ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งเต้านม MDA-MB-231 โดยสมุนไพรไทยในกลุ่มยาอายุวัฒนะ

นางสาวสุพัตรา ชวลิตพงษ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาสัตววิทยา ภาควิชาชีววิทยา
คณะวิทยาศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2555

ANTIPROLIFERATION ACTIVITY AGAINST BREAST CANCER CELLS MDA-MB-231 BY THAI REJUVENATING HERBS

Miss Supattra Chawalitpong

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology Faculty of Science
Chulalongkorn University
Academic Year 2012

Thesis Title	ANTIPROLIFERATION ACTIVITY AGAINST BREAST CANCER CELLS MDA-MB-231 BY THAI REJUVENATING HERBS
By	Miss Supattra Chawalitpong
Field of Study	Zoology
Thesis Advisor	Associate Professor Wichai Cherdshewasart, Ph.D.
Thesis Co-advisor	Chantragan Phiphobmongkol, Ph.D.

Accepted by the Faculty of Science, Chulalongkorn University in Partial Fulfillment of the Requirements for the Master's Degree

.....…… Dean of the Faculty of Science
(Professor Supot Hannongbua, Dr. rer. nat.)

THESIS COMMITTEE

…….. Chairman
(Associate Professor Kumthorn Thirakhupt, Ph.D.)

…….……. Thesis Advisor
(Associate Professor Wichai Cherdshewasart, Ph.D.)

..…….……. Thesis Co-advisor
(Chantragan Phiphobmongkol, Ph.D.)

…….. Examiner
(Associate Professor Chanpen Chanchao, Ph.D.)

…….. Examiner
(Associate Professor Polkit Sangvanich, Ph.D.)

.…….. External Examiner
(Suttijit Sriwatcharakul, Ph.D.)

สุพัตรา ชวลิตพงษ์: ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งเต้านม MDA-MB-231 โดยสมุนไพรไทยในกลุ่มยาอายุวัฒนะ. (ANTIPROLIFERATION ACTIVITY AGAINST BREAST CANCER CELLS MDA-MB-231 BY THE THAI REJUVENATING HERBS) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. วิชัย เชิดชีวศาสตร์, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม : ดร. จันทรกานต์ พิภพมงคล, 80 หน้า.

การศึกษาฤทธิ์การต้านการเพิ่มจำนวนของสารสกัดหยาบจากพืชสมุนไพรไทยในกลุ่มยาอายุวัฒนะ จำนวน 24 ชนิด ต่อเซลล์มะเร็งเต้านมชนิดที่ไม่มีตัวรับฮอร์โมนเอสโตรเจน (MDA-MB-231) ที่ความเข้มข้น 0.1, 1, 10, 100 และ 1,000 ไมโครกรัมต่อมิลลิลิตร ของสารสกัดหยาบ โดยใช้เทคนิค MTT assay ผลการทดลองพบว่า อัตราการรอดชีวิตของเซลล์มะเร็งเต้านมลดลงอย่างมีนัยสำคัญ เมื่อความเข้มข้นของสารสกัดเพิ่มขึ้น เปรียบเทียบจากค่า IC_{50} โดยสารสกัดหยาบที่มีศักยภาพดีที่สุดสามลำดับแรกคือ *Stephania venosa*, *Piper nigrum* (เมล็ด) และ *Stephania erecta*. โดยมีค่า IC_{50} เท่ากับ 24.85, 31.34 และ 32.42 ไมโครกรัมต่อมิลลิลิตร ตามลำดับ จากนั้นนำสารสกัดหยาบทั้งสามชนิดมาทำการศึกษาผลการเหนี่ยวนำการตายของเซลล์แบบอะโพโทซิส (apoptosis) ของสารสกัดหยาบต่อเซลล์มะเร็งโดยใช้เทคนิคโฟลไซโทรเมทรี พบว่าสารสกัดทั้งสามชนิดมีคุณสมบัติในการเหนี่ยวนำการตายของเซลล์แบบอะโพโทซิส และยืนยันกระบวนการตายแบบอะโพโทซิสโดยใช้เทคนิคเวสเทิร์นบลอท (Western blot) โดยพบการกระตุ้นการทำงานของโปรตีนชนิด caspase-3 ตรวจพบชิ้นส่วนของโปรตีน PARP ที่ถูกตัดโดย caspase-3 และพบการแสดงออกของโปรตีน Bcl-2 ลดลงในกลุ่มที่ทดสอบด้วยสารสกัดหยาบ นอกจากนั้นพบว่าผลของสารสกัดดังกล่าวไม่สามารถกระตุ้นโปรตีนชนิด caspase-8 และ BID ได้ จึงสามารถสรุปได้ว่า สารสกัดหยาบทั้ง 3 ชนิดมีประสิทธิภาพในการกระตุ้นการตายของเซลล์แบบอะโพโทซิสโดยผ่านทาง intrinsic pathway ผลการศึกษาวิจัยครั้งนี้ชี้ให้เห็นถึงความเป็นพิษของพืชสมุนไพรไทยในกลุ่มยาอายุวัฒนะบางชนิดที่มีศักยภาพในการยับยั้งการเพิ่มจำนวนของเซลล์มะเร็ง

ภาควิชา ชีววิทยา ลายมือชื่อนิสิต .....

สาขาวิชา สัตววิทยา ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลักร่วม .....

5372365623 : MAJOR ZOOLOGY
KEYWORDS : Antiproliferation / Apoptosis / Caspase-3 / ER-negative breast cancer cell / PARP / Thai rejuvenating herbs

SUPATTRA CHAWALITPONG : ANTIPROLIFERATION ACTIVITY AGAINST BREAST CANCER CELLS MDA-MB-231 BY THE THAI REJUVENATING HERBS. THESIS ADVISOR : ASSOC. PROF. WICHAI CHERDSHEWASART, Ph.D., CO-ADVISOR : CHANTRAGAN PHIPHOBMONGKOL, Ph.D., 80 pp.

The antiproliferative activity of the 24 plant ethanolic crude extracts of Thai rejuvenating herbal plants present in the traditional Thai medicinal recipe against the human estrogen receptor negative breast cancer cells (MDA-MB-231) at the concentration of 0.1, 1, 10, 100 and 1,000 µg/ml with the aid of MTT assay was done. The results revealed that the breast cancer cell survival rates were decreased significantly following the treatment with the increasing doses of the certain plant crude extracts as determined by the IC_{50} values. The top three of the most potent plant ethanolic crude extracts according to their IC_{50} values were *Stephania venosa*, *Piper nigrum* (seed) and *Stephania erecta*. (IC_{50}; 24.85, 31.34 and 32.42 µg/ml, respectively) The same set of plant samples were evaluated for apoptosis assay with the aid of flow cytometry. The plant ethanolic extract treatments exhibited typical apoptotic features. The apoptosis was confirmed with Western blot and the results showed activation of caspase-3 and cleavage of PARP by caspase-3, decrease expression of Bcl-2, in the 3 plant samples. However, caspase-8 and BID were not inactivated by 3 plant samples. The result indicated that the plant ethanolic extract can induces apoptosis via intrinsic pathway. The study confirms the potential of the three Thai rejuvenating herbal plants against the estrogen receptor negative breast cancer cells.

Department : BIOLOGY	Student's Signature .....
Field of Study : ZOOLOGY	Advisor's Signature .....
Academic Year : 2012	Co-advisor's Signature .....

ACKNOWLEDGEMENTS

I would like to express my deeply grateful thank to my advisor, Associate Professor Dr. Wichai Cherdshewasart, Department of Biology, for his valuable suggestion, guidance, kindness, encouragement and helps throughout this study. Especially, I would like to express my gratitude to my co-advisor Dr. Chantragan Phiphobmongkol, Laboratory of Biochemistry, Chulabhorn Research Institute; for her support and encouragement. I am thankful to my Examiners, Associate Professor Dr. Chanpen Chanchao, Associate Professor Dr. Polkit Sangvanich and Dr. Suttijit Sriwatcharakul.

Thankfulness would be given to all members, Miss. Chutima Chaisanit and Miss Kanokkorn Topan, in Kwao Krua Research Labolatory, Department of Biology, for Counseling and encouraging. Gratefulness would be given to Miss Penchatr Diskul-Na-Ayudthaya and Miss Daranee Chokchaichamnankit and all members of Laboratory of Biochemistry, Chulabhorn Research Institute, for permission to use the necessary instruments and laboratory facilities for my thesis.

Sinner thanks to all my friends and finally, the deepest sincere gratitude to my parent and my family for their love, support, understanding and encouragement.

CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
CO_2	Carbon dioxide
DMSO	Dimethylsulfoxide
DW	Distilled water
ED	Effective dose

EDTA	Ethylene diamine tetraacetic acid
ER	Estrogen receptor
FBS	Fetal bovine serum
g	Gram
g_{max}	Gravity max
IC_{50}	50% of Inhibition concentration
l	Liter
L-15	Leibovit's 15 medium
mg	Milligram
ml	Milliliter
mm	Millimeter
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
N/A	not applicable or not available
O.D.	Optical density
^oC	Degree Celsius
PBS	Phosphate-buffered saline
RH	Relative humidity
RO	Reverse osmosis
V	Volume
µg	Microgram
µl	Microliter

CHAPTER I

INTRODUCTION

Breast cancer is one of the most common cause of cancer-related death and the second leading incidence of cancer in Thailand women (Information Technology Division National Cancer Institute, 2010). While the ER-positive breast cancers

respond to anti-estrogen therapy but ER-negative breast cancers showed poor prognosis and less responded toward chemotherapeutic agents. Therefore, there is an urgent need for the development of efficient prevention and therapy strategies against this type of breast cancer. (Hsieh, Hernández-Ledesma and de Lumen, 2011)

There were evidences that phytochemicals exhibited potential alternative treatment for MDA-MB-231 breast cancer with low adverse effects (McEligot, Yang and Meyskens, 2005). For example, inhibition of the MEK5/ERK5/NF-κB pathway by genistein may be an important mechanism in the suppression of cell growth and induction of apoptosis (Li *et al.,* 2008). Induces apoptosis by Carvacrol via decrease in the mitochondrial membrane potential of the cells, resulting in release of cytochome C, caspase activation and cleavage of PARP (Arunasree, 2000). Inhibit effects on cell proliferation by cell cycle arrest at the G_0/G_1 phase and induces apoptosis, increased in the expression of Bax, Bak, and Bcl-Xs, but decreased the levels of Bcl-2 and Bcl-XL, and subsequently triggered mitochondria apoptotic pathway by Rugosin E was isolated from *Rosa rugosa* Thunb (Kuo *et al.,* 2007). And rhizomes of *Cimicifuga racemosa* can induces apoptosis by activation of caspases (Hostanska *et al.,* 2004).

The rejuvenating plants have long term been traditionally used for treatment of various diseases including infection, immunological disorders and cancers (Fiebig *et al.,* 1985). Furthermore, those with the efficacy of reversal of aging and repair of the damage are associated with aging (Govindarajan *et al*., 2005). Thai rejuvenating herbs are widely and legally used in traditional Thai medicine. The herbal plant in this group was rarely studied for cytotoxicity or anti-proliferation activity to cancer cells or induced cancers in animal model. However, anti-cancer properties of these plants were limited studied. In this study, we did screen and deeply analysis on the antiproliferative activity of the selected Thai rejuvenating herbal plants against human breast cancer cells MDA-MB-231.

Purposes of the study

1) To screen for the antiproliferative activity of 22 Thai rejuvenating herbal plant extracts against the breast cancer cells (MDA-MB-231), based on the MTT assay

2) To quantify and qualify the antiproliferative activity of the 3 strongest cytotoxic rejuvenating Thai herbal extracts against MDA-MB-231 cells with the aid of a flow cytometry
3) To establish molecular data at the protein level of the antiproliferative activity of the 3 strongest cytotoxic Thai rejuvenating herbal extracts on MDA-MB-231 cells with the aid of immunoblotting

CHAPTER II

LITERATURE REVIEW

2.1 Rejuvenating herbal plants

Plants are main sources for drug development. Currently, over 50% of drugs used in clinical trials for anticancer activity were related to or isolated from natural

sources (Newman and Cragg, 2007). Thailand locates in a tropical area with abundance of diverse herbal plants. Thai medicinal plants were long term traditionally consumed for treatment of certain diseases including infection, immunological disorders and cancers (Farnsworth and Bunyapraphatsara, 1992). The rejuvenating herbal plants with the efficacy of reversal of aging and repair of the damage are associated with aging (Govindarajan *et al*., 2005). The herbal plant in this group was rarely studied for cytotoxicity or anti-proliferation activity to cancer cells or induced cancers *in vitro*. We therefore select twenty-two plants used in Thai traditional rejuvenating remedies for screening of cytotoxicity against the human MDA-MB-231 prostate cancer cells.

2.1.1 *Stephania erecta* Craib

S. erecta, Thai name is "Buabokpa" (Family Menispermaceae) (Figure 2.1), is used in Thai folk remedies as an analgesic and tonic and a skeletal muscle relaxant (Nantawan and Arnuch, 2001). Cepharanthine, (+)-1,2-dehydrotelobine, (+)-dephnandrine, (+)-homoaromoline, (+)-isotetrandrine, (+)-2-N-methyltelobine, (+)-2-nor-cepharanthine, (+)-2-nor-isotetrandrine, (+)-nor-obaberine, (+)-2-nor thalrugosine, (+)-obaberine (+)-thalrugosine, (+)-stephibaberine, were isolated from *S. erecta* (Likhitwitayawuid *et al.*, 1993; Tamez *et al.*, 2005)

2.1.1.1 Anticancer activity

(+)-2-N-methyltelobine from *S. errecta* Craib showed potent cytotoxic activity against the KB (ED_{50} 3.6 µg/mL) and P-388 (ED_{50} 0.8 µg/mL) cell systems (Likhitwitayawuid *et al.*, 1993)

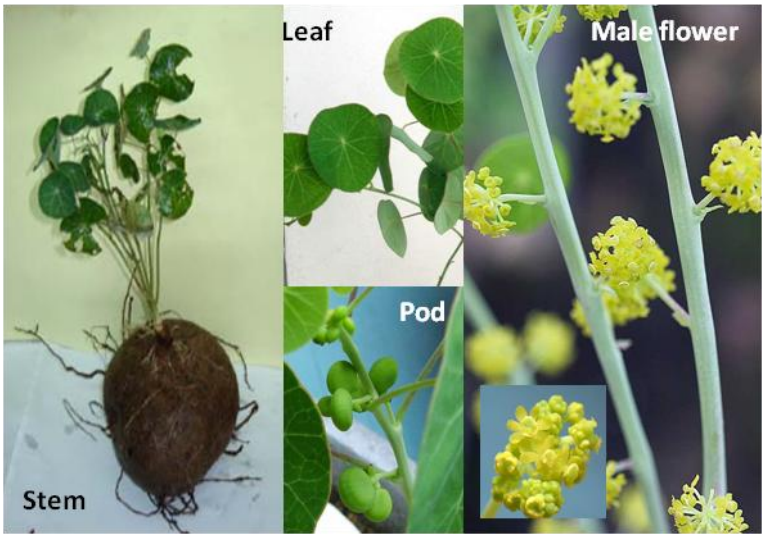

Figure 2.1 *Stephania erecta* Craib (http://public.fotki.com/plumo/stephania and http://www.toptropicals.com)

2.1.2 *Stephania venosa* (Blume) Spreng

S. venosa, Thai name are "Saboo-luad" or "Boraphet pungchang" (Family Menispermaceae) (Figure 2.2). (−)-crebanine, dehydrocrebanine, dehydrostephanine, kamaline, (−)-kikemanine, liriodenine, (+)-N-carboxamidostepharine, (−)-O-acetylsukhodianine, (−)-O-methylstepharinosine, oxoaporphin, oxocrebanine, oxostephanine, oxostephanosine, (−)-sukhodiamine, (−)-sukhodianine-β-N-oxide, (−)-stephadiolamine-β-N-oxide, (+)-stepharine, (−)-stepharinosine, (−)-tetrahydropalmatine, (−)-ushinsunine, (Guinaudeau *et al.*, 1981, 1982; Pharadai *et al.*, 1985; Charles *et al.*, 1987; Banerji *et al.*, 1994; Likhitwitayawuid *et al.*, 1999), stepharanine, cyclanoline and N-methyl stepholidine were isolated from *S. venosa* (Ingkaninan *et al.*, 2006). The genus Stephania could be a potential source of biologically active compounds which might be used as lead molecules for the development of new drugs (Semwal *et al.*, 2010).

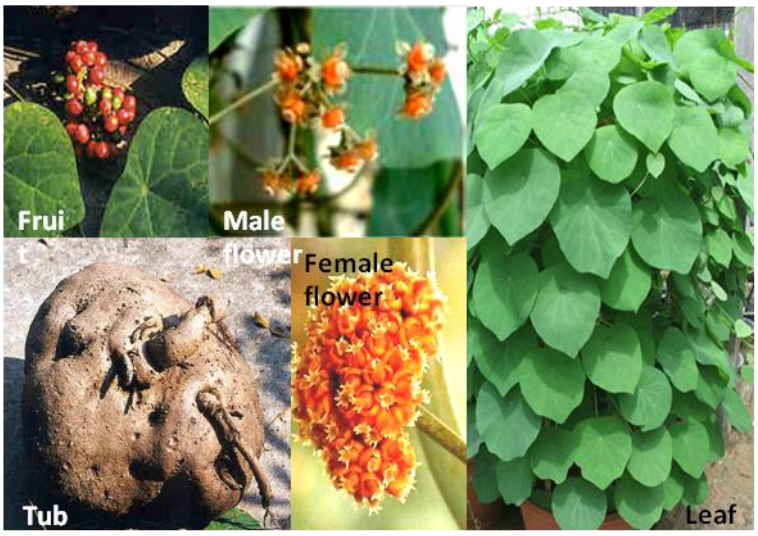

Figure 2.2 *Stephania venosa* (Blume) Spreng (http://www.tropicaflore.com and http://www.herblpg.com/thai/herb55.html)

2.1.2.1 Anticancer activity

The ethanolic extract from tuber showed anticancer activity in human small cell lung cancer (NCI-H187), human breast cancer (MCF-7) cells (Leewanich *et al.,* 2011) and SKBR3 human breast adenocarcinoma cell line (Moongkarndi *et al.,* 2004). The oxostephanine, against breast cancer and acute lymphoblastic leukemia cells (MOLT-3) with an IC_{50} of 0.24 and 0.71 µg/mL, respectively, and very low cytotoxicity against normal embryonic lung cells MRC-5 cells. Dehydrocrebanine against promyelocytic leukemia cells (HL-60) with an IC_{50} of 2.14 µg/mL (Makarasen *et al.,* 2011). Aporphine isolated from the tuber exhibited cytotoxic activity against SKOV3 human ovarian cancer cells (Montrirattigri *et al.*, 2008).

