$) of Hua Khao Yen extracts against cancer cell lines at exposure time 72 hours (n=3)

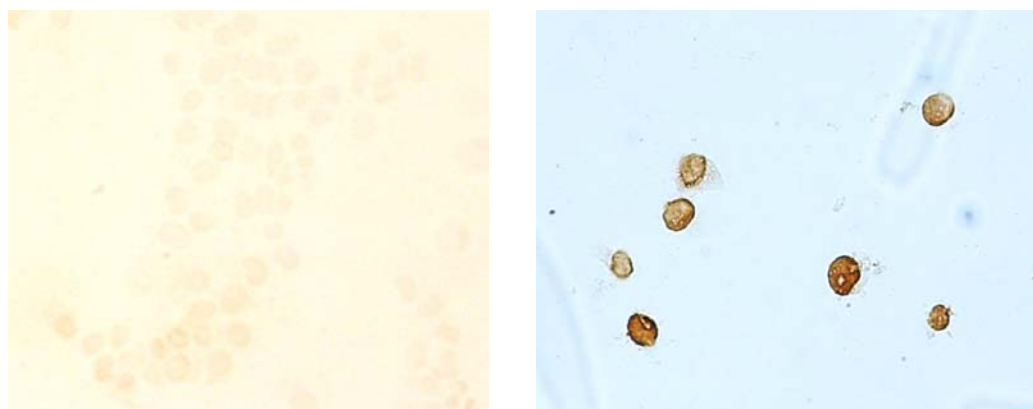
Plant Species	Extracts	Cell line		
		MCF-7	MDA-MB 468	HeLa
<i>Dioscorea membranacea</i> Pierre	EtOH	22.0 ± 0.2	55.0 ± 0.8	29.2 ± 0.2
	Water	5.5 ± 0.6	26.9 ± 0.8	50
<i>Dioscorea bimmanica</i>	EtOH	6.3 ± 0.8	27.2 ± 0.2	34.2 ± 3.0
	Water	50	50	50
<i>Smilax corbularia</i>	EtOH	50	9.8 ± 4.0	50
	Water	50	50	50
<i>Smilax glabra</i>	EtOH	50	30.7 ± 0.8	50
	Water	50	50	50
<i>Pygmeopremna herbacea</i>	EtOH	50	50	50
	Water	50	100	50

3.2 Induction of apoptosis by crude extracts of Hua Khao Yen

Apoptosis is characterized by prominent morphological features such as membrane blebbing, nuclear condensation and DNA fragmentation. Hence, TUNEL assay was performed to determine DNA fragmentation induced by the crude extract of Hua Khao Yen. Apoptotic nuclei are stained dark brown (Figure 06).

The water extract and ethanolic extract of *Dioscorea membranacea* and the ethanolic extract of *Dioscorea birmanica* exhibited the induction of apoptosis in HeLa and MCF-7 cells in a dose-dependent manner as shown in Figure 07 and 08. After HeLa cells were exposed to 0 and 50 µg/ml of the ethanolic extract of *D. birmanica*, the number of apoptotic cells increased to 8.48 and 8.89 folds compared to the control cells, respectively, and increased to 4.3 and 4.87 folds after treatment with 0 and 50 µg/ml of the ethanolic extract of *D. membranacea*, respectively.

In MCF-7, the ethanolic extract of *D. birmanica* significantly increased the number of apoptotic cells to 2.59 and 9.07 folds at a concentration of 0 and 50 µg/ml, respectively. After incubation with 0 and 50 µg/ml of the water extract of *D. membranacea*, the apoptotic cells significantly increased to 5.39 and 24.88 folds, respectively while the ethanolic extract of this plant showed 8 and 22.77-folded increases in the number of apoptotic cells after treatment with 0 and 50 µg/ml, respectively.



Control cells

Treated cells

Figure 16 The picture showed induction of apoptosis in MCF7 after treated with ethanolic extract of *Dioscorea membranacea*. The apoptotic cells showed brown-staining nuclei. (left picture, x200; right picture, x1000)

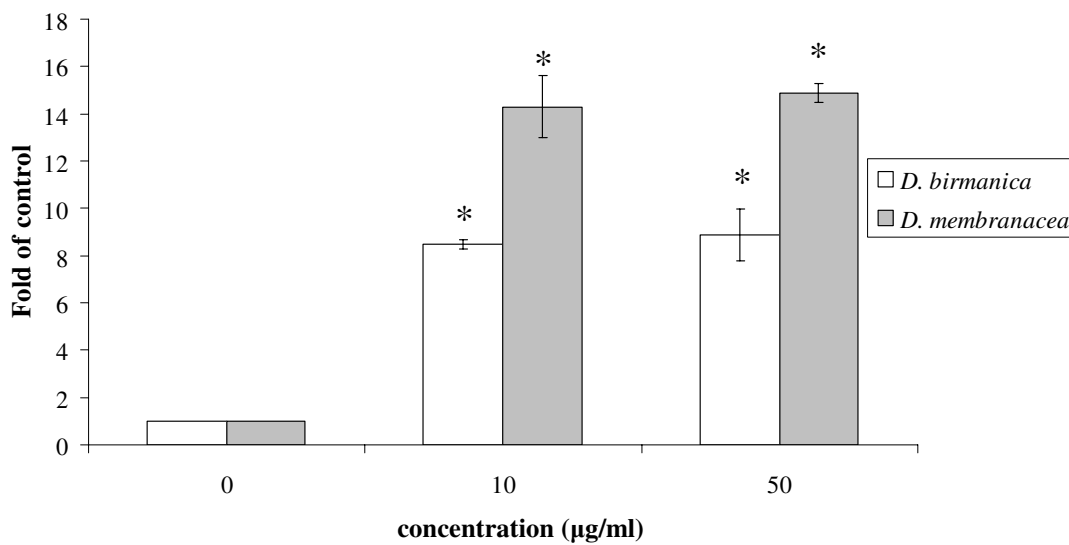


Figure 17 The fold of apoptotic cells was determined from three experiments (mean \pm SEM). HeLa cells were treated with the ethanolic extract of *Dioscorea birmanica* and *D. membranacea* or vehicle (control) for 72 h. The induction of apoptosis was assessed by TUNEL assay. Statistical analysis on log transformed data; asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

