ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งต่อมลูกหมาก PC-3 โดยสมุนไพรไทยในกลุ่มยาอายุวัฒนะ

นางสาวชุติมา ชัยสนิท

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIPROLIFERATION ACTIVITY AGAINST PROSTATE CANCER CELLS PC-3 BY THAI REJUVENATING HERBS

Miss Chutima Chaisanit

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Biotechnology

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Thesis Title	ANTIPROLIFERATION ACTIVITY AGAINST PROSTATE CANCER CELLS PC-3 BY THAI REJUVENATING HERBS
By	Miss Chutima Chaisanit
Field of Study	Biotechnology
Thesis Advisor	Associate Professor Wichai Cherdshewasart, D.Sc.

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

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ชุติมา ชัยสนิท: ฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งต่อมลูกหมาก PC-3 โดยสมุนไพรไทยในกลุ่มยาอายุวัฒนะ . (ANTIPROLIFERATION ACTIVITY AGAINST PROSTATE CANCER CELLS PC-3 BY THAI REJUVENATING HERBS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.วิชัย เชิดชีวศาสตร์, 117 หน้า.

การศึกษาฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็งต่อมลูกหมากชนิด PC-3 ของสารสกัดหยาบเอทานอลจากสมุนไพร ไทยซึ่งเป็นส่วนประกอบในตำรับยาอายุวัฒนะ 22 ชนิด โดยนำสารสกัดหยาบมาบ่มกับเซลล์มะเร็งต่อมลูกหมากเป็นเวลา 72 ชั่วโมง วัดระดับความเป็นพิษต่อเซลล์เปรียบเทียบกับชุดควบคุม DMSO และเจนิสไตน์ โดยวิธี MTT พบว่าสารสกัดหยาบที่มีฤทธิ์ต้านการเพิ่มจำนวนเซลล์มะเร็ง ต่อมลูกหมากแรงที่สุด 3 ลำดับแรก ได้แก่ บัวบกป่า บอระเพ็ดพุงช้าง และข่อย โดยมีค่า IC_{50} เท่ากับ 4.30 , 4.47 และ 10.78 ไมโครกรัม /มิลลิลิตร ตามลำดับ สารสกัดหยาบเอทานอลจากสมุนไพรมีฤทธิ์ยับยั้งการเพิ่มจำนวนเซลล์มะเร็งต่อมลูกหมากโดยเหนี่ยวนำให้เกิดอะพอพโทซิส เมื่อยืนยันผลโดยการตรวจวัดด้วยเครื่องโฟลไซโตมิเตอร์โดยใช้สีย้อม annexin V-FITC/propidium iodide การศึกษาเพื่อยืนยันผล อะพอพโทซิส ทำโดยวิธี immunoblot โดยนำสารสกัดหยาบมาบ่มกับเซลล์มะเร็งต่อมลูกหมากเป็นเวลา 48 ชั่วโมง พบว่าโปรตีน Bcl-2 มีการแสดงออกลดลง ในขณะที่โปรตีน Bax, caspase-3 และ PARP มีการแสดงออกเพิ่มขึ้น ศึกษาระดับการแสดงออกของโปรตีนโดยวิธีเจลอิเลคโตรโฟรีซีส 2 มิติ โดยใช้สารสกัดหยา บที่ค่า IC_{20} บ่มกับเซลล์มะเร็งต่อมลูกหมากเป็นเวลา 48 ชั่วโมง ชนิดของโปรตีนที่ แสดงออกแตกต่างกันจำแนกโดยใช้เทคนิค LC/MS/MS และ Mascot search engine และยืนยันผลการแสดงออกของโปรตีนด้วยวิธี immunoblot พบว่า 14-3-3 protein ในเซลล์มะเร็งต่อมลูกหมากชนิด PC-3 มีการแสดงออกลดลงในการตอบสนองต่อสารสกัดหยาบ จากพืชทั้ง 3 ชนิด เมื่อเปรียบเทียบกับชุดควบคุม DMSO ดังนั้นจึงอาจพิจารณาได้ว่า 14-3-3 protein เป็นเป้าหมายของการรักษา หรือตัวชี้วัดผลการวินิจฉัยโรคมะเร็งต่อมลูกหมากได้ นอกจากนี้มีความเป็นไปได้ที่จะ ใช้ 78 kDa glucose-regulated protein, annexin A5 และ tropomyosin alpha-3 chain เป็นดัชนีบ่งชี้ชนิดใหม่ ของโรคมะเร็งต่อมลูกหมาก จากการศึกษานี้พบสมุนไพรบางชนิดมี ศักยภาพสูง ในการยับยั้งการเพิ่มจำนวนเซลล์มะเร็งต่อมลูกหมาก

สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2554 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

5272281423 : MAJOR BIOTECHOLOGY
KEYWORDS : Prostate cancer / PC-3 / Antiproliferation / Apoptosis / Protein / Thai rejuvenating herbal plant

CHUTIMA CHAISANIT : ANTIPROLIFERATION ACTIVITY AGAINST PROSTATE CANCER CELLS PC-3 BY THAI REJUVENATING HERBS. ADVISOR : ASSOC. PROF. WICHAI CHERDSHEWASART, D.Sc., 117 pp.