2.1.3 *Piper nigrum* Linn.

P. nigrum (Family Piperaceae) locally known as "Phrikthai" or black pepper (Figure 2.3). The plant is native of Southern India and Sri Lanka. It is known to exhibit a variety of biological activities which include anti-pyretic, anti-inflammatory, anti-depressant, hepatoprotective and antitumor (Pathak and Khandelwal, 2006). Piperine has been documented to enhance the

bioavailability of a number of therapeutic drugs as well as phytochemicals by this very property, while it is non-genotoxic, has in fact been found to possess anti-mutagenic and anti-tumor influences (Srinivasan, 2007).

Figure 2.3 *Piper nigrum* Linn. **(**http://en.wikipedia.org/wiki/Black_pepper)

2.1.3.1 Anticancer activity

Pellitorine isolated from the roots of *P. nigrum* showed anticancer activity in HL60 (human promyelocytic leukemia) cell line and MCF-7 (breast cancer) cell line (Lian Ee *et al.,* 2010). Alkylamides and piperine are compounds derived from black pepper those suppressed TNF-induced NF-kB activation in human cancer cells. Piperine, The growth inhibition related to cell cycle arrest at the G_2/M phase, induction of apoptosis via activation of caspase 3 and decreasing the expression of metalloproteinases-9 (MMP-9) and MMP-13 in 4T1 cells (mouse mammary carcinoma) (Lai *et al.*, 2012).

2.2 Molecular targets of phytochemicals for cancer therapy

The possible protective molecular mechanisms initiated by the active components of dietary phytochemicals against cancer are the alternation of proteomic expression of the apoptotic proteins, anti-apoptotic proteins (Figure 2.4) (Aggarwal and Shishodia, 2006). Identification and characterization of functionally modulated proteins involved in molecular targets events should lead to a better understanding of the long-term action of these drugs at the molecular level and will contribute to the future development of novel therapeutic drug treatments based upon current therapies.

Figure 2.4 Molecular targets of dietary agents, modified from Aggarwal and Shishodia (2006)

2.2.1 The compounds have effects to MDA-MB-231 cell lines.

As showed in Table 2.1

Table 2.1 The compounds; drugs, pure compound and crude extracts those have effects to MDA-MB-231 cell lines

Name	Type of Compounds (Crude extracts; C, Drugs; D, Pure compounds; P, Toxin; T)	IC_{50}	Mechanism	References
Amlodipine	Dihydropyridine (P)	16.5 μM	Antiproliferation, related to induces apoptosis via decrease of the expression of Bcl-2 and PCNA.	Luo *et al.,* 2008
Anacardic acid and lunacin	6-pentadeca-8(Z), 11(Z), 14-trienylsalicylicacid, isolated from *Anacardium occidentale* and unique 43-amino acid peptide (P)	79 and 181 μM	Inhibit effects on cell proliferation by induces apoptosis and cell cycle arrest at the S phase.	Hsieh, Hernández-Ledesma and Lumen, 2011
Berberine	phenanthren alkaloid (P)		Induced cell cycle arrest at G_0/G_1 in the anoikis-resistant MCF-7 and MDA-MB-231 cells	Kim *et al.,* 2010

Name	Type of Compounds (Crude extracts; C, Drugs; D, Pure compounds; P Toxin; T)	IC_{50}	Mechanism	References
Carvacrol	essential oils (P)	100 μM	Induces apoptosis by decrease in the mitochondrial membrane potential of the cells, resulting in release of cytochome C, caspase activation and cleavage of PARP.	Arunasree, 2000
Centchroman	non steroidal anti-estrogen (D)	20 μM	Induces apoptosis and cell cycle arrest at G_0/G_1 phase.	Nigam *et al.,* 2008
Cimicifuga racemosa	rhizomes (isopropanolic and ethanolic) (C)	29.5±3.0 and 58.6±12.6 μg/ml	Induces apoptosis by activation of caspases.	Hostanska *et al.,* 2004
Genistein	Isoflavonoid (P)	10 μM	Inhibit NF-kB activity via the MEK5/ERK5 pathway. And down-regulates Bcl-2, up-regulates Bax; induces apoptosis.	Li *et al.,* 2008

Name	Type of Compounds (Crude extracts; C, Drugs; D, Pure compounds; P, Toxin; T)	IC_{50}	Mechanism	References
Goniothalamin	styryl-pyrone (P)	1.46 μM	Induces cell cycle arrest at G_2/M phase and apoptosis by disrupts intracellular redox balance and induces cdc25C degradation.	Chen *et al.*, 2005
Homokiol	Neolignan (P)	59.5 μM	The growth inhibition related to cell cycle arrest at the G_0/G_1 phase and induction of apoptosis via signal transduction pathway in the c-Src/EGFR and Akt/Mtor cascade signaling.	Park *et al.*, 2009
Pheophorbide A	(3S,4S)-9-Ethenyl-14-ethyl-21-(methoxycarbonyl)-4,8,13,18-tetramethyl-20-oxo-3-phorbinepropanoic acid isolated from *Scutellaria barbata* (P)	0.5 μM	Anti-tumor effects by the ERK-mediated autophagy and.the activation of mitochondria-mediated apoptosis	Bui-Xuana *et al.*, 2010

Name	Type of Compounds (Crude extracts; C, Drugs; D, Pure compounds; P, Toxin; T)	IC_{50}	Mechanism	References
Pinolenic acid	polyunsaturated fatty acid (P)	-	Inhibited cell metastasis by suppressing cell invasiveness and motility.	Chen *et al.,* 2011
Prenylated flavones	Isoflavonoid (P)	3.12 μM	Antiproliferative effect by an intense cytoplasmic vacuolization that could be associated with autophagy.	Pedro *et al.,* 2006
Rugosin E	Ellagitannin, is isolated from *Rosa rugosa* Thunb (P)		Inhibit effects on cell proliferation by cell cycle arrest at the G_0/G_1 phase and induces apoptosis, increased in the expression of Bax, Bak, and Bcl-Xs, but decreased the levels of Bcl-2 and Bcl-XL, and subsequently-	Kuo *et al.,* 2007

Name	Type of Compounds (Crude extracts; C, Drugs; D, Pure compounds; P, Toxin; T)	IC_{50}	Mechanism	References
			triggered mitochondria apoptotic pathway.	
γ-Tocotrienol	Isofrom of vitamin E (P)	20 μM	Induces apoptosis by involved initiation of the mitochondria-mediated death pathway, reduction of metalloproteinases (MPPs) and releasing of cytochrome C.	Takahashi and Loo, 2004

2.3 Mode of cell death

Cell death is an indicator in cancer prevention and cancer therapy (Schulte-Hermann *et al.*, 1997). There are two forms of cell death: apoptosis and necrosis (Figure 2.5) that have been defined on the basis of morphological criteria (Kanduc *et al.*, 2002).

2.3.1 Apoptosis

Apoptosis is a morphological alteration associated with programmed cell death and certain pathological processes, occurs during the normal development of multicellular organisms (Kresch *et al.*, 1998). Induction or inhibition of apoptosis could be initiated by stimuli that pass through the surface of receptor or penetrate such as stress factor, drug toxin and others (Table 2.2) (Staunton and Gaffney, 1998).

The apoptotic processes are including chromatin aggregation, nuclear and cytoplasmic condensation, partition of cytoplasm and nucleus into membrane bound-vesicles (apoptotic bodies) which contain ribosomes, morphologically intact mitochondria and nuclear material (Table 2.3). The apoptotic bodies are rapidly recognized and subsequently phagocytized by either adjacent epithelial cells or macrophages (Savill *et al.*, 1989). The apoptotic bodies and the remaining cell fragments will undergo swelling and lyse. The secondary necrosis is the terminal phase of cell death (Krähenbühl and Tschopp, 1991).

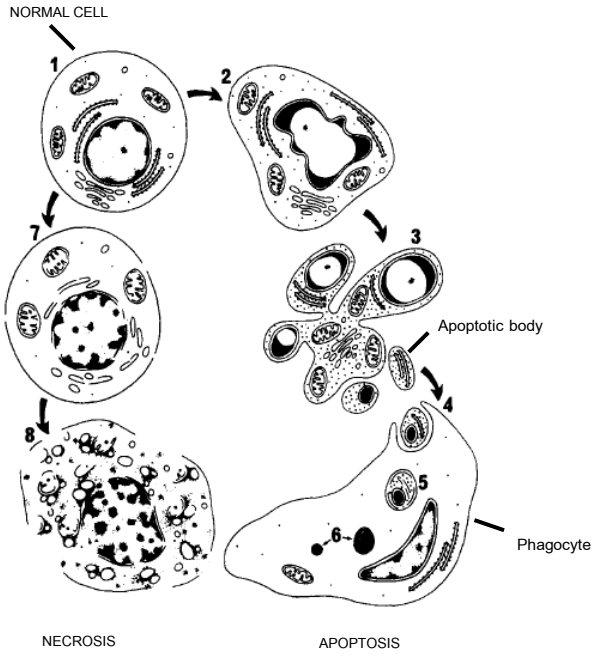

Figure 2.5 The different cell death pathway between necrosis and apoptosis (Kerr *et al.,* 1994)

The sequence of ultra structural changes in apoptosis and necrosis: (Figure 2.5; 2-6 show pathways of apoptosis, 7 and 8 show pathway of necrosis)

(1) Normal cell (early apoptosis)

(2) Early apoptosis: compaction of nuclear chromatin, condensation of cytoplasm, and convolution of nuclear and cell outlines.

(3) Late apoptosis: nuclear fragmentation, protuberances on the cell surface and produce apoptotic bodies.

(4) Phagocytosed of apoptotic bodies by macrophages.

(5 and 6) Degradation of apoptotic bodies within secondary lysosomes.

(7) Development of necrosis is associated with irregular clumping of chromatin, swelling of organelles and disruption of membranes.

(8) Disintegration of membrane.

Table 2.2 The apoptosis inducers and apoptosis inhibitors of apoptosis process (modifile by Staunton and Gaffney, 1998)

Apoptosis inducers	Apoptosis inhibitors
1) Physiologic activators	1) Physiologic inhibitors
1.1 loss of matrix attachment	1.1 estrogen, androgen
1.2 TNF family (Fas-L, TNF)	1.2 extracellular matrix
	1.3 growth factors
2) Damaged-related inducers	2) Viral agents
2.1 bacteria toxin	2.1 adenovirus E1B
2.2 cytotoxic T cells	2.2 African swine fever virus
2.3 free radicals	(LMW5-HL)
2.4 heat shock	2.3 Baculovirus IAP
2.5 oxidants	2.4 Baculovirus P53
2.6 tumor suppressor gene; *P53, bax, bad, bclXs* others.	2.5 Cowpox virus Crma
	2.6 Epstein-Barr-virus (EBV: *BHRF1, LMP1*)
2.7 viral infection	2.7 Herpesvirus γ 134-5
3) Therapy-associated agents	3) Pharmacological agents
3.1 chemotherapeutics, free radicals	3.1 calpain inhibitors
3.2 UV radiation, gamma ray	3.2 cysteine protease inhibitor
4) Toxins	3.3 tumor promoter; PMA
4.1 ethanol	Phenobarbital, α-hexachlorocyclohexane
4.2 β-amyloid peptide	

Table 2.3 Morphological and functional distinctions between apoptosis and necrosis

Apoptosis	Necrosis
active process	passive process
cellular shrinkage	cell swelling
evolutionarity conserved pathway	toxicant-specific biochemical mechanisms
intact membrane	membrane rupture
no inflammation	inflammation
nuclear chromatin condensation	nuclear chromatin clumping

2.3.2 Apoptosis pathways

The induction of programmed cell death occurs via two major pathways: the death receptor-dependent (extrinsic) pathway through tumor necrosis factor (TNF) family ligands, or via the mitochondrial (intrinsic) pathway induced by different factors such as UV radiation, chemotherapeutics, free radicals or DNA damage (Brouckaert *et al.*, 2005).

2.3.2.1 The death receptor-dependent (extrinsic) pathway

The extrinsic pathway of apoptosis was activated through the rapid binding of cytokine ligands to receptors of the TNF-superfamily (Blagosklonny and Darzynkiewicz, 2005). These event results could activate caspase-8 and triggering the proteolytic caspase cascade (Brouckaert *et al.*, 2005). The active caspase-8 could cleave the Bcl-2 homology domain 3 (BH3) of the protein BID into the active form tBid (truncated BID) (Khosravi-Far and Esposti, 2004).

2.3.2.2 The mitochondrial (intrinsic) pathway

The mitochondrial pathway was triggered by intracellular and extracellular stress signals and result in activation of pro-apoptotic. As

a result of activation/inactivation of Bcl- family proteins (Table 2.4) changes in the mitochondrial membrane lead to the dissipation of inner membrane potential and the permeabilization of the outer mitochondrial membrane which in turn induce the release of various proapoptotic proteins such as Smac, AIF (apoptosis inducing factor; AIF), cytochrome c (Barczyk *et al.*, 2005).

Table 2.4 Gene and molecular are regulate apoptosis, modified from Barczyk *et al.* (2005).

Gene/molecule	Effects
1) *bcl-2* subfamily	
(ced9 in *Caenorhabditis elegans*)	
bcl-2	promotes survival
bcl-xl	promotes survival
bcl-w	promotes survival
bcl-xs	promotes death
2) *bax* family	
bak	promotes death
bax	promotes death
bok	promotes death
3) *BH3* subfamily	
bad	promotes death
bid	promotes death
bimL	promotes death
bik	promotes death
blk	promotes death
HRK	promotes death
Caspases 1-13 (ced3 in *C. elegans*)	Cysteinyl aspartate-aspcific protease, central part of the apoptosis cascade.

Gene/molecule	Effects
apaf-1 (ced4 in *C. elegans*)	Promoting death or surviving remain to be defined.
P53 family of tumor suppressor genes	Apoptosis induces by agents that cause DNA damage.
Nitric oxide	This molecular prevent apoptosis by altering *bcl-2* expression and by nitrosylation of caspases.
Cytochrome C	Induction of apoptosis via caspase activation.

2.3.3 Roles of caspases-3 in apoptosis

Caspase 3 plays role in the death receptor pathway, either initiated by caspase 8, or the mitochondrial pathway, involving caspase 9 (Keane *et al.*, 1999). Caspase-3 is important for typical apoptosis; chromosome condensation and DNA fragmentation in all cell types. Furthermore, active caspase3 could mediate the apoptotic cascade and respond for the cleavage of the key cellular proteins that leads to the typical morphology changes observed in cells undergoing apoptosis (Porter and Jänicke, 1999).

Many protein targets of active caspases are apoptotic indicators of morphological and biochemical changes (Degterev *et al.* 2003). Poly-ADP-ribose polymerase (PARP) was cleaved by caspase-3 and -7 (Decker and Muller, 2002). The cleavage of PARP-1 initiates two fragments of 89 and 24 kDa which is the detection of apoptosis in many cell types. (Koh *et al.* 2005).

2.3.4 Flow cytometry and apoptosis assay

Apoptosis and necrosis could be identified base on biochemical, differences in morphological and molecular changes occurring in the dying cells. Cells undergoing apoptosis morphological changes (condensation of chromatin, cytoplasm and cell shrinkage) which are measurable by flow cytometry (Van Engeland *et al.*, 1998)

Flow cytometric was evaluated by annexin V and propridium iodide (PI) staining, used to determine the form of cell death by measuring the phosphatidylserine (PS). Induction of apoptosis is associated with plasma membrane changes whereas PS was translocated from the inner layer to outer leaflet of plasma membrane which can be assessed by measuring the binding of annexin V-conjugated to fluorescense to cells by flow cytometry (Savill *et al.*, 2000) Co-staining with PI allows differentiation between necrotic and apoptotic cells. Living cells, stained negative for both annexin-V and PI (An-/PI-). Annexin-V positive and PI-negative (An+/PI-) stained cells undergo early stages of apoptosis. The late stage of apoptosis, stained positive for both AnnexinV and PI (An+/PI+). Necrotic cells, PI positive and annexinV-negative (An-/PI+) stained cells (Figure 2.6) (Vermes *et al.*, 1995).