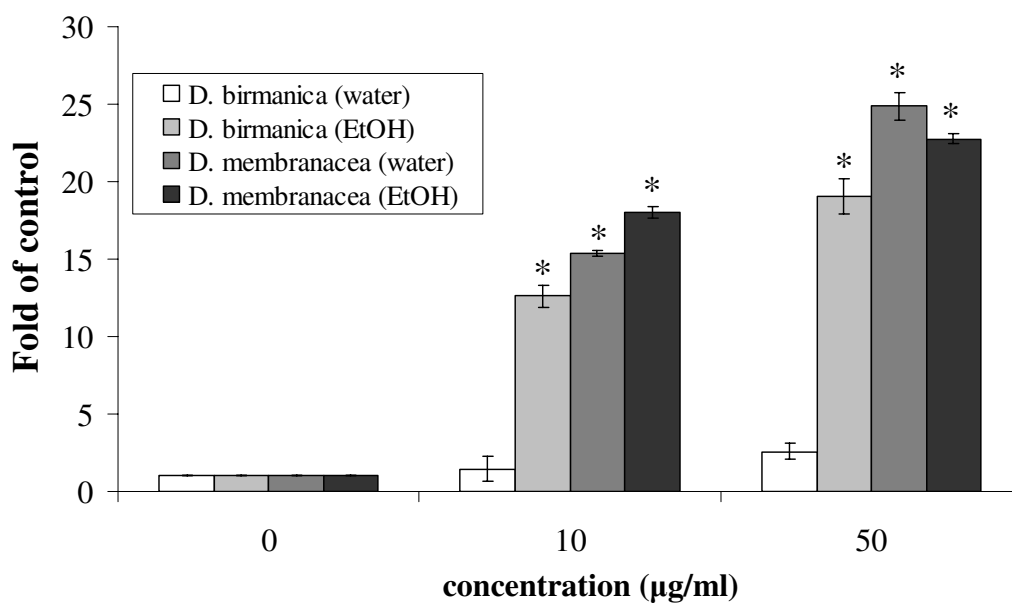


Figure 18 The fold of apoptotic cells was determined from three experiments (mean \pm SEM). MCF-7 cells were treated with the water and ethanolic extracts of *Dioscorea birmanica* and *D. membranacea* or vehicle (control) for 72 h. The induction of apoptosis was assessed by TUNEL assay. Statistical analysis on log transformed data; asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

3.3 Cytotoxic effect of Dioscorealide B on human breast cancer cells

Dioscorealide B is a pharmacologically active compound from the ethanol extract of *Dioscorea membranacea*. Therefore, in this study, the cytotoxic effect of Dioscorealide B on two human breast cancer cell lines: MCF-7 and MDA-Mb 468 was determined by SRB assay. Cells were treated at different concentrations of Dioscorealide B (0–50 μM) for 72 hr, the proportions of surviving cells were then estimated and IC_{50} values (concentrations leading to 50% inhibition of viability) were calculated (Table 7).

The data demonstrated that Dioscorealide B had a cytotoxic effect on MCF-7 with the IC_{50} value of 2.82 μM and showed less cytotoxic activity in MDA-MB 468 with IC_{50} value of 9.93 μM . In addition, the viability of MCF-7 cells showed a significant decrease after exposure of Dioscorealide B (3, 6 and 12 μM) for the indicated times. These results indicated that Dioscorealide B-induced growth inhibition presented with dose- and time-dependent manner on MCF-7 cells as shown in Figure 19 and 20.

Table 9 Cytotoxicity of Dioscorealide B (IC_{50} (μM) \pm SEM) against breast cancer cell lines, MCF-7 and MDA-MB 468

compound	IC_{50} (μM) \pm SEM	
	MCF-7	MDA-MB 468
Dioscorealide B	2.82 ± 0.36	9.93 ± 0.79

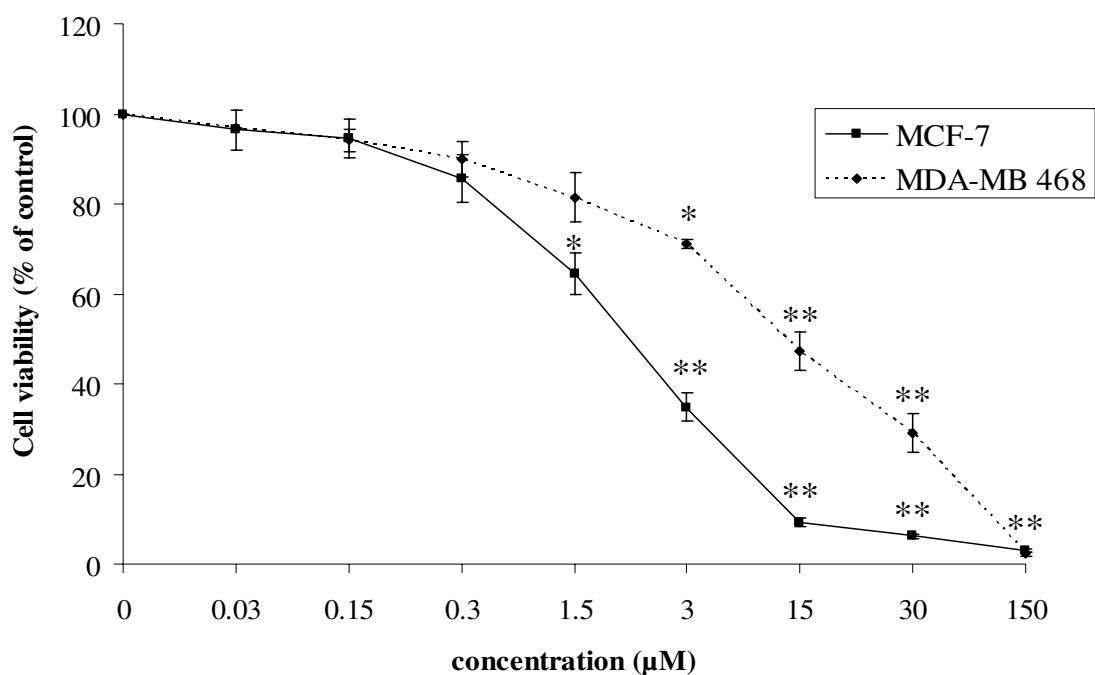


Figure 19 The antiproliferative effect of Dioscorealide B on MCF-7 and MDA-MB 468. MCF-7 and MDA-MB 46 were incubated with various concentrations of Dioscorealide B. After 72 hours, cell proliferation was determined by SRB assay. Results are expressed as a percentage of viable cell in the studied group as compared with those in the control group. (* $p < 0.05$ and ** $p < 0.01$)

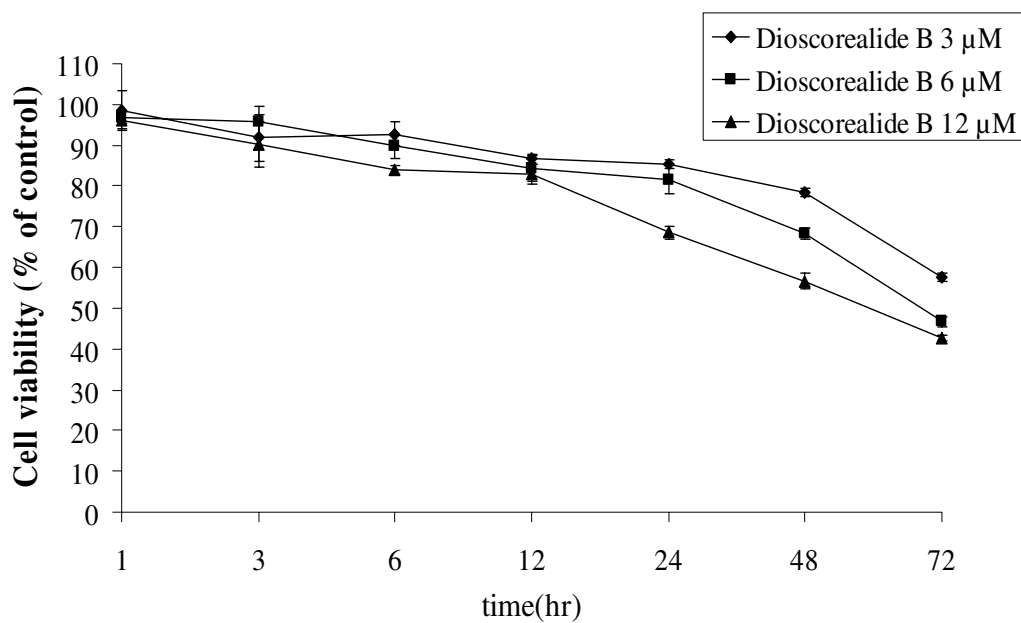


Figure 20 The effect of Dioscorealide B against MCF-7 cells. The cells were plated in 96-well plates (10^4 cells/well) prior to incubation with 3, 6 and 12 μ M Dioscorealide B at different time points. Cell proliferation was then determined by SRB assay. Results are expressed as a percentage of viable cell in the studied group as compared with those in the control group.