The antiproliferation activity potential of ethanolic crude extracts obtained from 22 traditional Thai rejuvenating herbal plants against PC-3 (human prostate cancer) cells were evaluated. The PC-3 cells were incubated with plant crude extracts for 72 h prior to MTT assay in comparison with the DMSO and genistein control. There were three ethanolic crude extracts with the strong antiproliferation activity including *S. erecta*, *S. venosa* and *S. asper* with IC_{50} values of 4.30, 4.47 and 10.78 μg/ml, respectively. The ethanolic crude extracts showed antiproliferation against PC-3 cells via apoptosis induction when confirmed by the flow cytometry with annexin V-FITC/propidium iodide double staining. Immunoblotting analysis was confirmed for apoptosis-related proteins at 48 h incubation period which resulted in underexpression of anti-apoptotic Bcl-2, overexpression of pro-apoptotic Bax, overexpression of caspase-3 and cleavage of poly (ADP-ribose) polymerase (PARP). The protein expression levels were studied with two-dimensional (2-DE) gel electrophoresis. The differential protein expressions were identified with LC/MS/MS along with Mascot search engine and confirmed with immunoblotting. PC-3 cells treated with the 3 plant ethanolic crude extracts exerted differences in protein expression in comparison with the control. The 14-3-3 protein in PC-3 cells was down-regulated in all treatment and thus might be considered as diagnostic markers or as therapeutic targets for prostate cancer. Besides, the 78 kDa glucose-regulated protein, annexin A5 and tropomyosin alpha-3 chain are possible to be novel prostate cancer marker candidates. The study demonstrates the high potential of certain Thai rejuvenating herbal plant against the prostate cancer cells.

Field of Study : Biotechnology Student's Signature .....

Academic Year : 2011 Advisor's Signature .....

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LIST OF ABBREVIATIONS

ATCC	American Type Culture Collection
APS	Ammonium persulfate
CHAPS	3-[(3-cholamidopropyl)-methylammonio] propanesulfonate
EDTA	Ethylenediamine tetraacetic acid
FBS	Fetal bovine serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
RPMI	Rosewell Park Memorial Institute
PBS	Phosphate-buffered saline
DMSO	Dimethylsulfoxide
DTT	Dithiothreitol
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide
2-DE	Two-dimensional polyacrylamide gel electrophoresis
IAA	Iodoacetamide
IEF	Isoelectric focusing
IPG	Immobiline^{TM} DryStrip
PAGE	Polyacrylamide Gel Electrophoresis
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TEMED	N, N, N', N'-Tetra-methyl ethylenediamine
O.D.	optical density
IC	inhibition concentration
CO_2	carbon dioxide
^oC	degree celsius
%	percentage

pI	isoelectric point
MW	molecular weight
µA	microampere
µg	microgram
µl	microliter
V	volt
Vhr	volt per hour
h	hour
min	minute
g	gram
mg	milligram
l	liter
ml	milliliter
rpm	revolution per minute
etc.	et ectera (Latin), other things
et al.	et alli (Latin), and other people

CHAPTER I

INTRODUCTION

Prostate cancer, the most common cancer worldwide is the second leading cause of male cancer death in the United States and is increased with age (Gittes, 1991). One in six men is probably harbor prostate cancer (Prostate Cancer Foundation, 2005). The cancer treatments are including surgery, radiation therapy, hormone therapy and chemotherapy. Chemotherapy can cause damage to normal cells (Stetler and Kleiner, 2001) and weakening of the immune system (Patel *et al*., 2006). Therefore, any discovery of anticancer agents should be effective against specific types of cancer cells but less toxic to normal cells (Pezzuto, 1997).

Phytotherapy is an alternative mean to reduce the adverse effect on the use of synthetic drugs. There has been traditionally consumption of natural products as anticancer drugs. Fifty per cents of the drugs used in clinical trials for anticancer were natural origins (Costa-Lotufo *et al*., 2005).

Herbal plant formulations have been used for the treatment of cancer in Asian countries. Some traditional Thai medicines have been consumed as anti-cancer drugs (Saetung *et al*., 2005). Besides, the plant crude showed potential for development of phytotherapy, for example, the crude extract of *Dioscorea membranacea* roots showed cytotoxic activity against lung, colon (Itharat *et al*., 2004) and prostate cancer cells (Saetung *et al*., 2005).