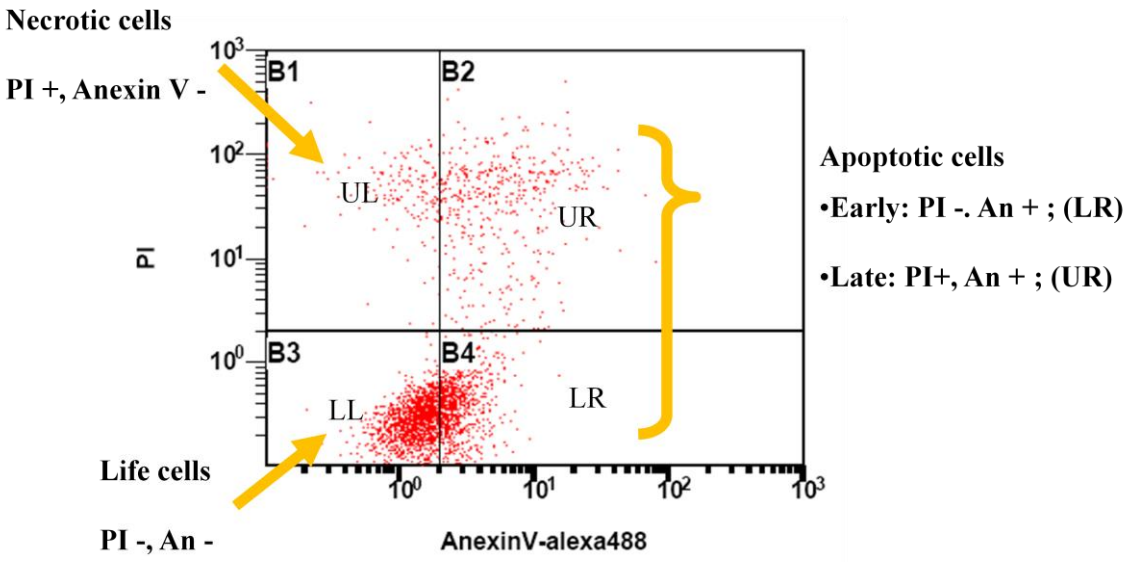

Figure 2.6 Data processing of the flow cytometry; Living cells (LL) are classified as cells stained negative for both annexin-V and PI (An-/PI-). Annexin-V positive and PI-negative (An+/PI-) stained cells undergo early stages of apoptosis (LR). AnnexinV-positive and PI-positive (An+/PI+) cells were found in the late stage of apoptosis (UR). Necrotic cells (UL) are only PI positive (An-/PI+)

2.3.5 Necrosis

Necrosis occurs when cells are exposed to extreme variance from chemical trauma and physical, resulting in damage of the cell. It is leading to an influx of water and extracellular ions. Cells swell and rupture of intracellular organelles (Table 2.3). The cytoplasmic contents including lysosomal enzymes are released into the extracellular fluid. Therefore, necrotic cell death is often associated with extensive tissue damage resulting in an intense inflammatory response (Van Furth and Van Zwet, 1988).

CHAPTER III

MATERIALS AND METHODS

3.1 Chemicals and Materials

The detail of materials and chemicals used in the study were described in Appendix A.

3.2 Preparation of crude plant extracts

3.2.1 Plant sample collection

Twenty two Thai herbal plants were selected based on their popular appearance in the rejuvenating Thai remedies. The plants were identified and authenticated by the medicinal expert of the largest traditional herbal plants in Bangkok, 1) the domestic traditional medicinal experts 2) the expert researcher; Supan Panriansaen[(1)], Thawhan Suwantemee[(1)] and Wichai Cherdshewasart[(2)]. The plant domestic names (Table 3.1) were referred to scientific name based on the reference book (Chuakul, Poani and Boonpleng, 2000). The plant samples are also being confirmed with the national herbarium.

3.2.2 Preparation of crude plant extracts

Fifty grams of plant powders, crushed and dried, were extracted with 500 ml 95% ethanol for 7 days in a blue-cap bottle with light-protect condition. The plant supernatants were filtered through No.1 filter paper. The precipitate was percolated two times with 500 ml 95% ethanol for 3 days. The total supernatants were evaporated *in vacuo*. The plant crude extracts were stored in light-protect bottle at 4°C until used (Figure 3.1).

3.2.3 Preparation of stock solution of the plant crude extracts

The plant ethanolic crude extracts (0.1 g) were mixed with 1,000 µl of dimethyl sulfoxide (DMSO) to establish the stock solution concentration of 1×10^5 µg/ml

3.2.3.1 Screening test concentration

Subsequently diluted to the working concentration of 0, 10, 100, 1,000, 10,000 and 100,000 µg/ml with DMSO

3.2.3.2 Full rang test concentration

Subsequently diluted to the working concentration of 0, 0.25, 0.75, 1.0, 1.25, 1.5, 1.75, 10, 25, 50, 75 and 100 µg/ml with DMSO

Table 3.1 The scientific name, plant family, part of plant used, sample collecting source, crude extract yield and IC_{50} of the 24 Thai rejuvenating plants in the cytotoxic assay against MDA-MB-231 cells.

	Scientific name	Domestic name	Family	Part used	Source
1.	*Acacia farnesiana*	Krathin Thet	Mimosaceae	root stem	Khon Kaen
2.	*Albizia procera*	Thingthon	Mimosaceae	bark	Khon Kaen
3.	*Anaxagorea luzonensis*	Kamlang Wua Thaloeng Kamlang	Annonaceae	whole stem whole	Chiang Mai
4.	*Betula alnoides*	Sueakhrong Kwaokrua	Betulaceae	stem tuberous	Chiang Mai
5.	*Butea superba*	Daeng	Papilionaceae	root	Chiang Mai
6.	*Cyperus rotundus*	Yha Haewmoo	Cyperaceae	rhizome stem	Bangkok
7.	*Diospyros rhodocalyx*	Takona Kamlang	Ebenaceae	bark whole	Khon Kaen
8.	*Dracaena conferta*	Hanuman	Agavaceae	stem whole	Chiang Mai
9.	*Fagraea fragrans*	Kankrao	Potaliaceae	stem	Khon Kaen
10.	*Kaempferia parviflora*	Krachai Dam	Zingiberaceae	rhizome	Bangkok
11.	*Leucaena leucocephala*	Krathin Thai	Mimosaceae	root whole	Bangkok
12.	*Melia azedarach*	Lian	Meliaceae	stem	Khon Kaen
13.	*Mucuna collettii*	Kwaokrua	Papilionaceae	whole	Chiang Mai

	Scientific name	Domestic name	Family	Part used	Source
		Dum		stem	
14.	*Phyllanthus emblica*	Makhampom	Euphorbiaceae	fruit	Sa Kaeo
15.	*Piper nigrum*	Phrikthai Kwaokrua	Piperaceae	Seed and fruit	Bangkok
16.	*Pueraria mirifica*	Khao	Papilionaceae	tuberous root	Chiang Mai
17.	*Stephania erecta*	Buabokpa	Menispermaceae	tuberous root	Chaiyaphum
18.	*Stephania venosa*	Boraphet Pungchang	Menispermaceae	tuberous root	Sakon Nakhon
19.	*Streblus asper*	Khoi	Moraceae	seed	Bangkok
20.	*Suregada multiflorum*	Khunthong phayabat	Euphorbiaceae	stem and leaves	Khon Kaen
21.	*Tinospora crispa*	Boraphet	Menispermaceae	whole stem	Khon Kaen
22.	*Vitex trifolia*	Khonthiso	Verbenaceae	stem bark	Khon Kaen

3.3 Cell line and cell culture

3.3.1 Cell line

MDA-MB-231 (ATCC **No.TCP-1002**) (Figure 3.2), the human estrogen receptor negative (ER-negative) breast cancer cell line was derived from pleural effusion of a Caucasian female 51 years old patient. The cells were epithelial cells of mammary gland. The cells were purchased from American Type Culture Collection (ATCC) in February 2010.

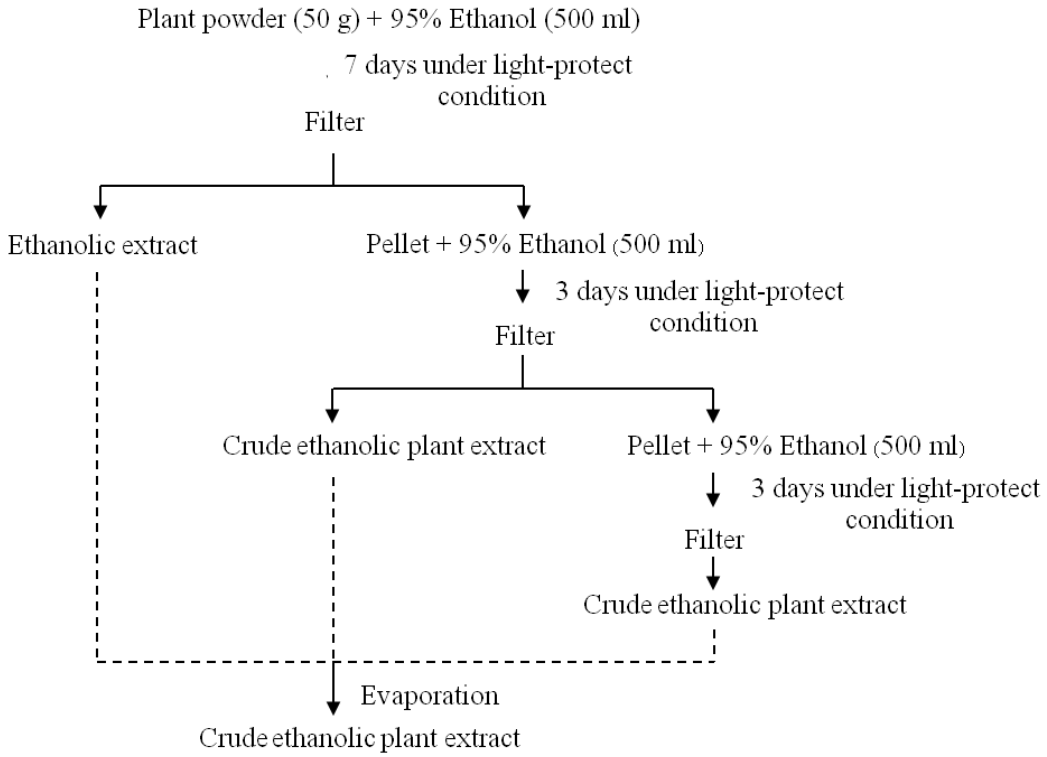

Figure 3.1 The preparation of crude ethanolic plant extracts

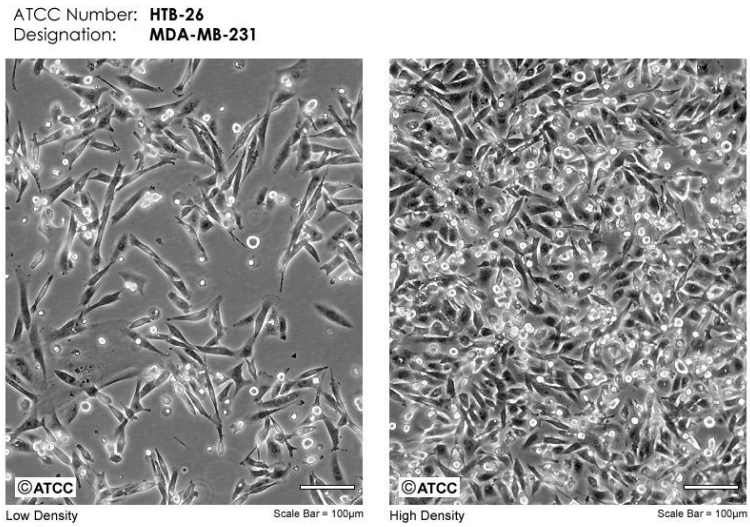

Figure 3.2 MDA-MB-231, the human estrogen receptor negative (ER-negative) breast cancer cell line (http://www.atcc.org)

3.3.2 Cell culture

3.3.2.1 Culture of MDA-MB-231 breast cancer cells

The human estrogen receptor negative (ER-negative) MDA-MB-231 breast cancer cells was grown in Leibovit's L-15 medium containing 10% FBS, 1% penicillin- streptomycin and 0.01% Amphotencin B in 1 liter of medium at 37 °C, 95% RH and 5% CO_2 incubator.

3.3.2.2 Subculture of MDA-MB-231 cells

The culture medium was removed from the culture flask and washed twice with 5 ml phosphate buffer saline (PBS). The cells were detached from the surface of the flask by trypsinization technique with 2.0 ml of 0.05% trypsin in 0.01% EDTA for 1-2 minutes. The supernatant was discarded. The cells were resuspended in a fresh culture medium and transferred into a new culture flask. The culture medium was added to the final volume of 10 ml and cultured in the 37ºC, 5% CO_2 incubator.

3.3.2.3 Cell suspension preparation for assay

MDA-MB-231 cells were propagated 3-4 days before starting of the experiment. The cells were rinsed with 5 ml of PBS followed by removal of the solution. The cells were detached from the surface of the flask with the aid of 2.5 ml 0.05% trypsin in 0.01% EDTA for 1-2 minutes. A fresh culture medium was added and aspirated gently with the aid of a pipette in order to dissociate the cell clumps into single cells. The cell suspensions were transferred to a 15 ml conical tube and centrifuged at 2,500 g_{max}, 4 C° for 10 minutes. The supernatant was discarded and the pellet was resuspended with a fresh culture medium.

3.3.2.4 Cell count

The cells were counted with the aid of a haemocytometer under an inverted microscope. The cell suspensions were diluted with culture medium (1:10, V/V) and the 20 µl cell suspension were mixed with 20 µl

0.4% Trypan blue solution with the aid of a Pasture pipette and allowed to stand for 10 min. Trypan blue will only penetrate through the membranes of the non-viable cells. Ten microliters of stained cells were placed in a hemocytometer and counted for the number of viable (unstained) cells in the 1 mm middle square and 1 mm four corner squares of the hemocytometer (Figure 3.3).

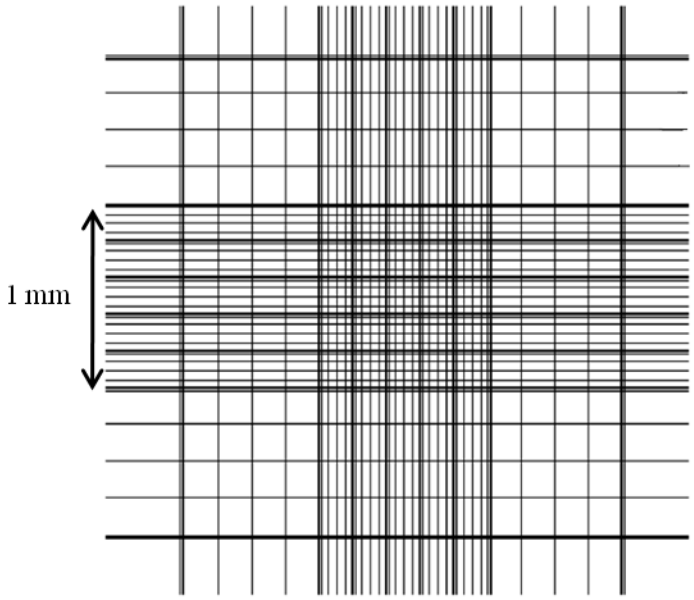

Figure 3.3 Magnified view of the cell counting chamber grid. The central 1 mm^2 area is divided into 25 smaller squares, 1/25 mm^2 each and are further subdivided into 16 squares, each 1/400 mm^2

The space between the slide and the cover slip was 0.1 mm. Each square of the haemacytometer was represented a total volume of 0.1 mm^3. The subsequent cell density per ml was calculated using the following equation:

Cell density = average cell count per square (1 mm) x dilution factor x 10^4; number of cells/ml

Cell/ml = (total cell count (from 5 square of 1 x 1 mm) /5) x (10 (cell dilution) x 2 (dilution factor from Trypan blue)) x 10^4 (a conversion volume from the surface area of 1x1 mm with a depth of 0.1 mm)

To start the experiment, the amount of cells used (desired cell density = 2.5 x 10^4 cells/ml) was calculated as follows; Dilution factor (x) = cell per ml/2.5 x 10^4

Diluted cell suspension with culture medium to desirable volume (y)

Media x-1 ml: Cell 1 ml

Media y ml: Cell z ml

(z = cell volume for dilution)

3.4 Antiperliferation assay

3.4.1 Inhibition concentration; IC

3.4.1.1 The stock solution of plant crude extracts (refer to 3.2.3) was mixed with L-15 medium containing 10% FBS. (2% V/V of substrate solution)

3.4.1.2 Cells were plated overnight at a density of 5×10^4 cells/well in the 96-well plate and subsequently treated with the final concentration of plant crude extracts (0, 0.1, 1, 10, 100 and 1,000 µg/ml; 1% V/V of substrate solution) for 3 days prior to MTT assay.

3.4.2 Cell viability test by MTT assay

Cell viability was evaluated by MTT assay which requires cellular metabolic activity to convert the colorless tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to the purple-color formazan. The 10 µl/well of MTT solution (0.5 mg/ml) were added and the plate was incubated for 4 hours at 37 °C. The supernatant was aspirated, and the formazan crystals formed were solubilized in 150 µl DMSO for 5 minutes. The absorbance of the solution was analyzed at 540 nm with the aid of a microplate reader. The results

were shown in line graph between the percentage of cell viability (Y-axis) and the concentrations of each sample (X-axis) and calculated the concentration of 50% cytotoxicity (IC_{50}).

Calculation of the percentage of cell viability

$$\text{The percentage of cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of vehicle cells}} \times 100$$

The IC_{50} value could calculate from this curve. It was defined as the 50% reduction of the absorbance or 50% of the percentage of cell viability compared with cells that were treated by DMSO as a negative control in MTT assay.

3.5 Annexin V binding assay to detect apoptosis cells (Van Engeland *et al.* (1996) and Casciola-Rosen *et al.* (1996))

3.5.1 Cell suspension and preparation for assay refer to 3.3.2.3

3.5.2 Preparation of the tested cells

3.5.2.1 Cells were plated overnight at a density of 5×10^4 cells/500 µl (1×10^5 cells/ml) into 6 well-plate and then treat with the three selected Thai rejuvenating herb at the IC_{50} values; *S. venosa, P. nigrum and S. erecta* which induced significant apoptosis of MDA-MB-231 cells at 48 and 72 hours incubation.

3.5.2.2 Cells were harvestd by trypsinization. The collected supernatants and cell pellets were washed twice with cold PBS.

3.5.3 Flow cytometry analysis

3.5.3.1 Cells were transferred (~1 x 10^5 cells) into a 1.5 ml centrifuge tube and 50 µl of 1x binding buffer solution containing 5 µl annexin V-Alexa Fluor®488 and 5 µl of propidium iodide (PI; 50 µg/ml) was added into the tube.

3.5.3.2 The cells were gently mixed and incubated for 30 minutes at room temperature in the dark

3.5.3.3 150 µl of 1x binding buffer solution was added to the tube.

3.5.3.4 The cell sample was analyzed by flow cytometry within 1 hour.

**Suggested controls for flow cytometric analysis of annexin V samples. The controls used to set up compensation and quadrants: Alexa Fluor®488, comprised;

1) Unstained cells
2) Cells stained with Annexin V- Alexa Fluor®488 alone (no PI)
3) Cells stained with PI alone (no Annexin V- Alexa Fluor®488)

3.6 Immunoblotting analysis

3.6.1 Preparation of the tested cells

Cells were plated overnight at a density of 1×10^5 cells/ml into 75 cm^2 culture flask and then treat with the three selected Thai rejuvenating herb; *S. venosa*, *P. nigrum and S. erecta* protein expression of MDA-MB-231 cells for 24, 48 and 72 hours (conc; IC_{50}).