3.4 Induction of apoptosis in MCF-7 cells by Dioscorealide B

To examine whether the antitumorigenic property against breast cancer cells is mediated through apoptosis induction after Dioscorealide B treatment. MCF-7 cells were stained with Annexin V-FITC and PI, and then subsequently analyzed by flow cytometry. This assay is based on the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the cell surface in the early apoptotic cells. MCF-7 cells were challenged with Dioscorealide B at 3, 6 and 12 μM for 24 h. The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (annexin V⁻PI⁻), the cells at the early apoptosis are in the lower right quadrant (annexin V⁺PI⁻), and the ones at the late apoptosis are in the upper right quadrant (annexin V⁺I⁺). The apoptotic cells were counted as late apoptotic cells (UR quadrant) and early apoptotic cells (LR quadrant), and represented as fold of control (Figure 20B). As shown in Figure 20, the result revealed that Dioscorealide B significantly induced the apoptosis in a dose-dependent manner compared with control cells. The number of apoptotic cells in MCF-7 increased to 7.02, 11.87 and 12.17 folds after 3, 6 and 12 μM Dioscorealide B treatment, respectively.

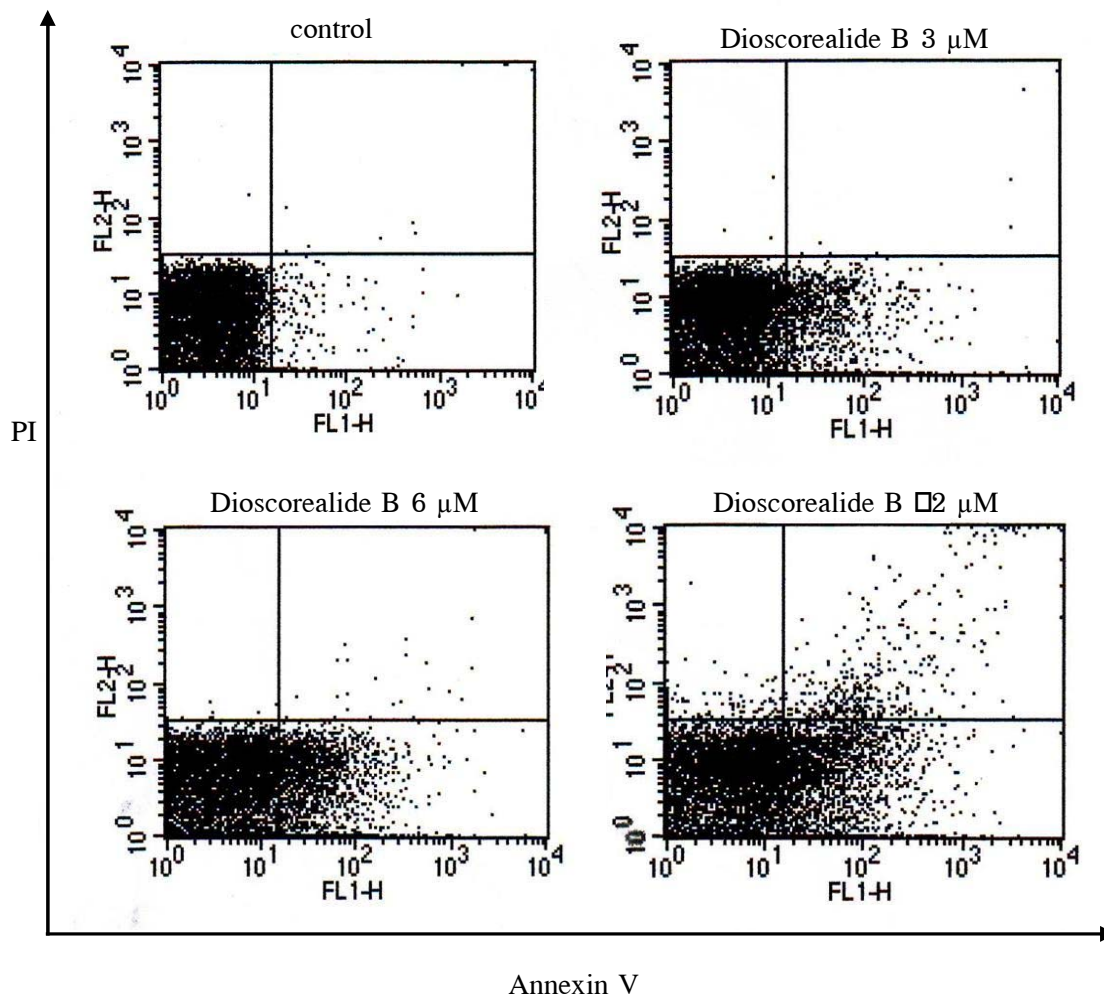


Figure 21 A. Representative dot plots after 24 hours of Dioscorealide B exposure. Dioscorealide B-induced apoptosis in MCF-7 cells assessed by annexin-V fluorescein isothiocyanate and propidium iodide double staining (An/PI). Apoptotic cells were detected as An⁺/PI⁻ cells, while viable cells were An⁻/PI⁻ cells and necrotic cells were PI⁺.

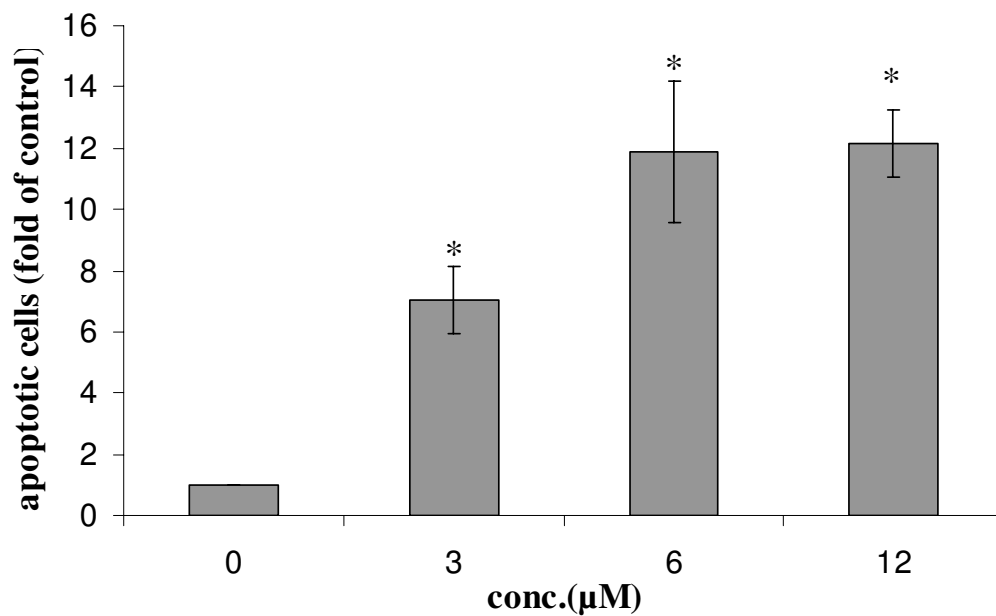


Figure 21 B. The fold of apoptotic cells was determined from three experiments (mean \pm SEM). MCF-7 cells were treated with Dioscorealide B or vehicle (control) for 24 h. The induction of apoptosis was assessed by annexin-V fluorescein isothiocyanate and propidium iodide double staining (An/PI). Statistical analysis on log transformed data; asterisks indicate ANOVA $p < 0.05$ versus corresponding control.