In the present study, the screening for the cytotoxic potential of the 24 ethanolic extracts from 22 Thai rejuvenating plants belonging to different plant families against PC-3 (human prostate cancer cell line) was investigated with the aid of MTT assay and then confirmed with a flow cytometry and immunoblotting as well as proteomic study. The plants with antiproliferative effects to the prostate cancer cells are interesting ones as they might become potential sources that could be developed into prostate anticancer drugs.

Purposes of the study:

To screen for Thai rejuvenating herbal plant extracts with antiproliferative activity against the human prostate cancer (PC-3) cells, based on the MTT assay.

To quantify and qualify the antiproliferative activity of the 3 most potent cytotoxic rejuvenating Thai herbal extracts against PC-3 cells with the aid of a flow cytometry.

To establish molecular data at the protein level of the antiproliferative activity of the 3 most potent cytotoxic Thai rejuvenating herbal extracts on PC-3 cells with the aid of immunoblotting and a two-dimentional electrophoresis techniques followed by LC/MS/MS identification of protein species.

CHAPTER II

REVIEW OF THE LITERATURE

2.1 Rejuvenating herbal plants

Medicinal plants have long term been traditionally used for treatment of various 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 benefit to aging populations (Govindarajan *et al*., 2005). Thailand locates in a sub-tropical area with abundance of diverse herbal plants. Thai rejuvenating herbal plants are widely used in traditional Thai medicine. The Thai herbal plant was rarely studied for cytotoxicity or anti-proliferation activity against cancer cells or induced cancers in animal model. We had selected twenty-two plants used in Thai traditional rejuvenating remedies to screen for cytotoxicity against the human PC-3 prostate cancer cells.

2.1.1 *Stephania erecta* Craib

S. erecta (Family Menispermaceae) is locally known as "Buabokpa". *S. erecta* is used in Thai folk remedies as a skeletal muscle relaxant and other diseases, as well as an analgesic and tonic (นันทวัน และ อรนุช, 2541 เล่ม 2). The plant produces a large number of alkaloids, including bisbenzylisoquinolines (Lin *et al.*, 1993). Several bisbenzylisoquinoline alkaloids have been shown to possess cytotoxicity against a number of human cancer cell lines (Buck, 1987).

2.1.1.1 Chemical constituents:

Cepharanthine and homoaromoline (Prawat *et al.*, 1982), obaberine, stephibaberin, telobine, tetradrine, thalrugosin, thalrugosine (Likhiwitayawuid *et al.*, 1993), methyltelobine, dehydrotelobine, isotetrandrine, thalrugosine, stephibaberine and dephnandrine (Saxena *et al.*, 2003) were isolated from the tubers of *S. erecta*.

2.1.1.2 Anticancer activity

There was no report on anticancer activity of *S. erecta*

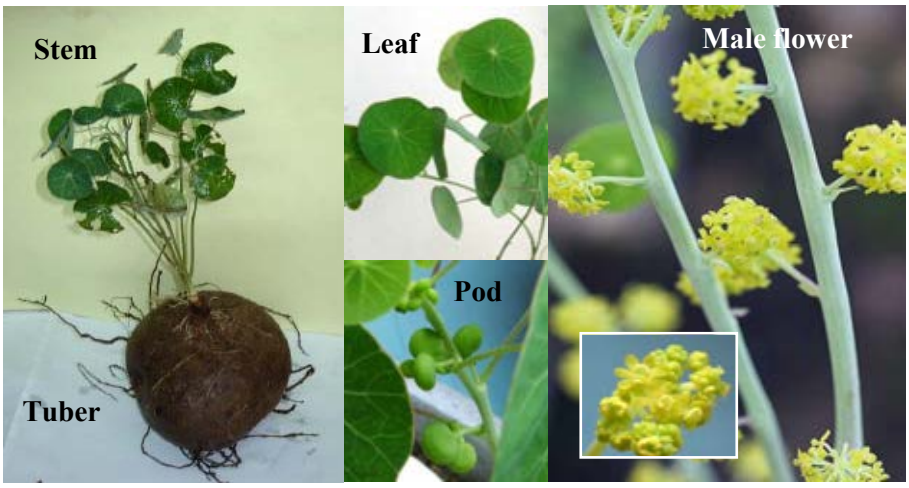


Figure 2.1 *S. erecta* Craib

Source of photos: http://public.fotki.com/plumo/stephania/
http://www.toptropicals.com

2.1.2 ***Stephania venosa*** (Blume) Spreng

S. venosa (Family Menispermaceae) is locally known as "Saboo luad" or "Boraphet pungchang". *Stephania* species which have been used in traditional medicine is an important source of isoquinoline alkaloids, one of the largest groups of natural products (Perry and Metzger, 1980).