3.6.2 Harvest cells and whole cell extracts

3.6.2.1 The medium within the culture flask was removed. The attach cells were washed three times with 2 ml of 1x PBS. The attach cells were imbedded in 150 µl of Radio Immune Precipitation

Assay (RIPA) buffer containing protease inhibitor cocktail (500:1, V/V) before harvest with the aid of a scraper of a size 25 cm. The harvested cells were incubated at room temp about 1 hour.

3.6.2.2 The cell suspension was vortex for 30 seconds and subsequently sonicated on ice to aid extraction of proteins and then centrifuged at 13,200 g_{max}, 4°C for 10 minutes. The supernatants were collected and transferred into a new 1.5 micro-centrifuge tube.

3.6.3 Measurment of protein concentration

3.6.3.1 Protein concentrations were duplicated measured by Bradford technique with a BSA standard curve reference.

3.6.3.2 The 15-20 µg proteins were mixed with sample running buffer and heated to 80-100 °C for 5 minutes. The protein samples could be kept at -80°C.

3.6.4 SDS-PAGE gel and Western blot

3.6.4.1 SDS-PAGE gel

Proteins were loaded onto 7.5% (for PARP), 12.5% SDS-PAGE at the constant 10 mA/gel for 2-2.5 hours and run on a SDS-PAGE until the green line (10 kDa) of protein marker is present at the bottom of the gel.

3.6.4.2 Western blot

3.6.4.2.1 In an electrophoretic transfer, the membrane and protein-containing gel were placed together with the filter paper between two electrodes (Figure 3.4). Proteins were migrated from gel to the membrane at 100 V for 1 hour at 4°C.

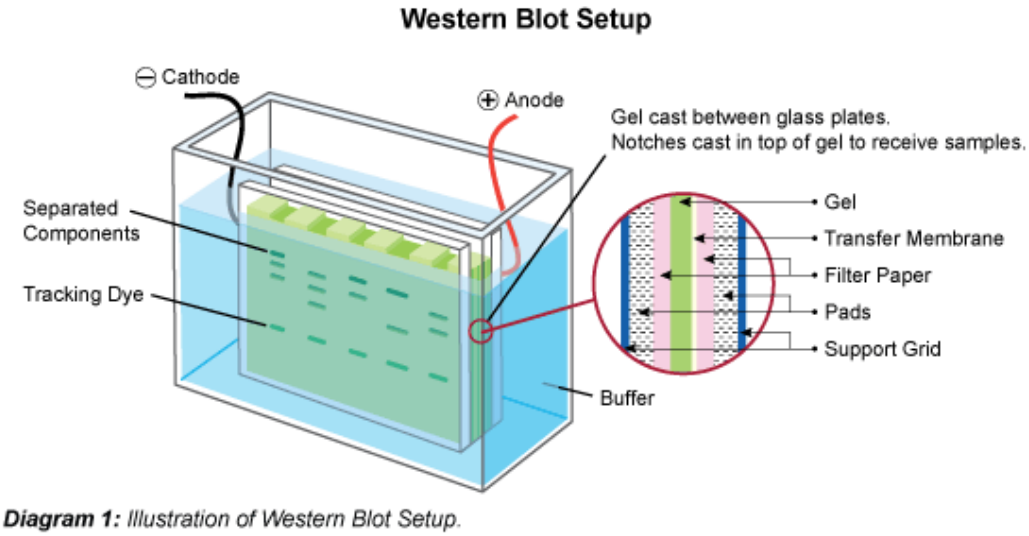

Diagram 1: *Illustration of Western Blot Setup.*

Figure 3.4 Assemble transfer sandwich by orientating cathode, fiber pad, filter paper, gel, membrane, filter paper, fiber pad and anode so protein transfer goes in the direction of cathode to anode (http://www.leinco.com/general_wb)

3.6.4.2.2 The membrane was blocked with the blocking solution no.1 (1x TBS/T (Tris-buffered saline + 0.1% Tween-20 + 10% BSA). The blocking solution (10-20 ml/membrane) was added and incubated for 1 hr in a small box on a shaker.

3.6.4.2.3 The membrane was blocked with the blocking solution no.2 for PARP (Tris-buffered saline + 0.1% Tween-20 + 5% skim milk). The blocking solution (10-20 ml/membrane) was added and incubated for 1 hr in a small box on a shaker.

3.6.4.2.4 The blocking solution was removed and washed three times with 1x TBS/T at RT in a small box on a shaker. (Used 10-15 minutes in ~50 ml 1x TBS/T)

3.6.4.2.5 The membrane was incubated with the primary antibody (The dilution of first antibody shown in Table 3.2) specific to protein of interest for overnight at 4°C

in a seal-a-meal bag. Membranes can be stacked back to back in one bag. The primary antibody mix can be re-used (store at 4ºC.)

3.6.4.2.6 The primary antibody was removed and washed three times with 1x TBS/T at RT in a small box on a shaker. (Used 10-15 minutes in ~50 ml 1x TBS/T)

3.6.4.2.7 The membrane was blocked with the blocking solution no.2 (1x TBS/T (Tris-buffered saline + 0.1% Tween-20 + 5% skim milk) and subsequently incubated with the secondary antibody (The dilution of secondary antibody shown in Table 3.2) for 45 minutes at RT.

3.6.4.2.8 The membrane was washed three times with 1x TBS/T at RT in a small box on a shaker. (Used 10-15 minutes in ~50 ml 1x TBS/T)

Table 3.2 The dilution of the first and second antibody in the immunoblotting assay

Protein	MW (kDa)	First antibody dilution	Secondary antibody	Secondary antibody dilution
Bcl-2	26	1 : 1,000	Rabbit	1 : 2,000
BID	15, 22	1 : 1,000	Rabbit	1 : 2,000
Caspase-3	17, 19, 35	1 : 500	Rabbit	1 : 2,000
Caspase-8	18, 43, 57	1 : 1,000	Mouse	1 : 2,000
PARP	24, 89, 116	1 : 1,000	Rabbit	1 : 2,000
α-tubulin	52	1 : 1,000	Mouse	1 : 2,000

3.6.4.2.9 The membrane was incubated for 5 minutes with the enhanced chemiluminescence reagent. The positive signals on the membranes were detected with the high-performance film.

3.7 Statistical analysis

The results were shown as mean ± standard deviation (S.D.) of five replicated experiments. Statistical analysis was performed using a one-way ANOVA for the analysis of the test results and Duncan analysis of variance at the significance levels of $p<0.05$ were considered significantly. (SPSS® version 14.0)

CHAPTER IV

RESULTS

4.1 Characteristics of the plant crude extracts

The plant crude extracts were successively obtained from the 22 plant samples after ethanolic extraction and evaporation *in vacuo*. The percentage yields and the characteristics of the 24 ethanolic plant crude ethanolic extracts are shown in Table 4.1.

Table 4.1 The percentage yields and characteristics of plant extracts

Scientific name	Yield of extract (%)	Crude extract characteristic
Acacia farnesiana	12.36	light brown sticky
Albizia procera	18.2	red-brown crystal
Anaxagorea luzonensis	4.72	yellow viscous
Betula alnoides	23.22	red-brown solid
Butea superba	15.14	brown sticky
Cyperus rotundus	4.9	red-brown viscous
Diospyros rhodocalyx	4.74	black sticky
Dracaena conferta	4.52	red powder
Fagraea fragrans	11.64	brown sticky
Kaempferia parviflora	2.64	green-yellow viscous
Leucaena leucocephala	4.9	red-brown sticky
Melia azedarach	18.84	black-brown powder
Mucuna collettii	4.76	red-brown viscous
Phyllanthus emblica	9.96	brown viscous

Scientific name	Yield of extract (%)	Crude extract characteristic
Piper nigrum (seed)	11.08	black-brown viscous
Piper nigrum (fruit)	9.63	yellow viscous
Pueraria mirifica	14.72	light yellow solid
Stephania erecta	10.82	red-brown powder
Stephania venosa	5.34	red-brown solid
Streblus asper	4.18	green-brown viscous
Suregada multiflorum (stem)	10.56	black sticky
Suregada multiflorum (leaf)	12.42	yellow viscous
Tinospora crispa	4.52	brown viscous
Vitex trifolia	5.26	yellow-brown solid

4.2 Anti-proliferative effects by ethanolic crude extracts treatment in MDA-MB-231

The anti-proliferative assay of the 24 plant ethanolic crude extracts against MDA-MB-231 cells revealed that the cell survival rates were decreased significantly following the treatment with certain plant crude extracts in a dose-dependent manner as determined by the 50% growth inhibitory concentration (IC_{50}) compared with a standard natural compound genistein, as show in (Table 4.2).

The 24 plant ethanolic crude extracts antiproliferation to the tested cells were categorized into three classes according to Balantyne *et al.* (1999) as follows; the potentially toxic, 10-100 µg/ml were *S. venosa*, *P. nigrum* (seed), *S. erecta*, *P. nigrum* (fruit) and *D. rhodocalyx* extracts respectively (Table 4.2; ranking no.1-5), the potentially harmful, 100-1000 µg/ml were *C. rotundus*, *S. multiflorum* (leaf), *S. asper*, *B. superba* and *S. multiflorum* (stem) and *A. luzonensis* extracts respectively (Table 4.2; ranking no.6-11) and the potentially non toxic, greater than 1,000 µg/ml, were *A. farnesiana*, *A. procera*, *B. alnoides*, *D. conferta*, *F. fragrans*, *K. parviflora*, *L.*

leucocephala, *M. azedarach*, *M. collettii*, *P. emblica*, *P. mirifica*, *T. crispa* and *V. Trifolia* extracts respectively (Table 4.2; ranking no.12-24).

Table 4.2 Ranking of antiproliferation of the plant ethanolic crude extracts against MDA-MB-231 cells determined with MTT assay presented as IC_{50} values (Mean ± S.D.) in comparison with genistein (positive control).

Ranking no.	Rejuvenating herbal plant	IC_{50} (µg/ml)
1	*Stephania venosa*	24.85±0.04
2	*Piper nigrum* (seed)	31.34±0.02
3	*Stephania erecta*	32.42±0.01
4	*Piper nigrum* (fruit)	32.74±0.02
5	*Diospyros rhodocalyx*	45.08±0.04
6	*Cyperus rotundus*	126.42±0.03
7	*Suregada multiflorum* (leaf)	154.08±0.01
8	*Streblus asper*	190.82±0.02
9	*Butea superba*	310.31±0.05
10	*Suregada multiflorum* (stem)	425.08±0.02
11	*Anaxagorea luzonensis*	731.57±0.03
12	*Acacia farnesiana*	>1,000
13	*Albizia procera*	>1,000
14	*Betula alnoides*	>1,000
15	*Dracaena conferta*	>1,000
16	*Fagraea fragrans*	>1,000
17	*Kaempferia parviflora*	>1,000
18	*Leucaena leucocephala*	>1,000
19	*Melia azedarach*	>1,000

Ranking no.	Rejuvenating herbal plant	IC_{50} (µg/ml)
20	*Mucuna collettii*	>1,000
21	*Phyllanthus emblica*	>1,000
22	*Pueraria mirifica*	>1,000
23	*Tinospora crispa*	>1,000
Positive control	Genistein	10×10^{-6} M

The growth curves of the MDA-MB-231 cells treated with the plant ethanolic crude extracts with the top three of the most potent according to their IC_{50} values from Table 4.2 are demonstrated in Figure 4.1-4.3. Genistein was used as a standard natural compound in the study. Genistein didn't showed dose-dependent antiproliferation activity on MDA-MB-231cells (data not show).

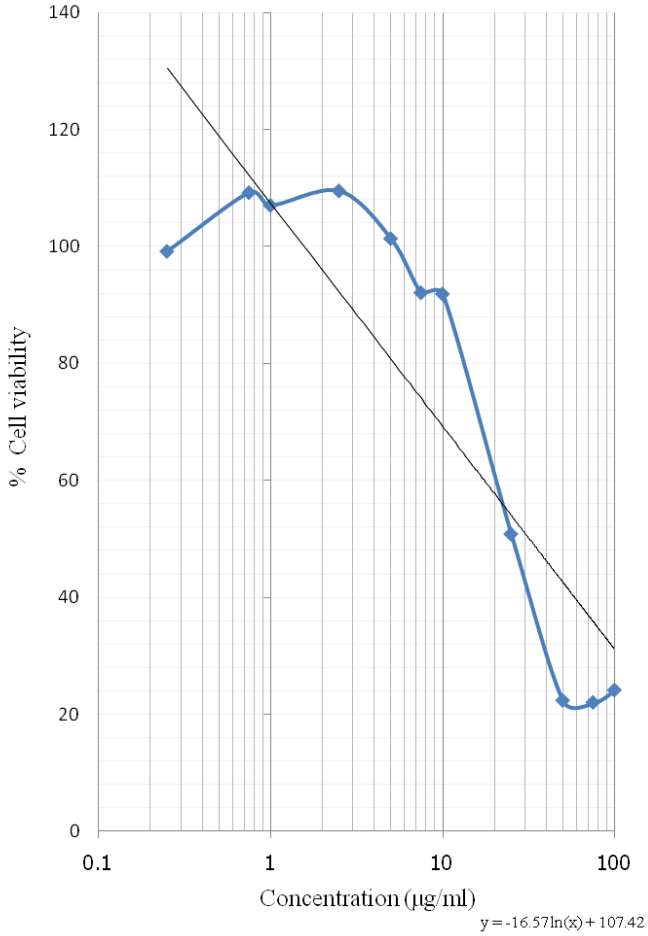

Figure 4.1 The anti-proliferation effect of *S. venosa* ethanolic crude extract against MDA-MB-231 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of ethanolic crude extracts**.**

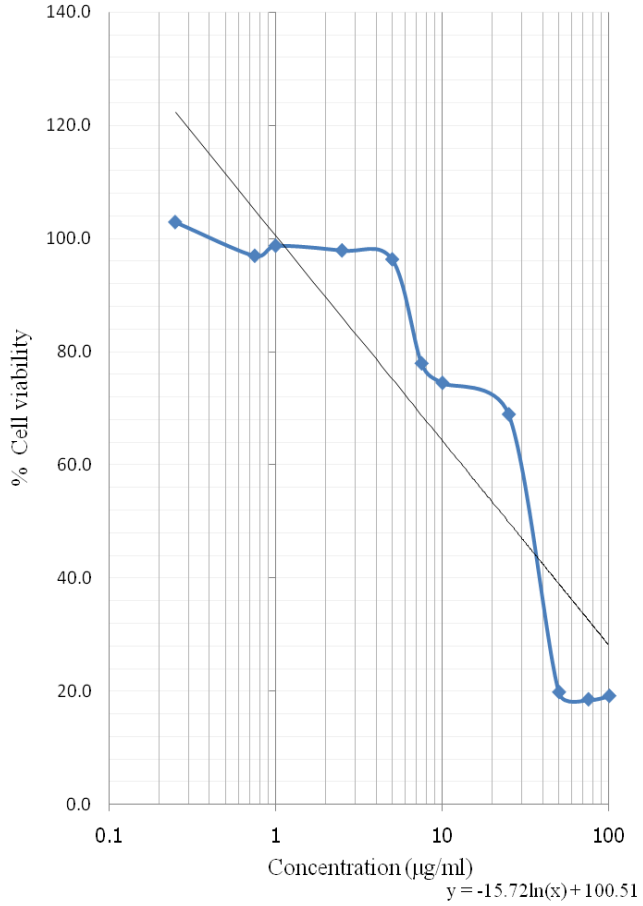

Figure 4.2 The anti-proliferation effect of *P. nigrum* (seed) ethanolic crude extract against MDA-MB-231 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of ethanolic crude extracts.

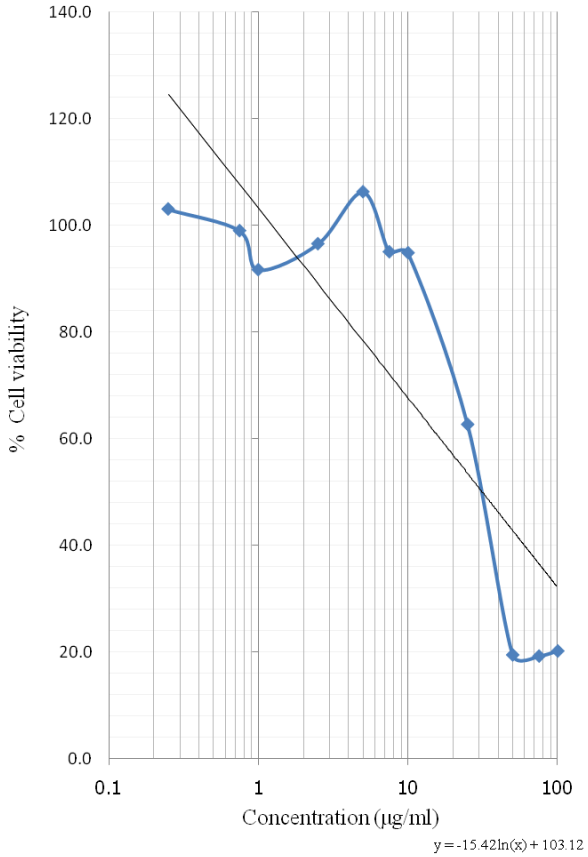

Figure 4.3 The anti-proliferation effect of *S. erecta* ethanolic crude extract against MDA-MB-231 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of ethanolic crude extracts**.**

4.3 Cell death assay analyzed by flow cytometry.

4.3.1 Morphology change after treat with the plant ethanolic crude extract

The morphology of MDA-MB-231 cells treated with the plant ethanolic crude extract were compared with the DMSO control cells (negative control) (Figure 4.4). Genistein, positive control for apoptotic cells (Figure 4.5A), and H_2O_2, positive control for necrotic cells (Figure 4.5B) under a visualization with the aid of a phase-contrast inverted microscope.