3.5 Dioscorealide B induce cell death via caspase cascade in MCF-7

Caspases are aspartate-specific cysteine proteases which play crucial roles in execution of apoptosis program. Activation of caspases results in cleavage and inactivation of key cellular proteins. Currently, there are two known pathways that activate the caspase cascade, the intrinsic and extrinsic pathways. Following the initial activation of caspase-9 and -8 in the intrinsic and extrinsic pathways, respectively, the activation of caspase-7, an effector caspase can be observed (Salvesen et al, 1997). Therefore, we examined whether these caspases are involved in Dioscorealide B-induced apoptosis.

Immunoblot analyses of lysates obtained from MCF-7 cells treated with Dioscorealide B at 3 μ M were examined. The data exhibited that cleaved form of caspase-7 was increased after 3 hours and the procaspase-7 was totally cleaved at 24 hours of treatment respectively (Figure 22). Caspase-7 activity was further measured by caspase-7 assay. The result showed that Dioscorealide B increased caspase-7 activity in a dose- and time dependent manner, as demonstrated in Figure 23 and 24. After treatment with 3 μ M of Dioscorealide B, the caspase-7 activity was significantly increased to 230.7% at 6 hours. These results suggested that this compound induced the activation of caspase-7.

Next, the effect of Dioscorealide B on caspase-8 and caspase-9 activities were determined (Figure 25). The data revealed that activation of caspase-9 was significantly increased after treating the cells with Dioscorealide B starting from 3 hour of exposure time; however, caspase-8 activity showed a significant elevation after 1 hour of treatment. For further definition, the role of caspase-8 and -9 in Dioscorealide B-induced apoptosis, MCF-7 cells were pretreated with 50 μ M of the caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK for 3 h prior to treatment with 3 μ M of Dioscorealide B. As shown in Figure 26, pretreatment of MCF-7 cells with either caspase-8 inhibitor or caspase-9 inhibitor significantly decreased the caspase-7 activity. These results suggested that this bioactive compound might be involved in both intrinsic and extrinsic apoptotic pathways.

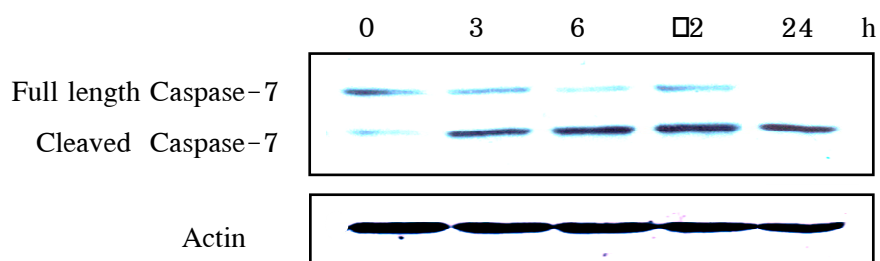


Figure 22 Immunoblotting for caspase-7 using lysates from MCF-7 cells treated with 3 μ M of Dioscorealide B for the indicated time periods. The blots were stripped and reprobbed with anti-actin antibody to normalize for differences in protein loading.

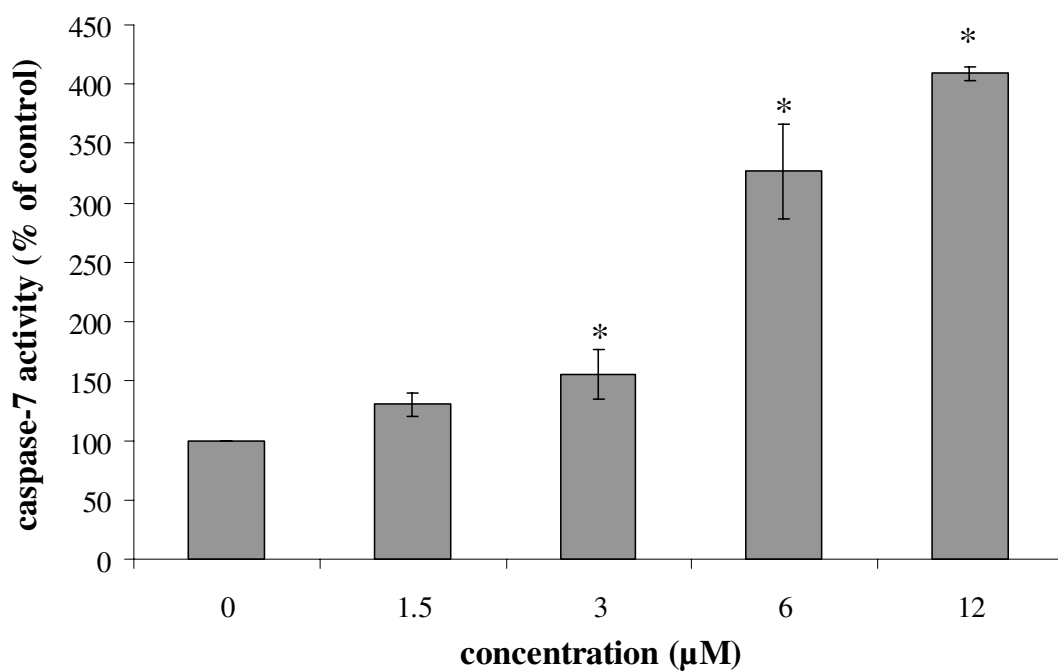


Figure 23 The effect of Dioscorealide B on caspase-7 activity in MCF-7 breast carcinomas. MCF-7 cells were treated with 0.5, 3, 6 and 12 µM of Dioscorealide B for 3 hr and induction of caspase-7 activity was measured. The data represented the average value of 3 replications from 2 independent experiments.