2.1.2.1 Chemical constituents

The most plant chemical compounds are alkaloid and alkaloid derivative, i.e. anonaine, asimilobine, glaziovine, kikemanine, reticuline, stepharine, stesakine, sukhodianine, ushinsunine, thalirugosamine, tudoranine (Charles *et al*., 1987) liriodenine, mecambroline, nuciferoline, stepharine, stephanosine, ushinsuninine (Pharadai *et al*., 1985) crebanine, sukhodianine, thailandine, uthongine (Guinaudeau *et al*., 1981), kamaline (Banerji *et al*., 1994), protoberberines (Ingkaninan *et al.*, 2001) and aporphine (Montrirattigri *et al.*, 2008).

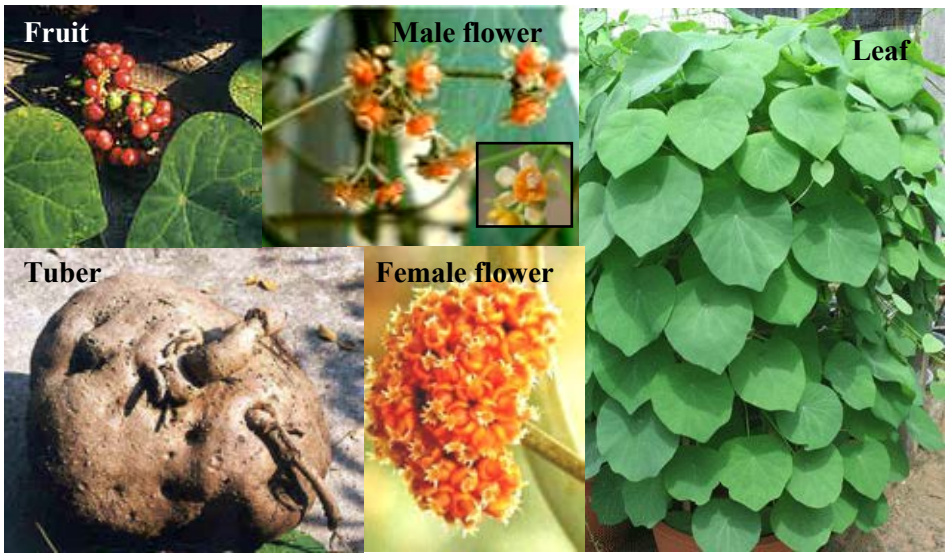


Figure 2.2 *S. venosa* (Blume) Spreng

Source of photos: http://www.herblpg.com/thai/herb55.html
http://www.tropicaflore.com

2.1.2.2 Anticancer activity

The ethanolic extract from tuber showed anticancer activity in human small cell lung cancer (NCI-H187) and human breast cancer (MCF-7) cells (Leewanich *et al*., 2011). The isolated berberine sulphate has anticancer activity (Keawpradub *et al*., 2001 and Simon *et al*., 1989). The isolated coralyne chloride has anti-leukemic activity on P-388 and L-1210 strain in mice (Pharadai *et al*., 1985 and Simon *et al*., 1989). The isolated aporphine exhibited cytotoxic activity against SKOV3 human ovarian cancer cells (Montrirattigri *et al*., 2008).

2.1.3 *Streblus asper* Lour.

S. asper (Family Moraceae) is locally known as "Khoi". Various parts of this plant are used for the treatment of cancer (Rastogi *et al*., 1990). *S. asper* contains different classes of chemical constituents listed in Table 2.1.

Figure 2.3 *S. asper* Lour.

Source of photos: http://en.wikipedia.org/wiki/Streblus_asper
http://thaiherb.most.go.th/plantdetail.php?id=366

Table 2.1 The classes of chemical constituents reported in *S. asper*

Class	Chemical constituents	Reference
cardiac glycoside	kamloside, asperoside, indroside, cannodimemoside, glucokamloside, glucogitodimethoside, glucostrebloside, strebloside, strophanolloside, sarmethoside, strophalloside, and 16-O-acetyl-glucogitomethoside	Khare *et al*., 1962 Manzetti and Reichstein, 1964
pregnane glycoside	sioraside, N-triacontane, tetraiacontan-3-one, β-sitosterol, stigmasterol, betulin and oleanolic	Chawla *et al*., 1990 Prakash *et al*., 1992
triterpene	α-amyrin acetate, lupeol acetate, lupeol, diol, strebloside and mansonin	Barua *et al*., 1968 Fiebig *et al*., 1985

2.1.3.1 Chemical constituents

The other constituents were α-copaene, α-cadinene, β-elemene, caryophyllene oxide, caryophyllene, geranyl acetone, germacrene, and 8-heptadecene (Phutdhawong *et al*., 2004). The major constituents of the volatile oil from *S. asper* leaf were α-farnesene, caryophyllene, phytol, trans-farnesyl acetate and trans-trans-α-farnesene (Phutdhawong *et al*., 2004).