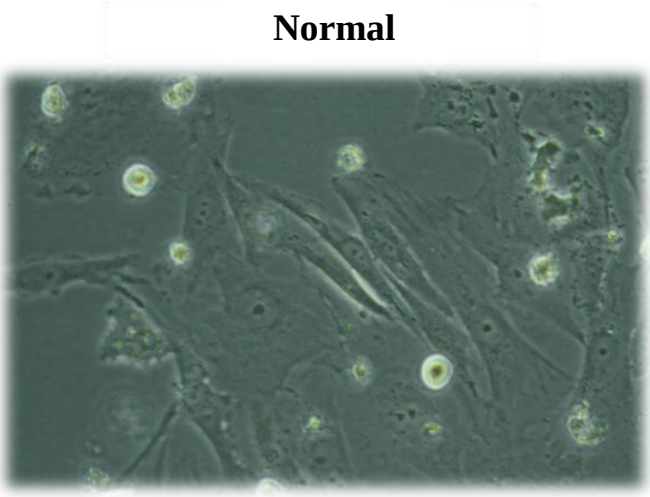

Figure 4.4 The morphology of MDA-MB-231 cells treated with DMSO, the negative control. Cells were photographed using an inverted microscopy and magnification ×200**.**

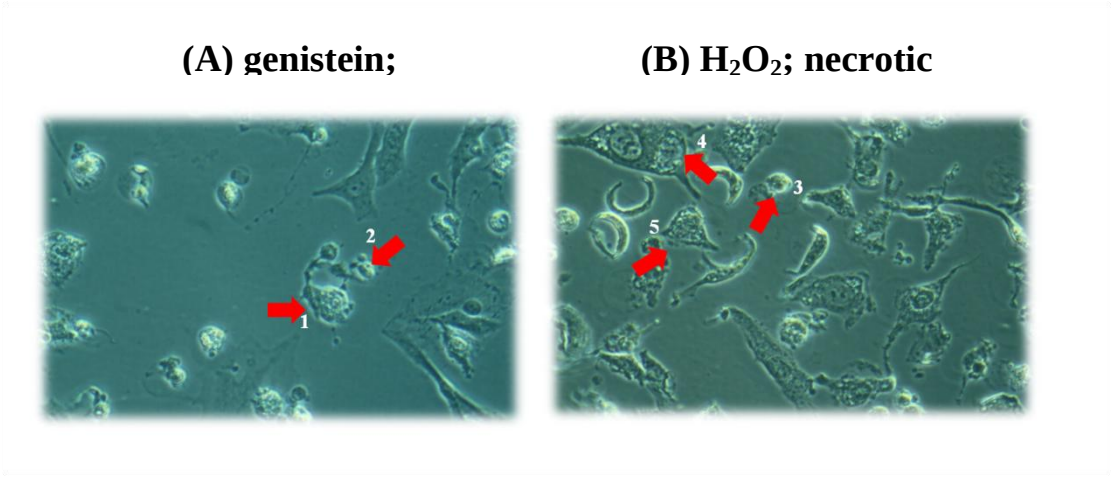

Figure 4.5 The morphology of MDA-MB-231 cells treated with genistein (A) and H_2O_2 (B), respectively. Poit: cell shrinkage(1), plasma membrane blebbing(2), lost of anchorage ability(3), cell swelling(4) and disruption of membranes(5). Cells were photographed using an inverted microscopy and magnification ×200.

The cell shrinkage(1), plasma membrane blebbing (apoptotic body)(2) round and lost their anchorage ability(3) were found in the genistein treated groups (Figure 4.5 (A)) conversely, the cell swelling(4) disruption of membranes(5) and lost of their anchorage ability(3) were found in the H_2O_2 treated groups (Figure 4.5 (B)). In the plant ethanolic crude extract treated groups; *S. venosa*, *S. erecta and P. nigrum* extracts as showed in Figure 4.6, 4.7 and 4.8, respectively. The morphological of the cells were changed by crude extract treat groups, cell shrinkage(1), plasma membrane blebbing (apoptotic body)(2) round and lost their anchorage ability(3).

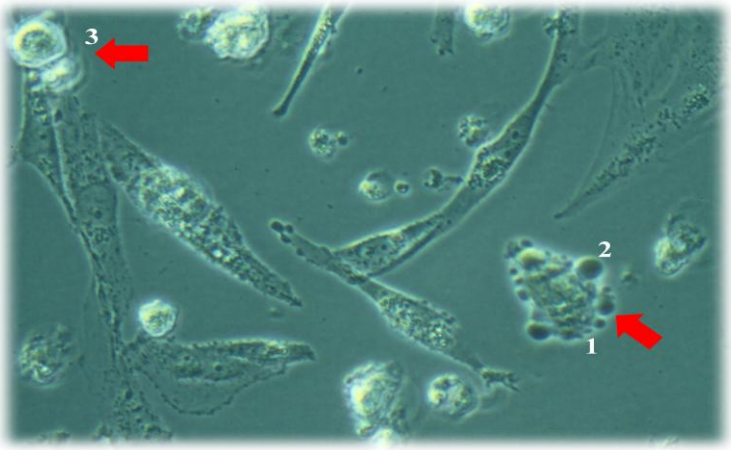

Figure 4.6 The morphology of MDA-MB-231 cells were treated with *S. venosa* ethanolic crude extract. Cells were photographed using an inverted microscopy and magnification ×200**.**

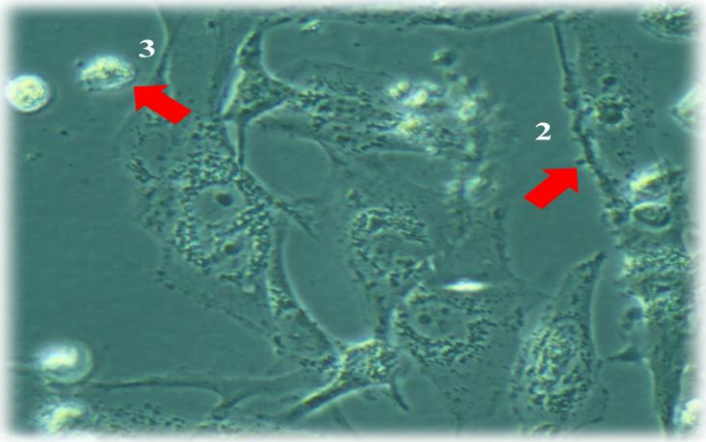

Figure 4.7 The morphology of MDA-MB-231 cells were treated with *S. erecta* ethanolic crude extract. Cells were photographed using an inverted microscopy and magnification ×200**.**

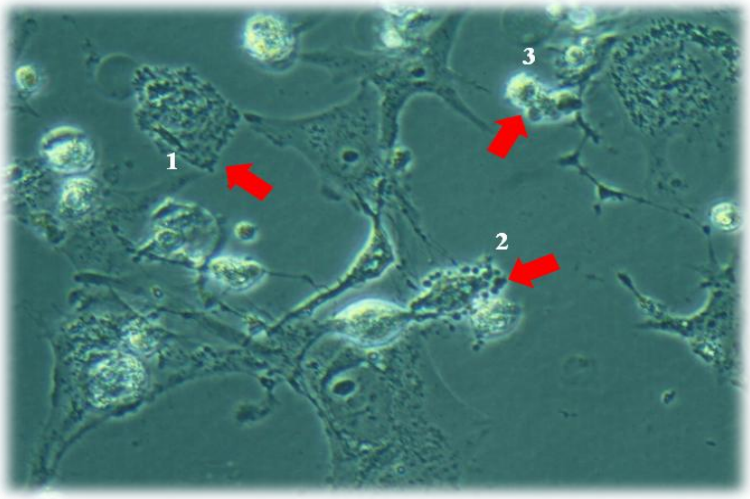

Figure 4.8 The morphology of MDA-MB-231 cells were treated with *P. nigrum* ethanolic crude extract. Cells were photographed using an inverted microscopy and magnification ×200**.**

4.3.2 Data processing of two-parameter (dual-color fluorescence) histograms

Two-parameter histograms of the apoptotic MDA-MB-231 cells derived from the exposure to the plant crude extracts for 48 h and 72 are shown in Figure 4.9 and 4.10, respectively. The percentage of apoptotic cells after treatment with 48 h and 72 h of plant ethanolic crude extract in quadrant analysis derived from the flow cytometric analysis are shown in Table 4.3.

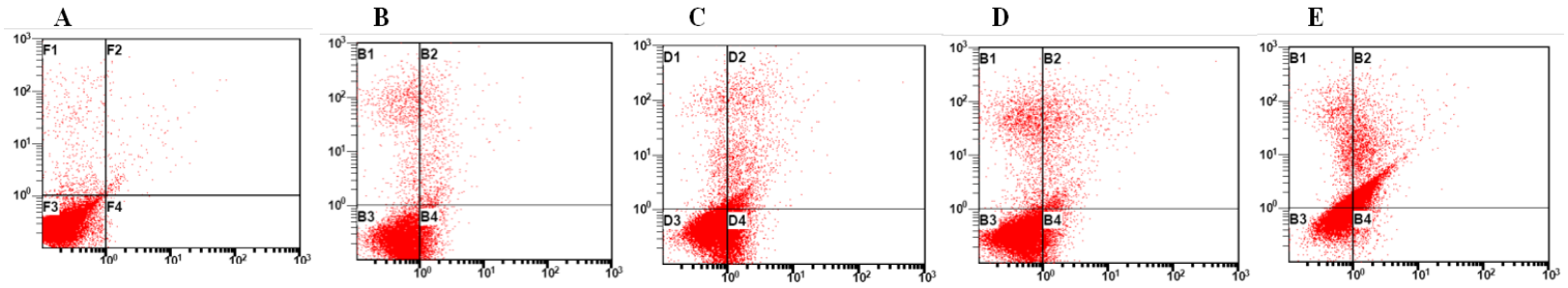

Figure 4.9 Two-parameter histograms; propridium iodide (Y-axis) and annexin V-Alexa Fluor®488 (X-axis). The cells were exposed to the plant crude extract *S. venosa* (C), *P. nigrum* (D) and *S. erecta* (E), respectively for 48 hours in comparison with DMSO; negative control (A) and genistein; positive control (B)

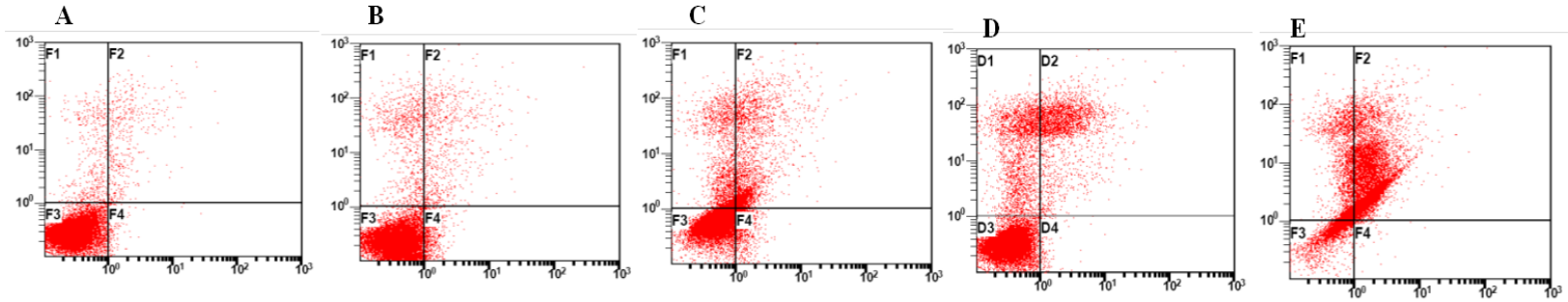

Figure 4.10 Two-parameter histograms; propridium iodide (Y-axis) and annexin V-Alexa Fluor®488 (X-axis). The cells were exposed to the plant crude extract *S. venosa* (C), *P. nigrum* (D) and *S. erecta* (E), respectively for 72 hours in comparison with DMSO; negative control (A) and genistein; positive control (B)

Table 4.3 The percentage of apoptotic MDA-MB-231 cells after treatment with plant ethanolic crude extract for 48 and 72 hours derived from the quadrant analysis of the flow cytometry

Treatment 48 hours	IC_{50} (µg/ml)	Viable cells (%)	Necrotic cells (%)	Total apoptotic cells (%)	Total apoptotic cells/necrotic cells
control (DMSO)	-	89.35 ± 0.06^{d}	3.21 ± 0.003^{a}	7.44 ± 0.06^{a}	2.32
Genistein	10 µM	73.20 ± 0.1^{bc}	7.03 ± 0.02^{b}	19.77 ± 0.11^{a}	2.81
S. venosa	24.85 ± 0.04	62.74 ± 0.01^{b}	4.81 ± 0.01^{ab}	32.45 ± 0.01^{b}	6.75
P. nigrum	31.34 ± 0.02	74.41 ± 0.03^{c}	10.07 ± 0.01^{c}	15.52 ± 0.04^{a}	1.54
S. erecta	32.42 ± 0.01	38.15 ± 0.04^{a}	24.78 ± 0.02^{d}	37.07 ± 0.05^{b}	1.50

Treatment 72 hours	IC_{50} (µg/ml)	Viable cells (%)	Necrotic cells (%)	Total apoptotic cells (%)	Total apoptotic cells/necrotic cells
control (DMSO)	-	89.97 ± 0.06^{c}	3.47 ± 0.01^{a}	6.56 ± 0.04^{a}	1.89
Genistein	10 µM	86.38 ± 0.06^{c}	4.22 ± 0.01^{a}	9.40 ± 0.05^{ab}	2.23
S. venosa	24.85 ± 0.04	60.97 ± 0.05^{b}	20.39 ± 0.05^{bc}	18.63 ± 0.02^{c}	0.91
P. nigrum	31.34 ± 0.02	66.11 ± 0.03^{a}	17.00 ± 0.01^{c}	16.89 ± 0.05^{d}	0.99
S. erecta	32.42 ± 0.01	16.10 ± 0.08^{b}	24.72 ± 0.05^{b}	59.18 ± 0.03^{bc}	2.39

4.4 Immunoblotting of apoptosis-related proteins

To confirm the effect of the ethanolic crude plant extract on apoptotic protein expression of MDA-MB-231 cells, immunoblotting (Western blot) analysis was performed for apoptosis- and transformation- related proteins to confirm for the expression patterns derived from proteome analysis. Six antibodies including caspase-3, PARP, Bcl-2, caspase-8 and BID were used against the internal control protein (α-tubulin).

Immunoblotting analyses were performed with the protein extracts from MDA-MB-231 cells treated with the plant ethanolic crude extracts at IC_{50} for 24 and 48 hours

4.4.1 Immunoblotting analysis (Western blot) of α-tubulin protein

α-tubulin protein was used as an internal loading control (Figure 4.11).

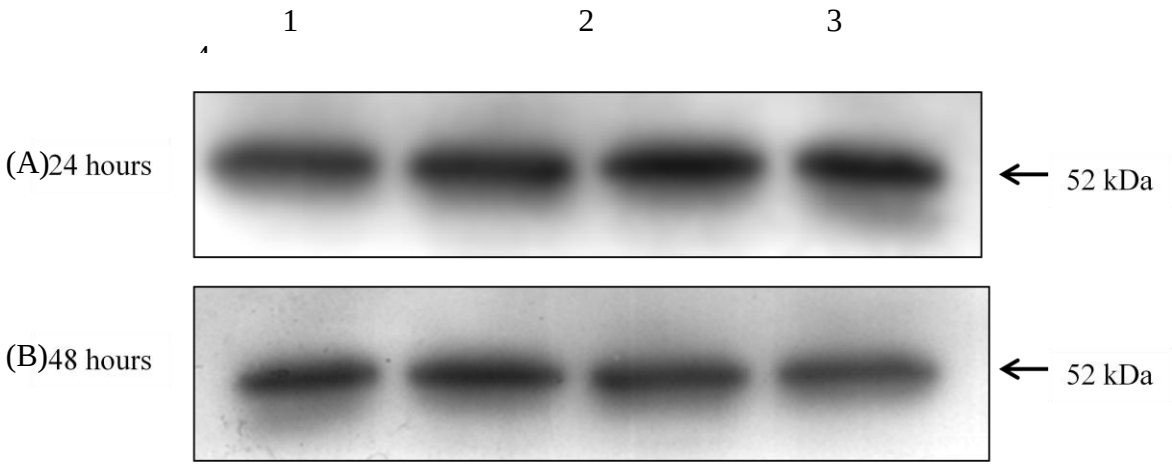

Figure 4.11 Immunoblotting analysis of the expression of α-tubulin protein in MDA-MB-231 cells after treatment with plant crude extract for 24[(A)] and 48 hours[(B)]. Lanes: DMSO control[(1)], *S. venosa* treated[(2)], *S. erecta* treated[(3)], *P. nigrum* treated[(4)] .

4.4.2 Apoptosis confirmation via activation of caspase-3 and cleavage of PARP

Caspase-3 is a typical hallmark of apoptosis. The active form of caspase-3 is responsible for the cleavage of PARP that will block DNA repair and cell becomes apoptosis. During apoptosis process, PARP was inactivated because it was cleaved by caspase-3 into two fragments. PARP cleavage is an established and reliable apoptosis indicator downstream of caspase activation (Poter and Jänicke, 1999).

4.4.2.1 Immunoblotting analysis (Western blot) of caspase-3

The expression and activation of procaspase-3 was presented by the plant ethanolic crude extract treatment for 24 and 48 hours. The 34 kDa of procaspase-3 was converted to cleaved caspase-3 of lower molecular weight, consisting of the 19 and 17 kDa active forms in a similar level.

The immunoblotting analysis for 24 hours showed the overexpression of cleaved caspase-3 after treatment with *S. erecta* and *P. nigrum* in a similar level (Figure 4.12).