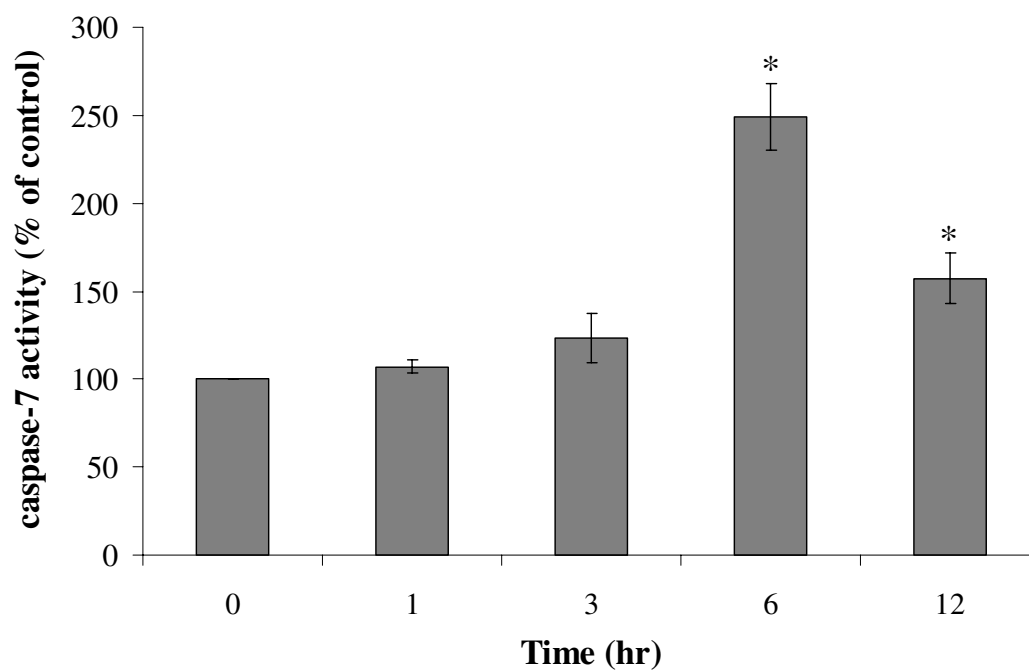


Figure 24 The activation of caspase-7 in MCF-7 by Dioscorealide B. MCF-7 cells were incubated with 3 μ M of Dioscorealide B for the indicated times. The data represented the average value of 3 replications from 2 independent experiments. Asterisks indicate ANOVA $p < 0.05$ versus the corresponding control cells.

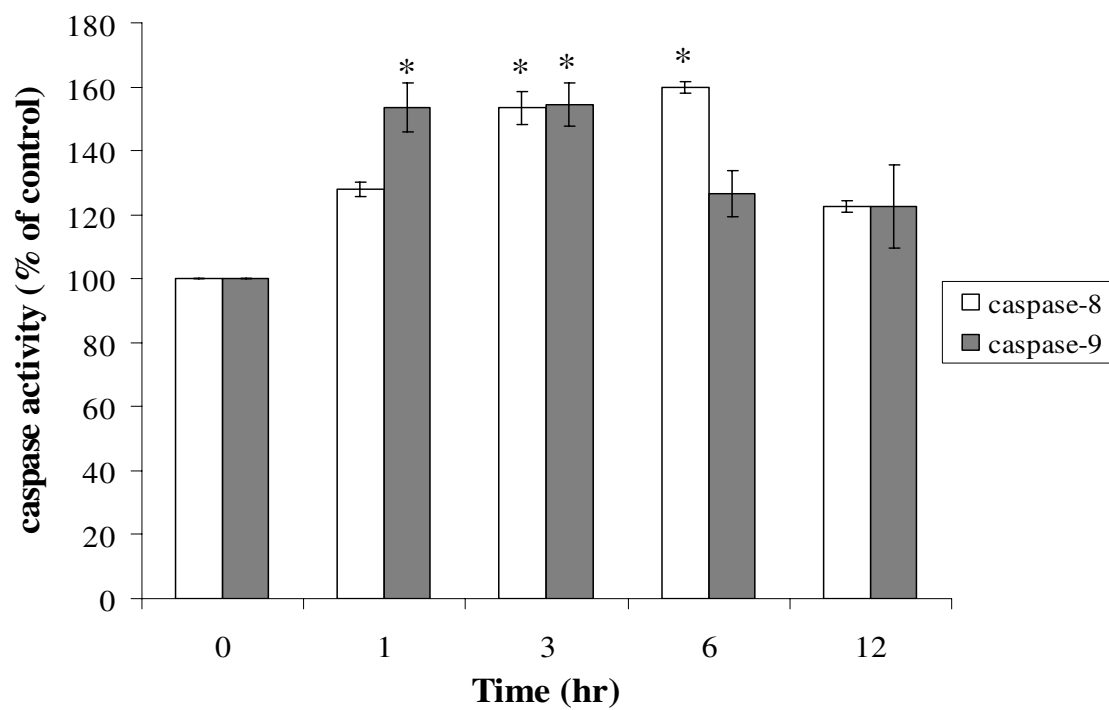


Figure 25 The activation of caspase-8 and -9 in MCF-7 by Dioscorealide B. MCF-7 cells were incubated with 3 μ M of Dioscorealide B at indicated time. Asterisks indicate ANOVA $p < 0.05$ versus the corresponding control cells.

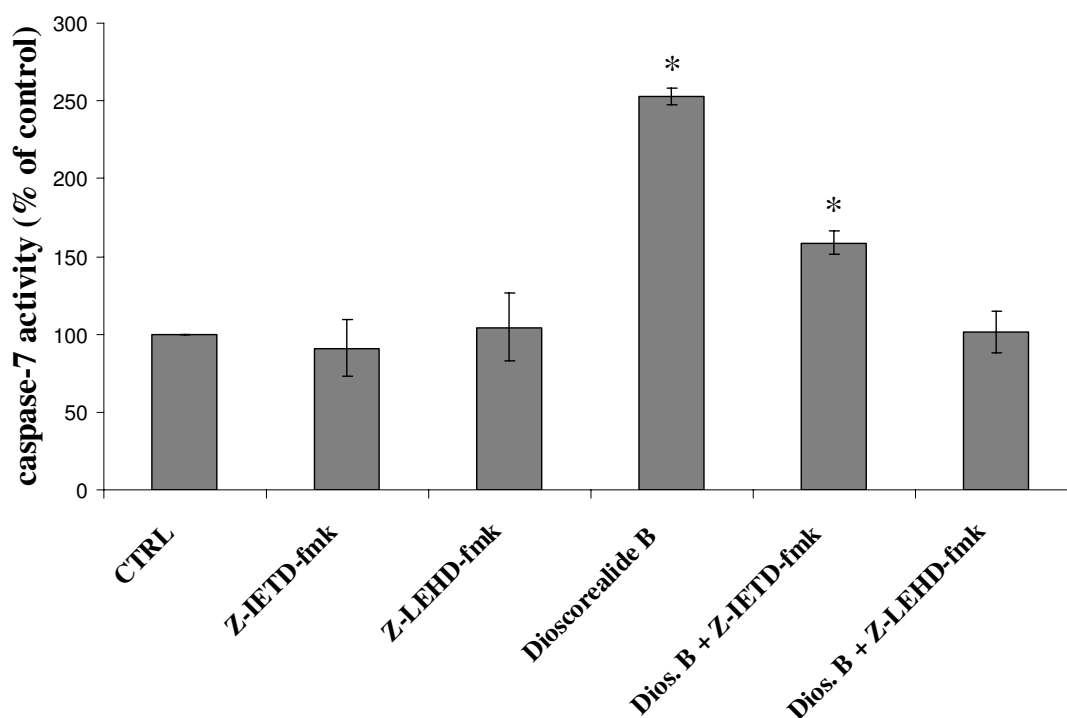


Figure 26 The effect of caspase-8 and -9 inhibitor on Dioscorealide B-induced apoptosis. MCF-7 cells were pre-incubated with 50 μ M of caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK for 3 h before challenge with 3 μ M Dioscorealide B. After 6 h of treatment, caspase-7 activity was measured by Caspase-Glo[®] assay. Each value is the mean \pm SD of three determinations. The asterisk indicates a significant difference between control and Dioscorealide B-treated cells, as analyzed by ANOVA $p < 0.05$.