2.1.3.2 Anticancer activity

Cardiac glycosides: strebloside and mansonin isolated from *S. asper* have significant anticancer activity in KB cell (human carcinoma of the nasopharynx) culture system (Fiebig *et al*., 1985). The volatile oil from fresh leaves of *S. asper* showed anticancer activity in P388 (mouse lymphocytic leukemia) cells (Phutdhawong *et al*., 2004).

2.1.4 ***Piper nigrum*** Linn.

P. nigrum (Family Piperaceae) is locally known as "Phrikthai" or black pepper. The plant contains piperidine and pyrrolidine alkamides (Parmar *et al*., 1997). The most important being piperine, known to possess a variety of biological properties like CNS stimulant, antipyretic, analgesic and antifeedant activities (Miyakado *et al*., 1979). In Iranian traditional medicine, black pepper is used to relieve menorrhagia in women (Craib, 1992).

2.1.4.1 Chemical constituents

The major constituent isolated from *P. nigrum* fruits is piperine, 8Z-N-isobutyleicosatrienamide, pellitorine, trachyone, pergumidiene, isopiperolein B (Venkat, *et al.*, 2004), piperidine, pyrrolidine amides (Parmar *et al*., 1997). The compounds 3, 4-dihydroxyphenyl ethanol glucoside and 3, 4-dihydroxy-6-(N-ethylamino) benzamide reported to be present in green pepper but absent in black pepper (Pradhan *et al*., 1999).

2.1.4.2 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) cells (Lian Ee *et al*., 2010).

Figure 2.4 *Piper nigrum* Linn.

Source of photos: http://www.manager.co.th
http://www.bloggang.com/viewdiary.phpid=nukoy-handmademonth=09-2008date=21group=5gblog=1
http://th.wikipedia.org/wiki.
http://www.nipahutgardens.com/products.aspcat=23

2.1.5 *Suregada multiflorum* Baill.

S. multiflorum (Family Euphorbiaceae) is locally known as "Khunthongphayabat". This plant is used for the traditional treatment of hepatic and gum diseases. The bark is used to treat inflammation and skin diseases (Choudhary *et al*., 2004). Several diterpene lactones, kaurane-type diterpenes, and flavonoids were isolated from the leaves, roots, and seeds of *S. multiflorum* (Choudhary *et al*., 2004). The triterpenoids isolated from the bark has been reported to contain GAP31, a protein which inhibits HIV-1 and is effective in a human breast (Bourinbaiar and Lee-Huang, 1996). *S. multiflorum* contains different classes of chemical constituents listed in Table 2.2.

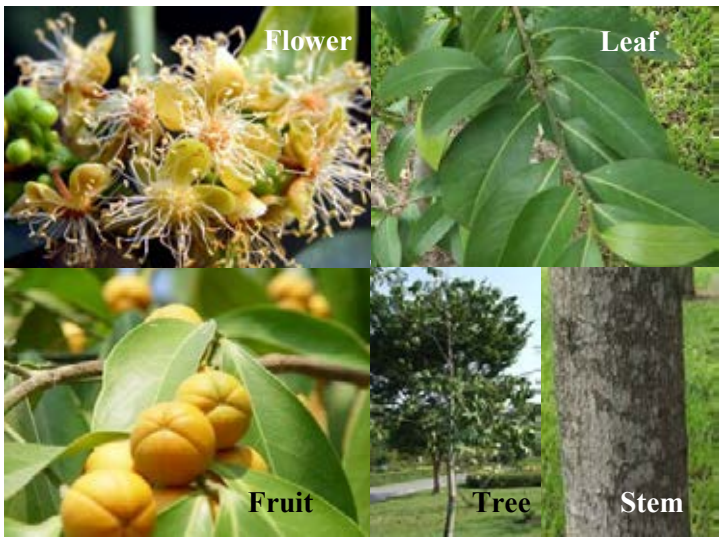


Figure 2.5 *S. multiflorum* Baill.

Source of photos: http://thaiherb.most.go.th/plantdetail.phpid=265
http://www.rspg.or.th/plants_data/kp_bot_garden/kpb_10-10.htm
http://www.nationaalherbarium.nl/ThaiEuph/ThSspecies/ThSuregada.htm

2.1.5.1 Chemical constituents

Table 2.2 The classes of chemical constituents reported in *S. multiflorum*

Class	Chemical constituents	Reference
terpene	gelomulide, jolkinolide B, multiflorenol, gelomusid A and gelomusid B	Talapatra *et al*., 1989 Das and Chakravarty, 1993 Choudhary *et al*., 2004
sterol	β-sitosterol	Talapatra *et al*., 1989
flavonoid	luteolin and scutellarein	Parveen and Khan, 1987 Das and Chakravarty, 1993

2.1.5.2 Anticancer activity

There was no report on anticancer activity of *S. multiflorum*

2.1.6 ***Tinospora crispa*** Miers ex Hook. f. & Thoms.