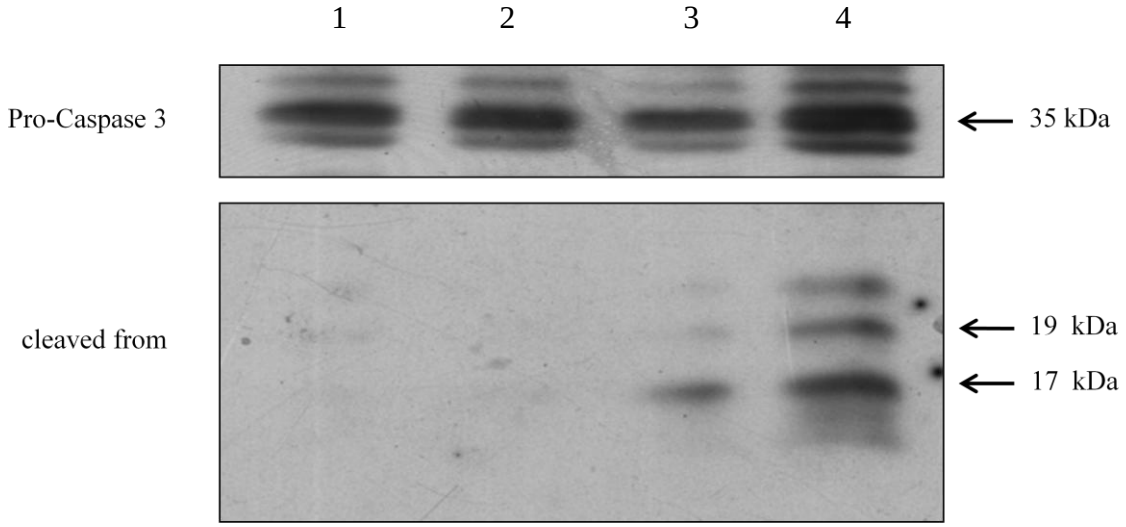

Figure 4.12 Immunoblotting analysis of the expression of caspase-3 protein in MDA-MB-231 cells after treatment with plant crude extract for 24 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

The immunoblotting analysis for 48 hours showed the overexpression of cleaved caspase-3 after treatment with *S. venosa* and *S. erecta* in a similar level (Figure 4.13).

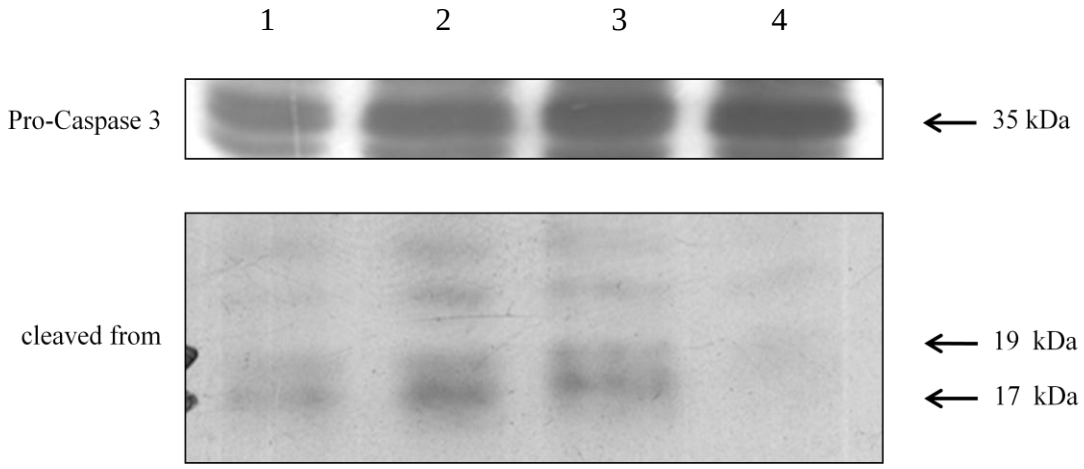

Figure 4.13 Immunoblotting analysis of the expression of caspase-3 protein in MDA-MB-231 cells after treatment with plant crude extract for 48 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

4.4.2.2 Immunoblotting analysis (Western blot) of PARP

The quantitative results of uncleaved 116 kDa pro-form of PARP was only seen in untreated control while in the plant ethanolic crude extract treatment resulted in appearance of the active 89 kDa cleaved fragments in a similar level.

The immunoblotting analysis for 24 hours showed the overexpression of cleaved PARP after treatment with *S. erecta* and *P. nigrum* in a similar level (Figure 4.14).

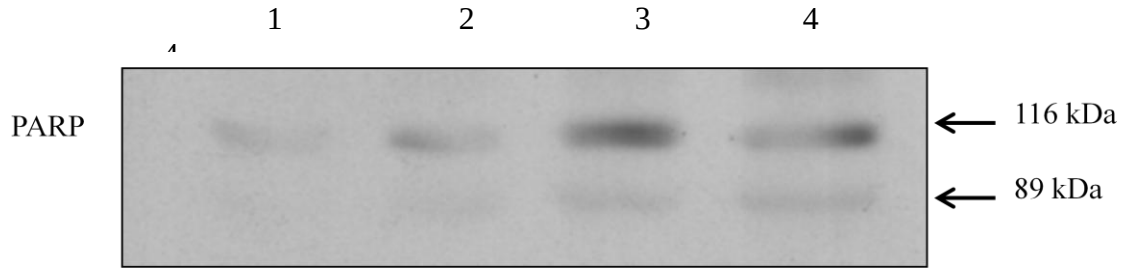

Figure 4.14 Immunoblotting analysis of the expression of PARP protein in MDA-MB-231 cells after treatment with plant crude extract for 24 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

The immunoblotting analysis for 24 hours showed the overexpression of cleaved PARP after treatment with *S. venosa, S. erecta* and *P. nigrum* in a similar level (Figure 4.15).

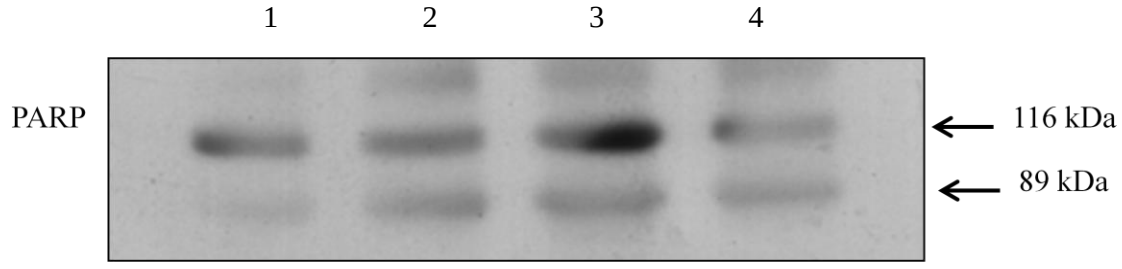

Figure 4.15 Immunoblotting analysis of the expression of PARP protein in MDA-MB-231 cells after treatment with plant crude extract for 48 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

4.4.3 Apoptosis confirmation via mitochondrial protein; Bcl-2

4.4.3.1 Immunoblotting analysis (Western blot) of Bcl-2

The immunoblotting analysis showed the underexpression of Bcl-2 after treatment with *S. venosa, S. erecta* and *P. nigrum* in a similar level (Figure 4.16).

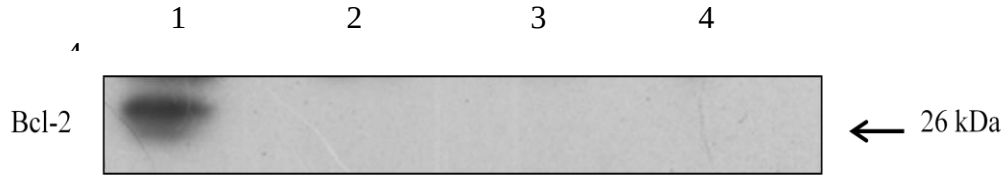

Figure 4.16 Immunoblotting analysis of the expression of Bcl-2 protein in MDA-MB-231 cells after treatment with plant crude extract for 24 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

The immunoblotting analysis showed the underexpression of Bcl-2 after treatment with *S. erecta* and *P. nigrum* but overexpression of Bcl-2 after treatment with *S. venosa* in a similar level (Figure 4.17).

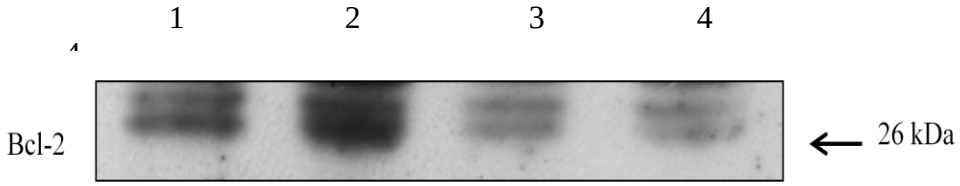

Figure 4.17 Immunoblotting analysis of the expression of Bcl-2 protein in MDA-MB-231 cells after treatment with plant crude extract for 48 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

4.4.4 Apoptosis confirmation via activation of caspase-8 and active from of BID on time- dependent (extrinsic pathway)

Activation of caspase-8 can cleave the protein BID into the active from tBid (tunicated BID) which provides a crosslink between the extrinsic and the intrinsic pathways (Khosravi-Far and Esposti, 2004)

4.4.4.1 Immunoblotting analysis (Western blot) of Caspase-8

The immunoblotting analysis of expression of caspase-8 for 24 hours not showed the expression of cleaved caspase-8 (43 and 18 kDa) after treatment with treatment with plant crude extract in a similar level (Figure 4.18).

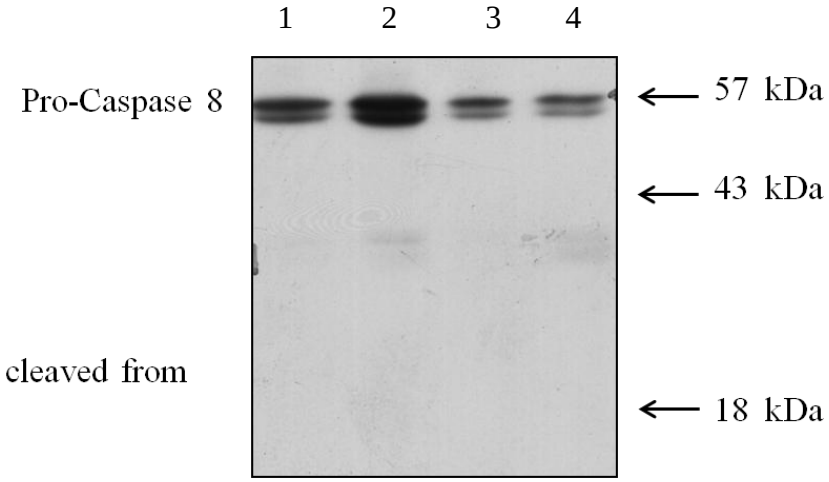

Figure 4.18 Immunoblotting analysis of the expression of caspase-8 protein in MDA-MB-231 cells after treatment with plant crude extract for 24 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

The immunoblotting analysis of expression of caspase-8 for 24 hours not showed the expression of cleaved caspase-8 (43 and 18 kDa) after treatment with treatment with plant crude extract in a similar level (Figure 4.19).

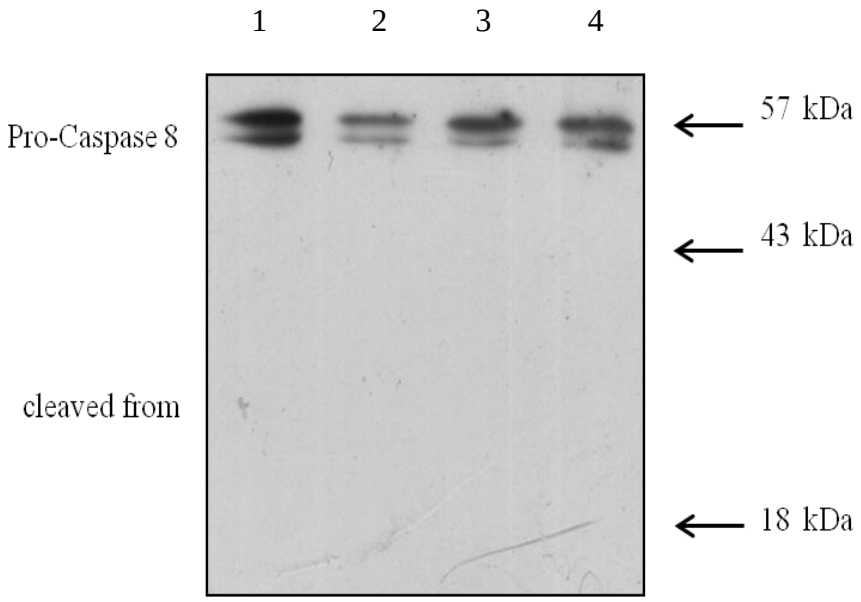

Figure 4.19 Immunoblotting analysis of the expression of caspase-8 protein in MDA-MB-231 cells after treatment with plant crude extract for 48 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

4.4.4.2 Immunoblotting analysis (Western blot) of BID

The immunoblotting analysis of expression of BID for 24 hours not showed the expression of tBID (15 kDa) after treatment with treatment with plant crude extract in a similar level (Figure 4.20).

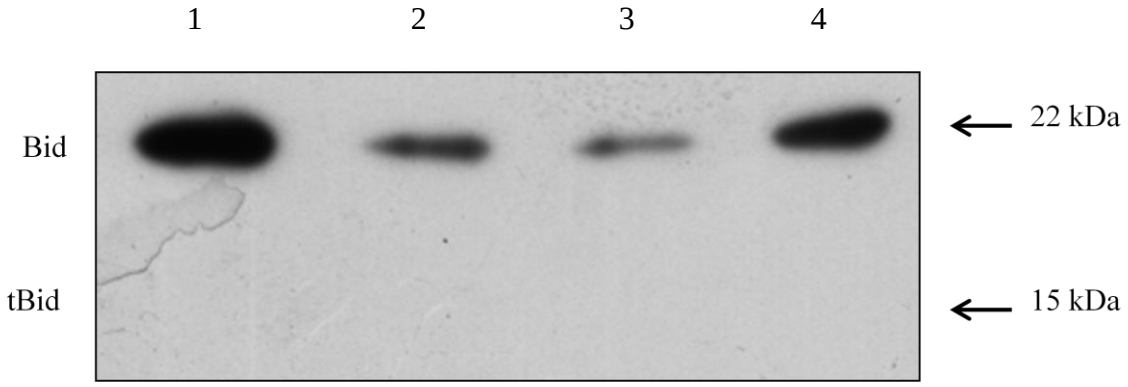

Figure 4.20 Immunoblotting analysis of the expression of BID in MDA-MB-231 cells after treatment with plant crude extract for 24 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

The immunoblotting analysis of expression of BID for 48 hours not showed the expression of tBID (15 kDa) after treatment with treatment with plant crude extract in a similar level (Figure 4.21).

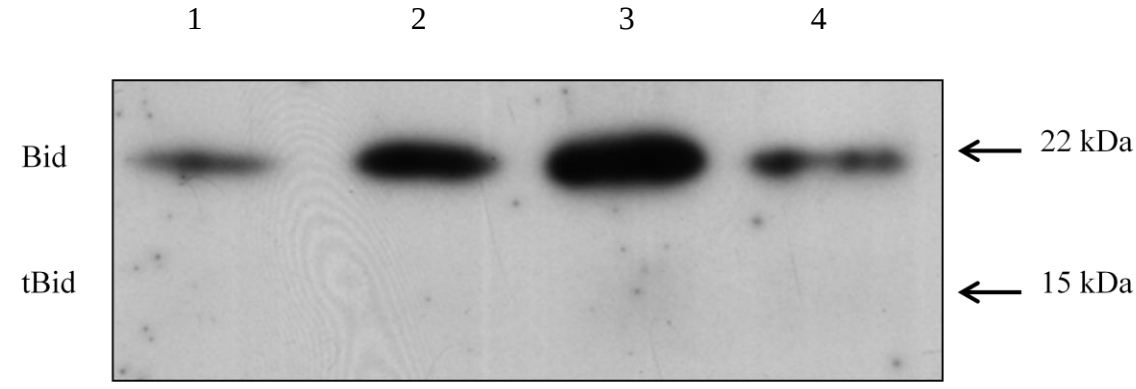

Figure 4.21 Immunoblotting analysis of the expression of BID in MDA-MB-231 cells after treatment with plant crude extract for 48 hours. Lanes: DMSO control(1), *S. venosa* treated(2), *S. erecta* treated(3), *P. nigrum* treated(4) .

CHAPTER V

DISCUSSION

Screening of a potential herbal or medicinal plant for anti-cancer is primarily based on anti-proliferation assay of the plant extract against the growth of the tested cancer cells. The plant sample that harbors strong IC_{50} is selected and submitted to further studies including analysis at protein differential level related to the cellular response to the phytochemical treatments.

The ethanolic crude extracts derived from 22 plants were evaluated for the antiproliferative effect against MDA-MB-231 cells. The results indicated that the test plant extracts expressed different degrees of antiproliferative activity against MDA-MB-231 cells according to the criteria of cytotoxicity for the crude extracts as established by the American National Cancer Institute (NCI); 50% inhibition values (IC_{50}) of proliferation at the less than 30 µg/ml were considered "active" in the preliminary assay (Suffness and Pezzuto, 1990).

The 24 plant ethanolic crude extracts antiproliferation to the tested cells were categorized into three classes according to Balantyne *et al.* (1999) as follows; the potentially non toxic, greater than 1,000 µg/ml against MDA-MB-231 cells, were including *A. farnesiana*, *A. procera*, *B. alnoides*, *D. conferta*, *F. fragrans*, *K. parviflora*, *L. leucocephala*, *M. azedarach*, *M. collettii*, *P. emblica*, *P. mirifica*, *T. crispa* and *V. Trifolia* extracts respectively. The potentially harmful, 100-1000 µg/ml against MDA-MB-231 cells were including *C. rotundus*, *S. multiflorum* (leaf), *S. asper*, *B. superba* and *S. multiflorum* (stem) and *A. luzonensis* extracts respectively. The potentially toxic, 10-100 µg/ml against MDA-MB-231 cells were including *S. venosa*, *P. nigrum* (seed), *S. erecta*, *P. nigrum* (fruit) and *D. rhodocalyx* extracts respectively.