3.6 Dioscorealide B up-regulate p53 and Bax expression and down-regulate bcl-2 expression in mRNA level

For evaluating the molecular pathway of apoptosis induced by Dioscorealide B, p53, Bax and bcl-2 were determined by RT-PCR. After the exposure of Dioscorealide B for \square hour, p53 and Bax showed an increase in their expressions, while bcl-2 expression was down-regulated in a time-dependent manner. In addition, we investigated the involvement of cell cycle arrest in the mechanism of this active compound via the expression of cyclin-kinase inhibitors genes: *p27*, *p57* as well as *p21* which is one of p53 target gene. The results demonstrated that the expression of *p21*, *p27* and *p57* were upregulated suggesting that the mechanism of Dioscorealide B might be also involved in cell cycle arrest (Figure 27).

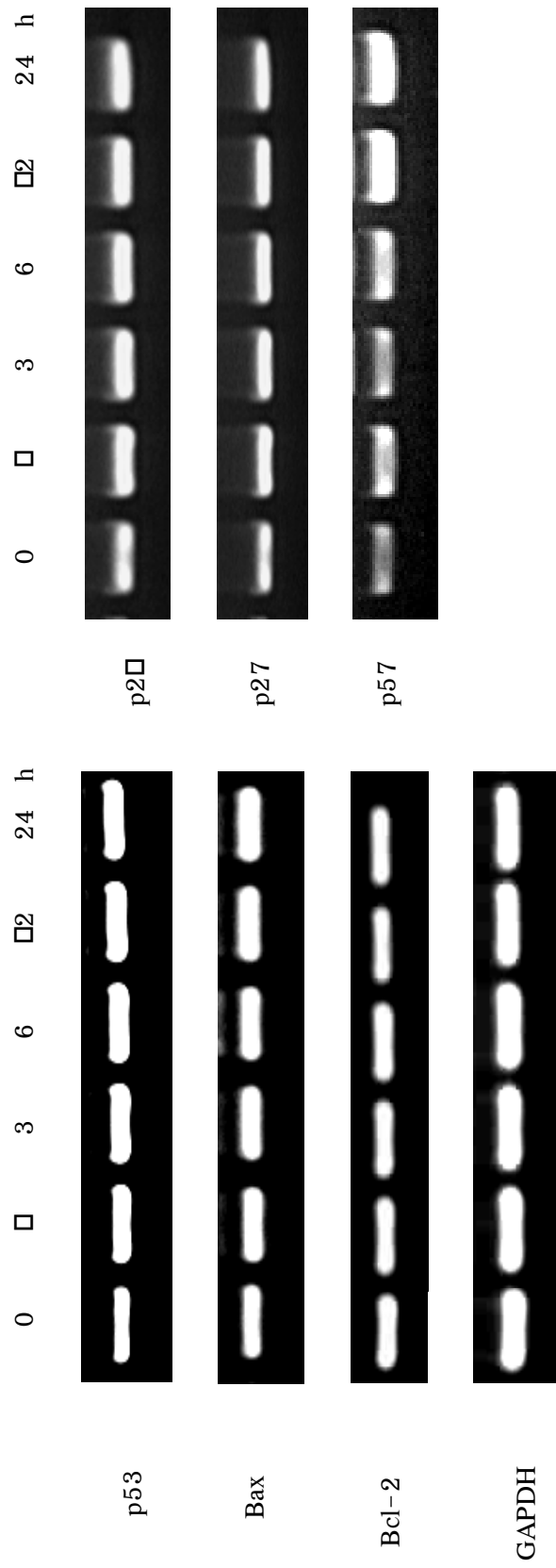


Figure 27 mRNA expression of the p53, Bax, Bcl-2, p21, p27 and p57 gene in human breast cancer cell lines, MCF-7, treated with 3 μ M of Dioscorealide B. mRNA expression of these genes were measured by RT-PCR. RT-PCR product was resolved in 2.5% agarose gel. GAPDH mRNA was analyzed as a control.

3.7 Dioscorealide B treatment decreases the expression of antiapoptotic protein Bcl-2 but increases the expression of proapoptotic protein Bax and Bak in MCF-7 Cells

The Bcl-2 family proteins play critical roles in regulation of apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process (Hockenbery et al, 1990). To gain insights into the mechanism of apoptosis induction in MCF-7 by Dioscorealide B, we determined the effect of Dioscorealide B treatment on levels of Bcl-2 family proteins by western blotting and the results are shown in Figure 28. Following treatment with 3 μ M of Dioscorealide B, anti-apoptotic protein Bcl-2 was down regulated after 3 hours, whereas the proapoptotic protein Bax expression was upregulated after 6 hours of the treatment. The Dioscorealide B treatment also caused an increase in the protein level of Bak as observed at 12 hours. This observation further supports the fact that induction of apoptosis in MCF-7 cells by Dioscorealide B is mediated through reduction of antiapoptotic protein Bcl-2 expression and induction of proapoptotic protein Bax and Bak expression which leads to the activation of caspase-9 and -7, respectively. In addition, the expression of *p21* was increased after 3 hours of the treatment but no change in the expression of *p27* was observed. These findings also supported the involvement of p53 in anticancer activity of Dioscorealide B in human breast cancer.

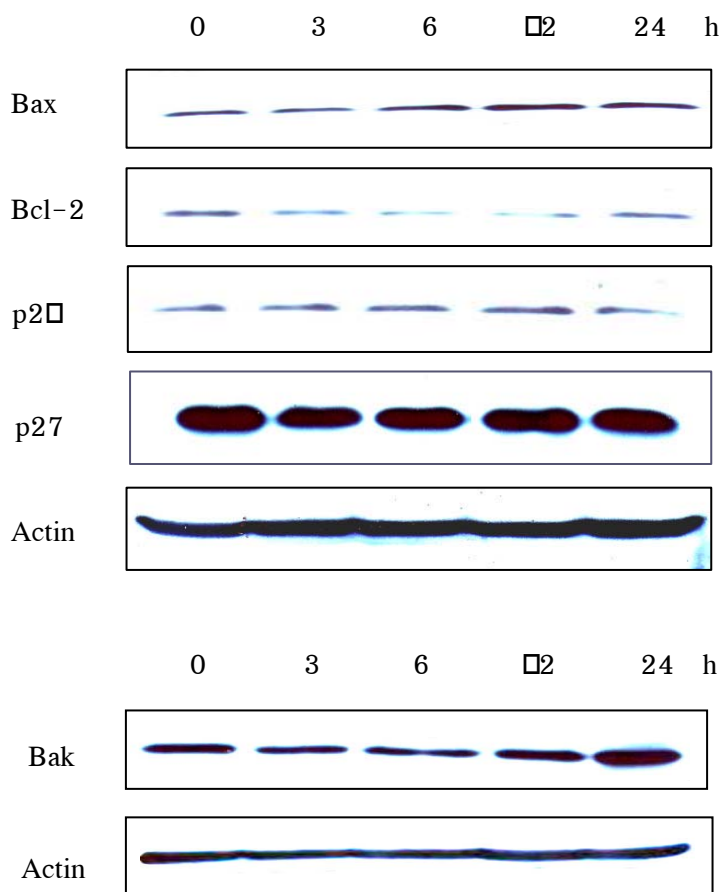


Figure 28 Immunoblotting for Bax, Bak, Bcl-2, p21 and p27 using lysates from MCF-7 cells treated with 3 μ M of Dioscorealide B for the indicated time periods. The blots were stripped and reprobbed with anti-actin antibody to normalize for differences in protein loading.

CHAPTER 4

DISCUSSION

Natural products are the organic molecules that are produced by living tissues from higher plants, fungi, microbes, marine organisms, and animals. They show a wide range of chemical diversity and biological properties. Natural resources have been used for combating human diseases for thousands of years. Over the last decade interest in natural products and their mechanisms of action has been revived. Naturally occurring substances play an increasing role in drug discovery and development. In fact, the majority of anticancer and anti-infectious agents are of natural origin (Newman et al, 2003; Fulda, 2009).