T. crispa (Family Menispermaceae) is locally known as "Boraphet". *T. crispa* is used as a hepatoprotectant (Adhvaryu *et al*., 2008). A decoction of the stems, leaves and roots is used to treat cholera, diabetes, fever, rheumatism and snake-bites (Sinsh *et al*.,

2003). The stem product is used for washing sore eyes. It also reduces thirst, internal inflammation, increases appetite (Sartori and Swift, 2003), useful as an anti-malarial and a wash for skin ulcers. An infusion is traditionally used to treat fever due to malaria and also in jaundice and for use against intestinal worms. The plant also contains antimalarial effect (Rahman *et al*., 1999). *T. crispa* contains different classes of chemical constituents listed in Table 2.3.

Figure 2.6 *T. crispa* Miers ex Hook. f. & Thoms.

Source of photos: http://www.sunglass.diaryis.com/
http://home.hiroshima-u.ac.jp/~shoyaku/photo/Malaysia/1023Tino.jpg

2.1.6.1 Chemical constituents

Table 2.3 The classes of chemical constituents reported in *T. crispa*

Class	Chemical constituents	Reference
terpenes	cycloeucalenol and cycloeucalenone	Kongkathip *et al*., 2002
terpenoid	β-carotene, *N*-formylanondine, *N*-formylnornuciferine, *N*-acetyl nornuciferine, picrotein, tinotubride	Pathak *et al*., 1995
flavonoid	flavone O-glycosides (apigenin), picroretoside, berberine, palmatine and picroretine	Umi and Noor, 1995 Cavin *et al.*, 1998

terpenes glucoside	borapetoside, tinotureride, tinotufolin, borapetol and tinocrisposide,	Fukuda *et al*., 1983 Pachaly *et al.*, 1992
sterol	γ-sitosterol	Pathak *et al*., 1995
alkaloid	colombine; glucoside, picroretine, berberine, palmatine, jatrorrhizine, tembetarine, choline and feruloyltyamine	Bisset and Nwaiwu, 1983 Cavin *et al*., 1998

2.1.6.2 Anticancer activity

There was no report on anticancer activity of *T. crispa*

2.1.7 *Albizia procera* Benth.

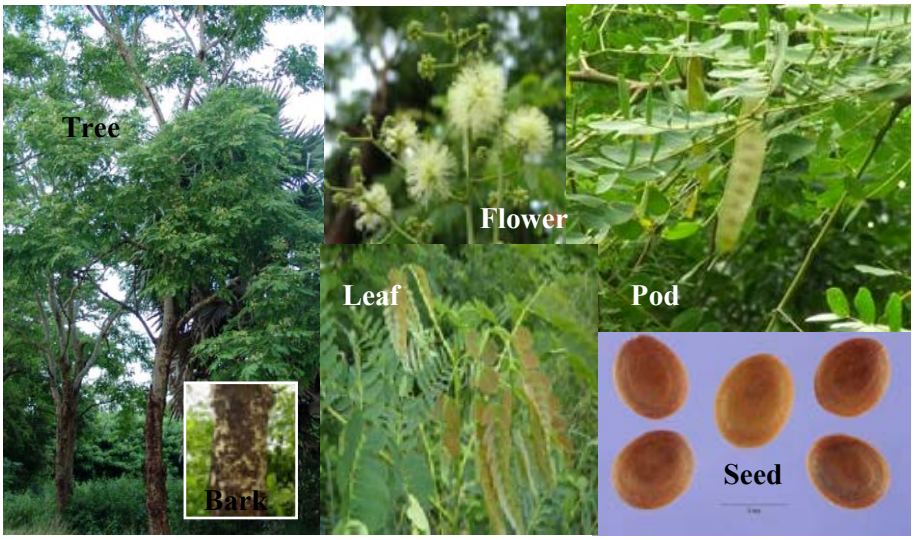


Figure 2.7 *A. procera* Benth.

Source of photos: http://thaiherb.most.go.th/plantdetail.phpid=350
http://commons.wikimedia.org/wiki/File:Albizia_procera_seeds.jpg
http://www.tistr.or.th

A. procera (Family Mimosaceae) is locally known as "Thingthon". *A. procera* bark contains tannins and a reddish gum. It can be used to make a poison, is considered

useful in pregnancy and stomachache (Melek *et al*., 2007). *A. procera* contains different classes of chemical constituents listed in Table 2.4.