In terms of quantitative analysis, the ethanolic crude extract of *S. venosa* (tuber), *P. nigrum* (seed) and *S. erecta* (tuberous root) exhibited the highest antiproliferation activity against MDA-MB-231 cells (IC_{50} = 24.85, 31.34 and 32.42 µg/ml, respectively).

The ethanolic extract from tuber of *S. venosa* showed antiproliferative activity against NCI-H187 (human small cell lung cancer), with IC_{50} of 4.88 µg/ml and MCF-7 human breast cancer cells with IC_{50} of 19.76 µg/ml (Leewanich *et al.*, 2011).

Palmatine and crebanine isolated from *S. venosa*, exhibited high cytotoxic activity against MCF-7 with IC_{50} values in the range of 5-6 µg/ml (Keawpradub *et al.*, 2001). Oxostephanine from *S. erecta* harboured cytotoxicity against breast cancer and acute lymphoblastic leukemia cells (MOLT-3) with an IC_{50} of 0.24 and 0.71 µg/mL, respectively, but very low cytotoxicity against normal embryonic lung cells MRC-5 cells. Dehydrocrebanine from *S. erecta* harboured cytotoxicity against promyelocytic leukemia cells (HL-60) with an IC_{50} of 2.14 µg/mL (Makarasen *et al.*, 2011). Aporphine isolated from the tubers of *S. erecta* exhibited cytotoxic activity against SKOV3 human ovarian cancer cells (Montrirattigri *et al.*, 2008).

Pellitorine isolated from the roots of *P. nigrum* showed anticancer activity against HL60 (Human promyelocytic leukemia) and MCF-7 (breast cancer) cells (Lian Ee *et al.*, 2010). Alkylamides and piperine isolated from black pepper suppressed TNF-induced NF-kB activation in human cancer cells. Piperine, acted as a growth inhibition related to cell cycle arrest at the G_2/M phase, induced apoptosis via activation of caspase 3 and decreased the expression of the migration-related proteins MMP-9 and MMP-13 in 4T1 cells (mouse mammary carcinoma) (Lai *et al.*, 2012).

The study on anti-proliferation activity of the plant ethanolic crude extract against MDA-MB-231 cells was verified by flow cytometric analysis. MDA-MB-231 cells were successively stained with both Annexin V-FITC and PI after exposured to plant ethanolic crude extracts prior to the flow cytometric analysis. The results demonstrated that treatment of the cells with the plant ethanolic crude extracts resulted in transformation from living cells to early and late apoptotic cells after the cell exposure to the plant ethanolic crude extracts. In terms of qualitative analysis, the 3 plant extracts exhibited the

survived cells at the different extent. However, the 3 plant extracts created higher percentage of early and late apoptotic cells than that of DMSO negative control and genistein positive control.

Even the 3 plant extracts caused the appearance of higher percentage of necrotic cells than that of DMSO negative control, though much lower than that of genistein. It implies that the 3 plant ethanolic crude extracts exhibited stronger anti-cancer activity than the tested dose of genistein. The anti-cancer potential of the 3 plant ethanolic crude extracts was also confirm with the early and late apoptotic/necrosis cells ratios in which appeared over 1 in all test samples. This implies that among the dead cells induced with the cytotoxic potential of the plant ethanolic crude extracts, the apoptotic cells were present in a greater number than the necrotic cell. This confirms that the anti-cancer activity of the 3 plant ethanolic crude extracts is rather favored to apoptosis than necrosis.

The experiment was performed in order to characterize the differential protein expression after exposure with the plant ethanolic crude extracts. There was no different in the intensity of α-tubulin protein in all tested cell samples as compared with the DMSO control. It implies that the immunoblooting system worked well in the test systems.

Caspase-3 is an indicator for chromosome condensation and DNA fragmentation in all cell types. The active form of caspase-3 is responsible for the cleavage of PARP that will block DNA repair and cell undergoing apoptosis (Porter and Jänicke, 1999; Nicholson *et al.*, 1995). There was a significant increased intensity of caspase-3 protein of the 3 tested samples in comparison with the control (Figure 4.12 and 4.13). This implies that caspase-3 is significantly up-regulated by the plant ethanolic crude extract treatment. Caspase-3-mediated PARP cleavage *in vitro* of cell subsets undergoing apoptosis after exposure to *S. venosa, S. erecta* and *P. nigrum* treated MDA-MB-231 cells. As the experiment of Chen *et al.* (2010), curcumin induced apoptosis via expression activation of caspase-9 and caspase-3.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in DNA repair in response to environmental stress (Satoh and Lindahl, 1992). PARP cleavage is an established and reliable apoptosis indicator downstream of caspase activation (Poter

and Jänicke, 1999) and serves as a marker of cells undergoing apoptosis (Oliver *et al.*, 1998). Furthermore, it is generally recognized as a substrate of both caspases-3 and -7 with a strong affinity for the latter (Germain et al. 1999), could be a very useful indicator of treatment-induced apoptosis probably by DNA damage. (Bressenot *et al.,* 2009). The results demonstrated cleavage of PARP in the 3 tested samples in comparison with the control (Figure 4.14 and 4.15).

Bcl-2 is a member of the Bcl-2 family of proteins involving in the regulation of apoptosis (Reed, 1996). The over expression of Bcl-2 enhances cell survival by suppressing apoptosis (Hasnan *et al.*, 2010). Inhibition of Bcl-2 proteins alters mitochondrial membrane permeability resulting in the release of cytochrome c into the cytosol, with activation of caspases and induction of cell death (Sharief *et al.*, 2003).

In this study there was a significant decreased intensity of Bcl-2 protein in the 3 tested samples within 24 hours in comparison with the control (Figure 4.16). However, at 48 hours, *S. venosa* initiated higher intensity of Bcl-2 protein than the others (Figure 4.17). It means that there might be a lower level of antiapoptosis protein expression within 24 hours and the cells undergone more apoptosis within 48 hour.

Activation of caspase-8 can cleave the protein BID into the active from tBid (tunicated BID) which provides a crosslink between the extrinsic and the intrinsic pathways (Khosravi-Far and Esposti, 2004). In this immunoblooting study, we applied caspase-8 and BID antibody for extrinsic pathway of apoptosis confirmation. The results indicated negative result in which active caspase-8 and tBID were not found (Figure 4.18, 4.19, 4.20 and 4.21). The results indicated that the tested plant materials could not induced apoptosis via the extrinsic pathway.

In conclusion, the ethanolic crude extracts of the herbal plants used in the Thai traditional rejuvenating remedies were screened for antiproliferative activity against MDA-MB-231 cells. Among the 24 plant extracts tested, 3 plant ethanolic crude extracts including *S. venosa*, *P, nigrun* and *S. erecta* exhibited a potential antiproliferation against human breast cancer cells (MDA-MB-231) The 3 plant ethanolic crude extracts were confirmed with immunoblotting to exert anti-cancer activity through inducing

cancer cell apoptosis. The plant materials in this study have long been presented as ingredients in the Thai traditional rejuvenating remedies. Hence, this is the strong proof that one of the rejuvenating efficacies of the plants might be related to their anti-proliferation effect to at least the ER-negative breast cancer cells.

REFERENCES

นันทวัน บุณยะประภัศร และอรนุช โชคชัยเจริญพร. 2541. สมุนไพรพื้นบ้าน. เล่ม 1. พิมพ์ครั้งที่ 1. กรุงเทพฯ : ประชาชน.

Aggarwal, B.B. and Shishodia, S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. Biochemical Pharmacology 71: 1397-1421.

American cancer Society. 2007. Cancer facts & Figures 2007; American cancer Society. Atlanta : GA.

Arunasree, K.M. 2000. Anti-proliferative effects of carvacrol on a human metastatic breast cancer cell line, MDA-MB 231. Phytomedicine 17: 581-588.

Balantyne, B., Marrs, T.C. and Syversen, T. 1999. General and Applied Toxicology. USA : Grove's dictionary's.

Banerji, J., Chatterjee, A., Patra, A., Bose, P., Das, R., Das, B., Shamma, M. and Tantisewie, B. 1994. Kamaline, an unusual aporphine alkaloid, from *Stephania venosa*. Phytochemistry 36: 1053-1056.

Barczyk, K., Kreuter, M., Pryjma, J., Booy, E.P., Maddika, S., Ghavami, S., Berdel, W.E., Roth, J. and Los, M. 2005. Serum cytochrome c indicates in vivo apoptosis and can serve as a prognostic marker during cancer therapy. International Journal of Cancer 116: 167-173.

Blagosklonny, M. V. and Darzynkiewicz, Z. 2005. Strategies of protection of normal cells during chemo- and radio-therapy based on modulation of cell cycle and apoptotic pathways. In: Los, M. and Gibson, S.B (Eds.), In Apoptotic Pathways as Target for Novel Therapies in Cancer and Other Diseases, pp. 357-391. New York : Springer Academic Press.

Bressenot, A., Zimmer, O., Marchal, S., Gauchotte, G., Montagne, K. and Plénat, F. 2009. Detection of apoptosis in vivo: comparison of different methods in

histological sections of subcutaneous xenografts of HT29 human colon adenocarcinoma. Annales de Phathologie 29: 370-375.

Brouckaert, G., Kalai, M., Saelens, X. and Vandenabeele, P. 2005. Apoptotic pathways and their regulation. In: Los, M. and Gilson, S.B. (Eds.), Apoptotic pathways as target for novel therapies in cancer and other diseases. New York : Springer Academic Press.

Bui-Xuana, N.H., Tanga, P.M.K., Wong, C.K. and Fung, K.P. 2010. Photo-activated pheophorbide-a, an active component of *Scutellaria barbata*, enhances apoptosis via the suppression of ERK-mediated autophagy in the estrogen receptor-negative human breast adenocarcinoma cells MDA-MB-231. Journal of Ethnopharmacology 131: 95-103.

Casciola-Rosen, L., Rosen, A., Petri, M. and Schlissel, M. 1996. Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. Proceedings of the National Academy of Science of the United Stated of America 93: 1624-1629.

Charles, B., Bruneton, J., Pharadai, K., Tantisewie, B., Guinaudeau, H. and Shamma, M. 1987. Some unusual proaporphine and aporphine alkaloids from *Stephania venosa*. Journal of Natural Products 50: 1113-1117.

Chen, Q.Y., Lu, G.H., Wu, Y.Q., Zheng, Y., Xu, K., Wu, L.J., Jiang, Z.Y., Feng, R. and Zhou, J.Y. 2010. Curcumin induces mitochondria pathway mediated cell apoptosis in A549 lung adenocarcinoma cells. Oncology Reports 23: 1285-1292.

Chen, S.J., Hsu, C.P., Li, C.W., Lu, J.H. and Chuang L.T. 2011. Pinolenic acid inhibits human breast cancer MDA-MB-231 cell metastasis in vitro. Food Chemistry 126: 1708-1715.

Chen, W.Y., Wu, C.C., Lan, Y.H., Chang, F.R., Teng, C.M. and Wu, Y.C. 2005. Goniothalamin induces cell cycle-specific apoptosis by modulating the redox status in MDA-MB-231 cells. European Journal of Pharmacology 522: 20-29.

Chuakul, W., Poanil, W. and Boonpleng, A. 2000. Survey on medicinal plants used at Khao Luang, Kirimas District, Sukhothai Province (Thailand). Mahidol Journal 7: 81-99.

Decker, P. and Muller, S. 2002. Modulating poly (ADP-ribose) polymerase activity: potential for the prevention and therapy of pathogenic situations involving DNA damage and oxidative stress. Current Pharmaceutical Biotechnology 3: 275-283.

Degterev, A., Boyce, M. and Yuan, J. 2003. A decade of caspases. Oncogene 22: 8543-8567.

Farnsworth, N.R. and Bunyapraphatsara, N. 1992. Thai medicinal plants. Recommented for primary health care system, pp. 402. Bangkok : MPIC,

Germain, M., Affar, E.B., D'Amours, D., Dixit, V.M., Salvesen, G.S. and Poirier, G.G. 1999. Cleavage of automodified poly (ADP-ribose) polymerase during apoptosis: evidence for involvement of caspase-7. Journal of Biological Chemistry 274: 28379-28384.

Govindarajan, R., Vijayakumar, M. and Pushpangadan, P. 2005. Antioxidant approach to disease management and the role of 'Rasayana' herbs of Ayurveda. Journal of Ethnopharmacology 99: 165-178.

Guinaudeau, H., Shamma, M., Tantisewie, B. and Pharadai, K. 1981. 4,5,6,6a-Tetradehydro-N-methyl-7-oxoaporphinium salts. Journal of the Chemical Society, Chemical Communications: 1118.

Guinaudeau, H., Shamma, M., Tantisewie, B. and Pharadai, K. 1982. Aporphine alkaloids oxygenated at C-7. Journal of Natural Products 45: 355-357.

Hasnan, J., Yusof, M.I., Damitri, T.D., Faridah, A.R., Adenan, A.S. and Norbaini, T.H. 2010. Relationship between apoptotic markers (Bax and Bcl-2) and biochemical markers in type 2 diabetes mellitus. Singapore Medical Journal 51: 51-55.

Hostanskal, K., Nisslein, T., Freudenstein, J., Reichling, J. and Saller, R. 2004. *Cimicifuga racemosa* extract inhibits proliferation of estrogen receptor-positive

and negative human breast carcinoma cell lines by induction of apoptosis. Breast Cancer Research and Treatment 84: 151-160.

Hsieh, C.C., Hernández-Ledesma, B. and De Lumen, B.O. 2011. Cell proliferation inhibitory and apoptosis-inducing properties of anacardic acid and lunasin in human breast cancer MDA-MB-231 cells. Food Chemistry 125: 630-636.

Information Technology Division National Cancer Institute. 2010. Hospital-Based Cancer Registry. Rumthaipress. Bangkok.

Ingkaninan, K., Phengpa, P., Yuenyongsawad, S., Khorana, N., 2006. Acetylcholinesterase inhibitors from *Stephania venosa* tuber. The Journal of Pharmacy and Pharmacology 58: 695-700.

Kanduc, D., Mittelman, A., Serpico, R., Sinigaglia, E., Sinha, A.A., Santacroce, R., Natale, C., Di Corcia, M.G., Lucchese, A., Dini, L., Pani, P., Santacroce, S., Simone, S., Bucci, R. and Farber, E. 2002. Cell death: apoptosis versus necrosis (review). International Journal of Oncology 21: 165-170.

Keane, M.M., Ettenberg, S.A., Nau, M.M., Russell, E.K. and Lipkowitz, S. 1999. Chemotherapy augments TRAIL-induced apoptosis in breast cell lines. Cancer Research 59: 734-741.

Keawpradub, N., Itharat, A., Tantikarnkul, A., Rugleng, S. and Inruspong, R. 2001. Cytotoxic alkaloids from the tuber of *Stephania venosa*. Songklanakarin Journal of Science and Technology 23: 225-234.

Kresch, M.J., Christian, C., Wu, F. and Hussain, N. 1998. Ontogeny of apoptosis during lung development. Pediatric Research 43: 426-431.

Kerr, J.F., Winterford, C.M. and Harmon, B.V. 1994. Apoptosis: its significance in cancer and cancer therapy. Cancer 73: 2013-2226.

Khosravi-Far, R. and Esposti, M.D. 2004. Death receptor signals to mitochondria. Cancer Biology Therapy 3: 1051-1057.

Kim, J.B., Yu, J.H., Ko, E., Lee, K.W., Song, A.K., Park, S.Y., Shin, I., Han, W. and Noh, D.Y. 2010. The alkaloid Berberine inhibits the growth of Anoikis-

resistant MCF-7 and MDA-MB-231 breast cancer cell lines by inducing cell cycle arrest. Phytomedicine 17: 436-440.

Koh, D.W., Dawson, T.M. and Dawson, V.L. 2005. Mediation of cell death by poly (ADP-ribose) polymerase-1. Pharmacological Research 52: 5-14.

Krähenbühl, O. and Tschopp, J. 1991. Debate: the mechanism of lymphocyte-mediated killing. Perforin-induced pore formation. Immunology Today 12: 399-402.

Kuo, P.L., Hsu, Y.L., Lin, T.C., Tzeng, W.S., Chen, Y.Y. and Lin, C.C. 2007. Rugosin E, an ellagitannin, inhibits MDA-MB-231 human breast cancer cell proliferation and induces apoptosis by inhibiting nuclear factor-κB signaling pathway. Cancer Letters 248: 280-291.

Lai, L.H., Fu, Q.H., Liu, Y., Jiang, K., Guo, Q.M., Chen, Q.Y., Yan, B., Wang, Q.Q. and Shen, J.G. 2012. Piperine suppresses tumor growth and metastasis in vitro and in vivo in a 4T1 murine breast cancer model. Acta Pharmacologica Sinica 33: 523-530.

Leewanich, P., Worachartcheewan, A., Prachayasittikul, S. and Prachayasittikul, V. 2011. Anticancer and antioxidative activities of *Stephania venosa*. European Journal of Scientific Research 51: 150-156.

Li, Z., Li, J., Mob, B., Hub, C., Liu, H., Qi, H., Wanga, X. and Xu, J. 2008. Genistein induces cell apoptosis in MDA-MB-231 breast cancer cells via the mitogen-activated protein kinase pathway. Toxicology in Vitro 22: 1749-1753.

Lian Ee, G.C., Lim, C.M., Rahmani, M., Shaari, K. and Bong, C.F.J. 2010. Pellitorine, a potential anti-cancer lead compound against HL60 and MCF-7 cell lines and microbial transformation of piperine from Piper nigrum. Molecules 15: 2398-2404.

Likhitwitayawuid, K., Angerhofer, C.K., Cordell, G.A., Pezzuto, J.M. and Ruangrungsi, N. 1993. Cytotoxic and antimalarial bisbenzylisoquinoline alkaloids from *Stephania errecta*. Journal of Natural Products 56: 30-38.

Likhitwitayawuid, K., Dej-ajisai, S. and Jong-bunprasert, V. 1999. Antimalarials from

Stephania venosa, Prismatomeris sessiliflora Diospyros montana and Murraya siamensis. Planta Medica 65: 754-756.

Luo, L., Xu, X., Sun, W. and Dong, L. 2008. Effect of amlodipine on apoptosis of human breast carcinoma MDA-MB-231 cells. Journal of Medical Colleges of PLA 23: 358-363.