Plants have formed the basis for traditional medicine systems that have been used for thousands of years in countries with ancient civilizations such as China (Chang and But, 1986), India (Kapoor, 1990) and Thailand (Subchareon, 1998). Plant-derived substances have traditionally played important roles in the treatment of human diseases including cancer. Over recent years, there has been considerable interest in exploiting the cytostatic and cytotoxic effects of phytochemicals in the treatment of human cancers (Butt et al, 2006).

Thai traditional doctors in the southern part of Thailand commonly used Hua Khao Yen as the ingredients in remedies for cancer (Itharat et al, 1998). And it was found that Hua Khao Yen were the rhizomes of 5 species, i.e. *Dioscorea membranacea* Pierre (Dioscoreaceae), *Dioscorea birmanica* Prain ex Burkill, *Smilax corbularia* Kunth (Smilacaceae), *Smilax glabra* Roxb. and *Pygmeopremna herbacea* Prain et Burkill (Verbenaceae) (Itharat et al., 1999).

In this study, the cytotoxicity of five species of Hua Khao Yen in cervical cancer cell line (HeLa), estrogen receptor positive (MCF-7) and estrogen receptor negative (MDA-MB 458) human breast carcinoma cell were first investigated. The data demonstrated that the water extract of *Dioscorea membranacea* had potent cytotoxic activity in MCF-7 and MDA-MB 468, however, it had no effect on HeLa cells. The ethanolic extract of *D. membranacea* showed cytotoxic activity against HeLa, MCF-7 and MDA-MB 468 cells as well as *D. birmanica*. The water and ethanolic extracts of the other species exhibited no cytotoxic activity against HeLa and MCF-7. These data recapitulate the previous report by Itharat (2004) which showed that the ethanolic extracts of *D.*

membranacea and *D. birmanica* had cytotoxic activity against three cancer cell lines (lung, colon, breast cancer cell line), however, *Smilax corbularia*, *Smilax glabra* and *Pygmaeopremna herbacea* exhibited no cytotoxic activity.

TUNEL assay was then performed to examine whether these active extracts can induce apoptosis in human cancer cells. It was found that the ethanolic extract of *D. membranacea* and *D. birmanica* caused DNA fragmentation in HeLa cells suggesting apoptosis-inducing property of this extract. Furthermore, the results revealed that the water extract of *D. membranacea* and ethanolic extract of *D. membranacea* and *D. birmanica* inhibited the *in vitro* growth of MCF-7 via the induction of apoptosis.

Another previous study revealed that Dioscorealide B, one of the isolated compounds from the rhizome of *D. membranacea*, had cytotoxic activity against MCF-7, human breast cancer cells (Itharat, 2002). However, the molecular mechanism of action of Dioscorealide B in human breast carcinoma was poorly understood. The results of the present study confirmed that Dioscorealide B was able to inhibit *in vitro* growth of MCF-7. Intriguingly, it was found that Dioscorealide B had stronger cytotoxic effect on MCF-7 than MDA-MB 468 ($IC_{50} = 2.82$ and $9.93 \mu\text{M}$, respectively).

A major complication of chemotherapy is toxicity to normal cells, which is due to the inability of drugs to differentiate between normal and malignant cells. This often impacts the efficacy of the treatment and even makes it impossible to cure the patients. One of the requisite of cancer chemotherapeutic agent is the elimination of damaged or malignant cell through cell cycle inhibition or induction of apoptosis without or with less toxicity in normal cells (Stolarska et al, 2006).

Apoptosis is a conserved mechanism of cell death controlling the development and homeostasis of multicellular organisms (Dennis et al, 2000; Simstein et al, 2003). A family of cytosolic proteases, the caspases, stored in most cells as zymogens, play a crucial role in the execution of apoptosis. Proteolytic cleavage activates the initiating zymogen, which in turn triggers sequential proteolytic activation of each successive procaspase in the apoptosis cascade (Salvesen, 1997). Two major pathways have been identified according to their initiator caspase: the death receptor pathway involving caspase-8 (Medema et al, 1997) and the mitochondrial pathway, in which various stimuli can trigger the release of harmful proteins by mitochondria into the cytoplasm, leading to activation of caspase-9 and downstream cleavage of caspase-3, -7 or -6 (Green, 1998; Grutter, 2000). Caspase-3 activation has been shown to be critically important for the

enactment of this apoptotic pathway. However, MCF-7 human breast carcinoma cells do not express caspase-3 due to a deletion in the *casp-3* gene, suggesting the existence of caspase-3-independent apoptotic pathways. Previous studies showed that MCF-7 cells can still undergo apoptosis by the sequential activation of caspase-9, -7 and -6 (Liang et al, 2001; Simstein et al, 2003).

In this study, the apoptosis induced by Dioscorealide B was indicated by an increase in the number of Annexin V-positive cells. The data showed that Dioscorealide B induced the activation of caspase-7 which coincides with activation of caspase-9 and caspase-8. In addition, the pretreatment of MCF-7 cells with either caspase-8 inhibitor Z-IETD-FMK or the caspase-9 inhibitor Z-LEHD-FMK significantly decreased the caspase-7 activity. These results suggested that Dioscorealide B-induced apoptosis in MCF-7 cells is probably mediated by both intrinsic and extrinsic apoptotic pathways.

The Bcl-2 family proteins have emerged as critical regulators of the mitochondria-mediated apoptosis by functioning as either promoters (e.g., Bax and Bak) or inhibitors (e.g., Bcl-2 and Bcl-xL) of the cell death process. Antiapoptotic Bcl-2 family members (Bcl-2 and Bcl-xL) possess four conserved BH domains (BH1-BH4) and mainly prevent the release of apoptogenic molecules namely cytochrome c from mitochondria to the cytosol by forming heterodimer with proapoptotic proteins, such as Bax (Chao et al, 1998; Adam, 1998). The proapoptotic Bcl-2 family proteins, which can be subdivided into the Bax subfamily of multidomain proteins (e.g., Bax and Bak) or BH3-only subfamily (e.g., Bid and Bim), induce mitochondrial membrane permeabilization and release of apoptogenic molecules from mitochondria to the cytosol. (Ruvolo et al, 2001; Yin, 2003). In addition, various forms of cellular stress such as DNA damage, can induce up-regulation of p53, an important tumor suppressor, which in turn triggers the mitochondrial apoptotic pathway via regulating transcription Bcl-2 family member, leading to up-regulated expression of the proapoptotic Bax and down-regulated expression of the antiapoptotic Bcl-2. Also, the p53 is essential for the checkpoint control which arrests human cells with damaged DNA in G1 by transactivating target gene especially, *p21^{WAF1/CIP1}* encoding cyclin-dependent kinase inhibitor.