2.1.7.1 Chemical constituents

Table 2.4 The classes of chemical constituents reported in *A. procera*

Class	Chemical constituents	Reference
triterpenoid saponin	oleanoliic acid,	Varshney and Badhwar, 1972
	echinocystic acid,	Banerji *et al*., 1979
	proceranin A	Melek *et al*., 2007
	and proceric acid	
amino acid	albizzin	Gmelin *et al.* 1958
flavonoid	biochanin A, daidzein,	Deshpande and Shastri, 1977
	genistein, formononetin	
sterol	β-sitosterol and	Varshney *et al*., 1965
	α-spinasterol	Banerji *et al*., 1979

2.1.7.2 Anticancer activity

Saponins isolated from the bark of *A. procera* exhibited cytotoxicity against HepG2 cell line with IC_{50} value 9.13 μg/ml (Melek *et al*., 2007).

2.1.8 ***Kaempferia parviflora*** Wall. Ex. Baker

K. parviflora (Family Zingiberaceae) is locally known as "Krachaidam". The rhizome flavonoids exhibited antifungal, antiplasmodial and antimycobacterial activities. The plant exhibits no cytotoxicity against KB, (oral human epidermoid carcinoma), BC (breast cancer), and NCI-H187 (human, small cell lung cancer) cell lines (Yenjai *et al.*, 2004), anti-peptic ulcer (Rujjanawate *et al.*, 2005) and anti-allergic (Tewtrakul and Subhadhirasakul, 2007) as well as modulators of multidrug resistance in cancer cells. The rhizome flavone inhibitted P-gp function, which may be useful for overcoming P-gp-mediated multidrug resistance and improving the oral bioavailability of anticancer agents (Patanasethanont *et al.*, 2007). *K. parviflora* contains different classes of chemical constituents listed in Table 2.5.

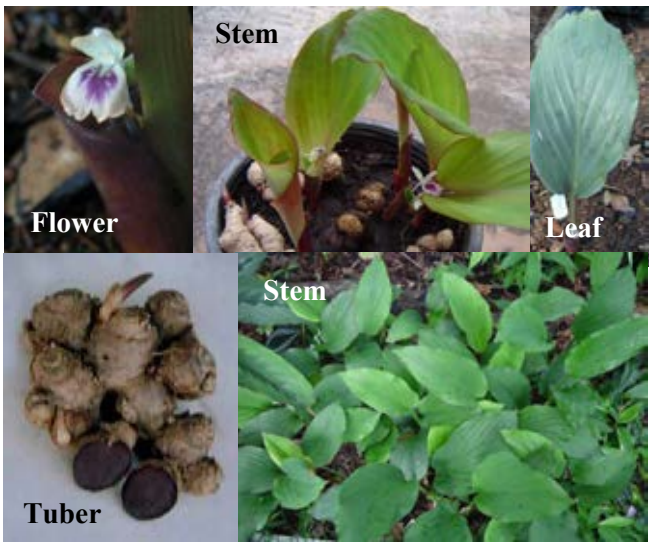


Figure 2.8 *K. parviflora* Wall. Ex. Baker

Source of photos: http://gotoknow.org/file/jannoniramai/view/79836
http://www.gingersrus.com/DataSheet.php?PID=4747

2.1.8.1 Chemical constituents

Table 2.5 The classes of chemical constituents reported in *K. parviflora*

Class	Chemical constituents	Reference
Flavonoid	5-hydroxy-3, 7-dimethoxyflavone, 5-hydroxy-7-methoxyflavone, 5-hydroxy-7,49-dimethoxyflavone, 5-hydroxy-3,7,49-trimethoxyflavone, 5-hydroxy-3,7,39,49-tetramethoxyflavone, 3,5,7-trimethoxyflavone, 3,5,7,49-tetramethoxyflavone, 5,7,49-trimethoxyflavone and 5,7,39,49-tetramethoxyflavone	Yenjai *et al.*, 2004
Chalcone	hydroxypanduratin A and panduratin A	Tuchinda *et al.*, 2002

2.1.8.2 Anticancer activity

There was no report on anticancer activity of *K. parviflora*

2.1.9 ***Diospyros rhodocalyx*** Kurz

D. rhodocalyx (Family Ebenaceae) is locally known as "Takona". Treatments of diarrhea, bleeding, abdominal discomfort, parasitic infestation abscess and renal disease are successive with the fruit products. The relief of leucorrhea and as antidiuretic are successive with the bark products. (Sutthivaiyakit *et al.*, 1995).

Figure 2.9 *Diospyros rhodocalyx* Kurz

Source of photos: http://thaiherb.most.go.th/plantdetail.php?id=223

2.1.9.1 Chemical constituents

The major phytochemical of *Diospyros* species are triterpenoids, naphthoquinones (Mallavadhani *et al*., 1998), lupeol, β-sitosterol, stigmasterol, diospyrin and betulinaldehyde (Theerachayanan *et al*., 2007).

2.1.9.2 Anticancer activity

There was no report on anticancer activity of *D. rhodocalyx*

2.1.10 *Betula alnoides* Buch.-Ham.