Makarasen, A., Sirithana, W., Mogkhuntod, S., Khunnawutmanotham, N., Chimnoi, N. and Techasakul, S. 2011. Cytotoxic and antimicrobial activities of aporphine alkaloids isolated from *Stephania venosa* (Blume) Spreng. Planta Medica 77: 1519-1524.

McEligot, A.J., Yang, S. and Meyskens, F.J. 2005. Redox regulation by intrinsic species and extrinsic nutrients in normal and cancer cells. Annual Review Nutrition 25: 261-295.

Montririttigri, K., Moongkarndi, P., Joongsomboonkusol, S., Chitkul, B. and Pattanapanyasat, K. 2008. Apoptotic activity of aporphine from *Stephania venosa* on human ovarian cancer cells. Mahidol University Journal of Pharmaceutical Sciences 35: 52-56.

Moongkarndi, P., Kosem, N., Luanratana, O., Jongsomboonkusol, S. and Pongpan, N. 2004. Antiproliferative activity of Thai medicinal plant extracts on human breast adenocarcinoma cell line. Fitoterapia 75: 375-377.

Newman, D.J. and Cragg, G.M. 2007. Natural products as sources of new drugs over the last 25 years. Journal of Natural Products 70: 461-477.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K. and Gallant, M. 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 376: 37-43.

Nigam, M., Ranjan, V., Srivastava, S., Sharma, R. and Balapure, A.K. 2008. Centchroman induces G_0/G_1 arrest and caspase-dependent apoptosis involving mitochondrial membrane depolarization in MCF-7 and MDA MB-231 human breast cancer cells. Life Sciences 82: 577-590.

Oliver, F.J., de la Rubia, G., Rolli, V., Ruiz-Ruiz, M.C., de Murcia, G. and Murcia, J.M. 1998. Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis. Lesson from an uncleavable mutant. The Journal of Biological Chemistry 273: 33533-33539.

Park, E.J., Min, H.Y., Chung, H.J., Hong, J.Y., Kang, Y.J., Hung, T.M., Youn, U.J., Kim, Y.S., Bae, K., Kang, S.S. and Lee, S.K. 2009. Down-regulation of c-Src/EGFR-mediated signaling activation is involved in the honokiol-induced cell cycle arrest and apoptosis in MDA-MB-231 human breast cancer cells. Cancer Letters 277: 133-140.

Pathak, N. and Khandelwal, S. 2006. Modulation of cadmium induced alterations in murine thymocytes by piperine: Oxidative stress, apoptosis, phenotyping and blastogenesis. Biochemical Pharmacology 72: 486-497.

Pedro, M., Lourenço, C.F., Cidade, H., Kijjoa, A., Pinto, M. and Nascimento, M.S.J. 2006. Effects of natural prenylated flavones in the phenotypical $ER^{(+)}$ MCF-7 and $ER^{(-)}$ MDA-MB-231 human breast cancer cells. Toxicology Letters 164: 24-36.

Pharadai, K., Pharadai, T., Tantisewie, B. and Guinaudeau, H. 1985. (−)-O-acetylsukhodianine and oxostephanosine: two new aporphinoids from *Stephania venosa*. Journal of Natural Products 48: 658-659.

Porter, A.G. and Jänicke, R.U. 1999. Emerging roles of caspase-3 in apoptosis. Cell Death and Differentiation 6: 99-104.

Reed, J.C. 1996. Mechanisms of Bcl-2 family protein function and disfunction in health and disease. Boehring Institute Mitteilungen 97: 72-100.

Satoh, M.S. and Lindahl, T. 1992. Role of poly (ADP-ribose) formation in DNA repair. Nature 356: 356-358.

Savill, J. and Fadok, V. 2000. Corpse clearance defines the meaning of cell death. Nature 407: 784-788.

Savill, J.S., Wyllie, A.H., Henson, J.E., Walport, M.J., Henson, P.M. and Haslett, C. 1989. Macrophage phagocytosis of aging neutrophils in inflammation.

Programmed cell death in the neutrophil leads to its recognition by macrophages. *Journal of Clinical Investigation* 83: 865-875.

Schulte-Hermann, R., Bursch, W., Grasl-Kraupp, B., Marian, B., Török, L., Kahl-Rainer, P. and Ellinger, A. 1997. Concepts of cell death and application to carcinogenesis. Toxicologic Pathology journal 25: 89-93.

Semwal, D.K., Badonib, R., Semwalc, R., Kothiyalb, S.K., Singha, G.J.P. and Rawatb, U. 2010. The genus *Stephania* (Menispermaceae): chemical and pharmacological perspectives. Journal of Ethnopharmacology 132: 369-383.

Sharief, M.K. Matthews, H. and Noori, M.A. 2003. Expression ratios of the Bcl-2 family proteins and disease activity in multiple sclerosis. Journal of Neuroimmunology 134: 158-165.

Srinivasan, K. 2007. Black pepper and its pungent principle-piperine: a review of diverse physiological effects. Critical Reviews Food Science and Nutrition 47: 735-748.

Staunton, J.M. and Gaffney, F.E, 1998. Apoptosis: basic concept and potential significances in human cancer. Archives of Pathology and Laboratory Medicine 122: 310-309.

Suffness, M. and Pezzuto, J.M. 1990. Assays related to cancer drug discovery, In: Hostettmann, K. (Ed), Methods Plant Biochemistry: Assays for Bioactivity, pp. 71-133. London : Academic Press 6,

Takahashi, K. and Loo, G. 2004. Disruption of mitochondria during tocotrienol-induced apoptosis in MDA-MB-231 human breast cancer cells. Biochemical Pharmacology 67: 315-324.

Tamez, P.A., Lantvit, D., Lim, E. and Pezzuto, J.M. 2005. Chemosensitizing action of cepharenthine against drug-resistant human malaria, Plasmodium falciparum. Journal of Ethnopharmacology 98: 137-142.

Van Engeland, M., Nieland, L.J.W., Ramaekerss, F.C.S., Schutte, B. and Reutelingsperger, C.P.M. 1998. Annexin-V affinity assay: a review on an

apoptosis detection system based on phosphatidylserine exposure. Cytometry 31: 1-9.

Van Engeland, M., Ramaekers, F.C., Schutte, B. and Reutelingsperger, C.P. 1996. A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 24: 131-139.

Van Furth, R. and Van Zwet, T.L. 1988. Immunocytochemical detection of 5-bromo-2-deoxyuridine incorporation in individual cells. Journal of Immunological Methods 108: 45-51.

Vermes, I., Haanen, C., Nakken, H.S. and Reutelingsperger, C. 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine early apoptotic cells using fluorescein labeled Annexin V. Journal of Immunological Methods 184: 39-51.

APPENDICES

Appendix A: Chemicals

1) Chemicals used in cell culture

Chemical	Company
0.89% Normal saline	
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma, USA
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	Sigma, USA
Antibiotics/Antimycotics	Gibco-Invitrogen, USA
$CaCl_2$	
Dimethy sulfoxide (DMSO)	E.MERK, Germany
Dimethy sulfoxide (DMSO) (cell culture grade)	Sigma, USA
Fetal Bovine Serum (FBS)	Gibco-Invitrogen, USA
KCl	
KH_2PO_4	
Leibovit's L-15 medium	Gibco-Invitrogen, USA
Na_2HPO_4	
NaCl	
Penicillin/Streptomycin	Gibco-Invitrogen, USA
Sodium Bicarbonate	Sigma-Aldrich, Germany
Sodium Hypoclorite	Clorox, USA
Trypan blue	Sigma-Aldrich, Germany
Trypsin EDTA	Gibco-Invitrogen, USA

2) Chemicals used in flow cytometry

Chemical	Company
Annexin-V- anexin V Alexa Fluor®488	Gibco-Invitrogen, USA
$CaCl_2$	
HEAPES	Gibco-Invitrogen, USA
NaCl	
Propidium Iodide (PI)	Gibco-Invitrogen, USA

3) Chemicals used in flow immunoblotting

Chemical	MW (g/mol)	Company
Absolute ethanol (C_2H_5OH)	46.07	E. Merck, Germany
Analytical reagent grade methanol (CH_3OH**)**	32.04	Fisher Scientific, USA
Glacial acetic acid (CH_3COOH**)**	60.05	Lab-Scan, Thailand
Acrylamide (C_3H_5NO**)**	71.08	Bio-Rad, USA
Agarose		CAMBREX, USA
Bradford protein assay		Bio-Rad, USA
Coomassie brilliant blue R-250	826.00	SERVA, Germany
ECL plus Western blotting detection system	-	GE Healthcare, UK

Chemical	MW (g/mol)	Company
Ethylenediamine tetraacetic acid (EDTA)	372.24	E. Merck, Germany
Glycine $H_2NCH_2CO_2H$	75.27	USB, USA
Hydrochloric acid (HCl)	36.50	E. Merck,Germany
Instant non-fat milk powder (skim milk)	-	Mission, Thailand
Low molecular weight calibration kit for SDS	-	GE Healthcare, UK
Polysorbate 20 (Tween-20) ($C_{58}H_{114}O_{26}$)	1,227.54	Sigma, USA
Protease inhibitor cocktail	-	Sigma-Aldrich, Germany
Sodium chloride NaCl	58.44	RANKEM, India
Sodium dodecyl sulfate (SDS) ($C_{12}H_{25}O_4SNa$)	288.38	Sigma, USA
Tris (Hydroxymethyl aminomethane) ($H_2NC(CH_2OH)_3$)	121.10	USB, USA

Appendix B: Instrument

1) Instruments and consumables used in cell culture

Instruments and consumables	Company
1.5 ml Microtube	Axygen, USA
15 ml centrifuge tube	Cloning, Mexico
50 ml centrifuge tube	Cloning, Mexico
6-well plate	Nunc, Denmark
96-well plate	Nunc, Denmark
25 cm^2 tissue culture flask	Nunc, Denmark
75 cm^2 tissue culture flask	Nunc, Denmark
Automatic Pipettes	Gilson, USA
Centrifuge	Jouan, France
CO_2 incubator	ThermoForma, USA
Phase-contrast inverted microscope	Nikon, Japan
Digital camera	Nikon, Japan
Heating magnetic stirrer	Mandel, Canada
Hemocytometer	HBG, Germany
Incubathing bath	Julabo, Japan
Biosafty cabinet class II	NuAir, USA
Membrane filter	Pall, USA
Multichannel autopipette	SOCOREX, Switzerland
pH merter	Mettler Toledo, USA
Pipetboy	IBS, Switzerland
Rocking shaker	IKA, Germany
Velp Vortex Mixers	Progen Scientific, UK
UV-visible Spectrophotometer	Molecular devices, USA

2) Instruments used in flow cytometry

Instruments and consumables	Company
1.5 ml Microtube	Axygen, USA
1.5 ml Microtube	Cloning, Mexico
5 ml Microtube	Cloning, Mexico
15 ml centrifuge tube	Cloning, Mexico
Auto Pipettes	Gilson, USA
Centrifuge	Heraeus, UK
Flow cytometer FC500	Beckman Coulter, USA

3) Instruments used in immunoblotting

Instruments and consumables	Company
0.5 ml Microtube	Axygen, USA
1.5 ml Microtube	Axygen, USA
15 ml centrifuge tube	Cloning, Mexico
50 ml centrifuge tube	Cloning, Mexico
Auto Pipettes	Gilson, USA
Freezer (-80°C)	Thermo Scientific, USA
Centrifuge	Heraeus, UK
Refrigerated circulating bath	Julabo, Germany
Electrophoresis power suppl	Bio-Rad, USA
Ettan IPGphor III IEF System	GE Healthcare, USA
High performance chemiluminescen film	GE Healthcare, UK

Instruments and consumables	Company
High Speed Micro Refrigerated Centrifuge	Sigma, USA
Magnetic stirrers hotplate	IKA, Germany
Milli-Q plus	Millipore, USA
0.45 PVDF membrane	Immobilop, USA
pH meter	Mettler Teledo, USA
Rocking shaker	Cleaver, UK
Scanner	GE Healthcare, USA
Sonicator	IKA, Germany
Spectrophotometer	Shimadzu, Japan
Speed Vacuum	Labconco, USA
Thermomixer	Eppendorf, Germany
Ultrasonic bath	Elma, Germany
Vortex mixer, Vortex Gene 2	Scientific Industries, USA

Appendix C: Reagents

1. Reagents used in cell culture

1) L-15 medium (Leibovit's L-15 medium)

L-15 medium powder medium (Gibgo)	9.58 g
HEPES	1.5 g
10,000 units/ml penicillin and 10,000 µg/ml streptomycin	10 ml
Amphotencin B	100 µl
DI water	1000 ml

Adjust pH to 7.2 - 7.4 with 1 N HCl or 1 N NaOH before adjust volume with water and sterilize by nitrocellulose membrane (pore size 0.2 µm). Dispense the filtrate into bottles. All bottled mediums are stored in 4°C until use.

2) Phosphate buffer saline (PBS)

NaCl	8 g
KCl	0.2 g
Na_2HPO_4	1.5 g
KH_2PO_4	0.1 g
DI water	1 L

Adjust pH to 7.4 with 1 N HCl or 1 N NaOH before adjust volume with water. Dispense into bottles and autoclave for 20 minute, storage at 4ºC

3) Trypsin EDTA (1x)

10x trypsin EDTA	1 ml
PBS	9 ml

Prepare this solution within laminar flow and storage at 4ºC

4) 0.4% Trypan blue dye

Trypan bue	1.6 g
NaCl	3.24 g
KH_2PO_4	0.24 g
Distilled water	400 ml

All ingredients were mixed altogether, heat and stirred with magnetic stirre until completely dissolved. Adjust pH to 7.2-7.3 (by add 7.5% $NaHCO_3$ and/or 1% HCl).Then dispensed into light protecting bottles.

5) MTT solution

MTT 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide	5 mg
0.89% Normal saline	1 ml

Add MTT 5 mg into 1 ml of 0.89% normal saline. All ingredients were mixed and sterilized by filter. Dispense into light protecting bottles and freshly prepared for every experiment.

2. Reagents used in flow cytometry

1) Binding buffer (1x)

HEAPES	2.383 g
NaCl	8.181 g
$CaCl_2$	0.2775 g
DI water	1,000 ml

Adjust pH to 7.4 with 1 N HCl or 1 N NaOH before adjust volume with water. Dispense into bottles and autoclave for 20 minute, storage at 4ºC

2) Anexin V Alexa Fluor®488 + propidiun iodide solution (for only one sample)

binding buffer	50 µl
anexin V Alexa Fluor®488 1 µg/ml	1 µl
propidium iodide (PI) 1 µg/ml	5 µl

Freshly prepare this solution

3. Reagents used in immunoblotting

1) 5X Blotting buffer (transfer buffer)

Glycine	72.00 g
Tris	15.00 g

Dissolved in Milli Q water and adjusted the volume to 1 L

2) 10X Tris-buffered saline (TBS), pH 7.6

Tris	24.20 g
NaCl	80.00 g

Dissolved in Milli Q water and adjusted the volume to 1 L

3) 1X Tris-buffered saline/0.1% Tween (TBS/T)

10X TBS	100.00 ml
Tween 20	1.00 ml
Milli Q water	899.00 ml

4) 10% Skim milk (1° and 2° antibody)

Skim milk	1.00 g
TBS/T	10.00 ml

5) 5X Running buffer

Glycine	72.00 g
Tris	15.00 g
SDS	5.00 g

Dissolved in Milli Q water and adjusted the volume to 1 L

6) 12.5% separating gel (11 ml/gel)

Milli Q water	3.616 ml
1.5 M Tris-HCl, pH 8.8	2.75 ml
10% SDS	110 µl
30% Acrylamide/0.8% N, N′- Methylenebis-acrylamide	4.464 ml
10% Ammonium persulfate	55.00 µl
TEMED	5.50 µl

7) 7.5 % separating gel (11 ml/gel)

Milli Q water	5.4015 ml
1.5 M Tris-HCl, pH 8.8	2.75 ml
10% SDS	110 µl
30% Acrylamide/0.8% N, N′- Methylenebis-acrylamide	2.678 ml
10% Ammonium persulfate	55 µl
TEMED	5.50 µl

8) 12.5% stacking gel (1.267 ml/gel)

Milli Q water	0.76 ml
0.5 M Tris-HCl, pH 6.8	0.31 ml
SDS	12.50 µl
30% Acrylamide/0.8% N, N′- Methylenebis-acrylamide	0.16 ml
10% Ammonium persulfate	7.80 µl
TEMED	1.25 µl

9) Coomassie blue R-250 (100 ml)

Coomassie blue R-250	0.10 g
MeOH (analytical grade)	40.00 ml
Acetic acid	10.00 ml
Distilled water	50.00 ml

Appendix D: Antibodies used in immunoblotting

Antibody	Company
Bcl-2	Cell Signaling Tech, USA
BID	Cell Signaling Tech, USA
PARP	Cell Signaling Tech,USA
Caspase-8	Cell Signaling Tech,USA
Caspase-3	Cell Signaling Tech,USA
α-tubulin	Sigma, USA
Polyclonal swine anti-rabbit immunoglobulins	Dako, Denmark
Polyclonal rabbit anti-mouse immunoglobulins	Dako, Denmark

BIOGRAPHY

Miss Supattra Chawalitpong was born on December 5th, 1986 in Nakhonsawan, Thailand. She was graduated with a Zoology degree of Science in Biology, Faculty of Science, Chiang Mai University in 2008. She has enrolled in the Graduate school, Chulalongkorn University for Master Degree of Science in Biology during 2010-2012.

Research presentation

Chawalitpong, S., Srisomsap, C., Panriansaen, R. and Cherdshewasart, W. 2012. Cytotoxicity of Thai rejuvenating herbs on colon cancer cells. Proceeding the 50th Kasetsart University Annual Conference, 31st January 3rd February, Kasetsart University, Bangkok, Thailand.

Chawalitpong, S., Srisomsap, C., Panriansaen, R. and Cherdshewasart, W. 2011. Cytotoxic against MDA-MB-231 breast cancer cells by the rejuvenating Thai herbal plants. Abstract the 16th Biological Sciences Graduate Congress, 12th-13th December, National University of Singapore, Singapore.