The present study showed that Dioscorealide B treatment caused an increase in the expression of Bax, but a decrease the expression of *BCL-2* gene in both mRNA and protein level. Moreover, MCF-7 treated with Dioscorealide B showed the upregulation of Bak protein. These data suggested that the mechanism of Dioscorealide B involved the

modulation of Bcl-2 family expression which leads to the activation of caspase-9 and -7, respectively. In this study, the expression of p53 protein in MCF-7 treated with Dioscorealide B was failed to be detected by western blot analysis, however, the expression of *p53* gene was upregulated in transcription level. In addition, the expression of *p21*, a p53 targeted gene, was increased in both mRNA and protein level after treatment with Dioscorealide B. The results of cytotoxic test revealed that MDA-MB 468 which is p53 mutant cell lines (Ho et al, 2007), displayed the lower susceptibility to Dioscorealide B than MCF-7 which harbor a functional p53 gene (Stoff-Khalili et al, 2006; Rebecca et al, 2003). Taken together, these findings suggested that p53 was likely involved in the apoptosis induction pathway initiated by Dioscorealide B. Moreover, Dioscorealide B can upregulate the expression of other genes encoding cyclin-dependent kinase inhibitors: *p27* and *p57* suggesting that the antitumorigenic property of Dioscorealide B might be attributable to cell cycle arrest. However, further study would be needed to refine the molecular mechanism underlying this cytotoxic effect.

CHAPTER 5

CONCLUSION

Apoptosis is a programmed cell death and a highly organized physiological mechanism to destroy injured or abnormal cells. The induction of apoptosis in tumor cells is considered to be very useful in the management and therapy of human cancers. A wide variety of natural compounds have been recognized to have the ability to induce apoptosis in various tumor cells of human origin. In this study, we investigated the anti-tumor activity and the underlying mechanism of Dioscorealide B. The results of the present study indicated that induction of apoptosis in MCF-7 cells by Dioscorealide B is mediated through the extrinsic apoptotic pathway via the activation of caspase-8 and the intrinsic apoptotic pathway via the downregulation of antiapoptotic *BCL-2* gene and upregulation of *p53* at mRNA level. Furthermore, Dioscorealide B treatment in MCF-7 caused the increases in the level of proapoptotic protein Bax and Bak and the decrease in Bcl-2 protein level which then led to the activation of caspase-9 and -7, respectively. In addition, the mechanism of action of Dioscorealide B in human breast cancer might be attributable to cell cycle arrest. These studies thus provide the molecular basis for the further development of Dioscorealide B as a novel chemotherapeutic agent for breast cancer therapy.

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APPENDIX

APPENDIX A

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method of SDS-PAGE was performed as described by Laemmli, 1970. The gel solution was prepared as shown in Table 4. Electrophoresis was carried out the descending direction on the Tris-glycine buffer (25 mM Tris-HCl, pH 6.8, 192 mM glycine and 0.1% (w/v) SDS) using a constant 100 v for 30 min and 200 v for 2 h or until the tracking dye reached the edge of the gel.

Table 4 Preparation of SDS-polyacrylamide gel

Solution	4% Stacking gel	12% Resolution gel
Water	1.88 ml	3.23 ml
40% Acrylamide (Acrylamide: N,N'-methylenebisacrylamide, 29:1)	0.3 ml	2.1 ml
1.5 M Tris-HCl, pH 8.8	-	1.52 ml
0.5 M Tris-HCl, pH 6.8	0.75 ml	-
10% SDS	0.03 ml	0.07 ml
10% APS	0.03 ml	0.07 ml
TEMED	0.01 ml	0.01 ml

SDS = Sodium Dodecyl Sulfate

APS = Ammonium Persulfate

TEMED = N,N,N',N'-tetramethylethylenediamine

APPENDIX B

1. Chemical stock solution and buffer

5 M Tris-HCl

Tris-base	6	g
Distilled water	100	ml

Adjust the pH to 8.8 by adding concentrated HCl. Adjust the volume of the solution to 100 ml with distilled water and sterilize by autoclaving.

5 M Tris-HCl

Tris-base	6	g
Distilled water	100	ml

Adjust the pH to 6.8 by adding concentrated HCl. Adjust the volume of the solution to 100 ml with distilled water and sterilize by autoclaving.

10% SDS (w/v)

SDS	10	g
Distilled water	90	ml

Heat to 68°C and stir with a magnetic stirrer to assist dissolution. Adjust the volume to 100 ml with distilled water and store at room temperature. Sterilization is not necessary.

10% APS (w/v)

Ammonium persulfate	1	g
Distilled water	10	ml

Phosphate-buffered Saline (PBS) : 1x

NaCl	8	g
KCl	0.2	g
Na ₂ HPO ₄	1.44	g
KH ₂ PO ₄	0.24	g

Dissolve the ingredients in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add distilled water to 1000 ml. Sterilize the buffer by autoclaving and store at room temperature or 4 °C.

Ponceau S

Ponceau S	10	g
Acetic acid	50	ml
Deionized water to	1000	ml

2. Solutions for SDS-PAGE

Tris-glycine buffer, Running Buffer : 10X

Tris-base	30.28 g
Glycine	144.13g
SDS	10 g

Dissolve the ingredients in 800 ml of distilled water. Adjust the volume to 1000 ml with distilled water.

SDS Gel loading buffer: 4X

200 mM Tris-HCl, pH 6.8
8% (w/v) SDS
0.4% (w/v) glycerol
8% (v/v) 2-B-mercaptoethanol
400 mM DTT

Adjust the volume of the solution to 50 ml with distilled water and store at -80°C .

Comassie blue staining

Comassie Brilliant Blue R250	0.025 g
Methanol	43 ml
Acetic acid	7 ml
Distilled water to	100 ml

Destain solution

Methanol	30 ml
Acetic acid	7 ml
Distilled water	63 ml

3. Solution for Western blot analysis

Electroblotting buffer: 1X

Tris-base	5.8	g
Glycine	7.9	g
Methanol	200	ml

Dissolve the ingredients in distilled water and bring up to volume 1000 ml with in distilled water.

TBS-T buffer: 5X

Tween [®] 20	5	ml
NaCl	45	g
Tris-HCl	31.75	g
Tris-base	5.8	g

Dissolve the ingredients in distilled water and bring up to volume 1000 ml with in distilled water.

Blocking buffer:

Low fat dry milk	5	g
1X TBS-T	100	ml

Washing buffer:

Low fat dry milk	10	g
1X TBS-T	1000	ml

Stripping buffer:

100 mM β -mercaptoethanol (4.3 M stock)	
2% (w/v) SDS	10 ml
62.5 mM Tris-HCl, pH 6.8	

Incubate the membrane at 60 °C 30 min and then wash with TBS-T, 2x10 min.

4. Solution for sulphorhodamine B (SRB) assay

10% (w/v) TCA (Trichloroacetic acid):

Dissolve 10 g TCA in 100 ml distilled water. Store at 4°C.

0.4% (w/v) sulphorhodamine B solution:

Dissolve 0.4 g sulphorhodamine B in 100 ml of 1% acetic acid. Store at 4°C.

1% acetic acid:

Add 1 ml of glacial acetic acid in 99 ml distilled water.

10 mM Tris-base solution:

Dissolve 1.2 g Tris-base in 1 L distilled water.

VITAE

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List of Publication and Proceeding

Saekoo, J., Dechsukum, C., Itharat, A. and Graidist, P. Anticancer Effect of Dioscorealide B On Human Breast Cancer Cell Line (MCF-7). Oral presentation at The 34th Congress on Science and Technology of Thailand. October 31-November 2, 2008. Bangkok, Thailand.

Saekoo, J., Dechsukum, C., Itharat, A. and Graidist, P. Cytotoxic activity and Mechanism of Action of Dioscorealide B in Human Breast Cancer Cell Line (MCF-7). Poster presentation at The 2nd International Conference on Natural Products for Health and Beauty. December 17-19, 2008. Phayao, Thailand.