B. alnoides (Family Betulaceae) is locally known as “Kamlang Sueakhrong”.

1.1.10.1 Chemical constituents

The known chemical compounds are terpenes (Kamperdick *et al*., 1995)

1.1.10.2 Anticancer activity

There was no report on anticancer activity of *B. alnoides*

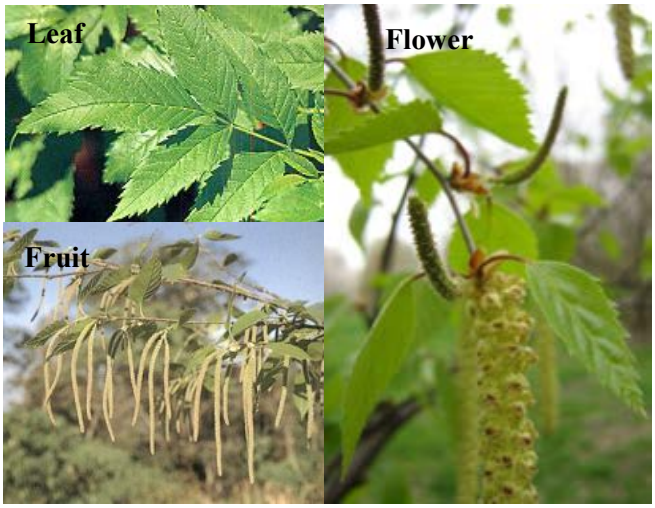


Figure 2.10 *B. alnoides* Buch.-Ham.

Source of photos: http://www.geocities.com/sawasdeenan/aboutnan.htm
http://herba.msu.ru/shipunov/w-album/20080427_boston/thumb.html

2.1.11 *Acacia farnesiana* Willd.

A. farnesiana (Family Mimosaceae, subfamily of Leguminosae) is locally known as “Krathinthet”. The flowers are the source of “cassia oil”. The bark is rich in tannin (Siegler *et al*., 1986). The flowers are used to treat headache and indigestion, whereas a decoction of the green pods is used to treat dysentery and skin inflammations. The bark, heartwood, and leaves are used to treat a variety of ailments (Parrotta, 2001). The pulp surrounding the seeds is used as a plaster for treating tumors and furuncle (Watt and Breyer-Brandwijk, 1962). *A. farnesiana* contains different classes of chemical constituents listed in Table 2.6.

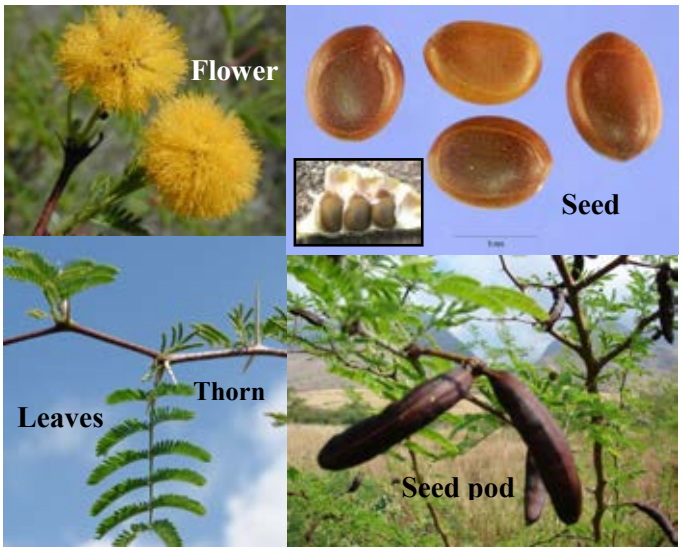


Figure 2.11 *A. farnesiana* Willd.

Source of photos: http://www.hear.org/starr/plants/images/species/?q=acacia+farnesiana

2.1.11.1 Chemical constituents

Table 2.6 The classes of chemical constituents reported in *A. farnesiana*

Class	Chemical constituents	Reference
cyanogenic glycosides	linamarin and lotaustralin	Seigler and Ebinger, 1987
benzenoid	salicylic acid, palmitic acid	El Sissi *et al*., 1973
	gallic acid, m-digallic acid,	Duke, 1981
	ellagic acid and methyl gallate	
phenylpropanoids	methyl-eugenol and eugenol	Duke, 1981
ketones	coumarin and	Duke, 1981
	hydroxyacetophenone	
flavonoids	kaempferol, atomadendrin	El Sissi *et al*., 1973
	and farnesol	Siegler *et al*., 1986
flavonoids glycosides	naringin	El Sissi *et al*., 1973
terpenoid	terpineol, nerolidol and	Siegler *et al*., 1986
	geraniol	

2.1.11.2 Anticancer activity

There was no report on anticancer activity of *A. farnesiana*

2.1.12 *Leucaena leucocephala* de Wit

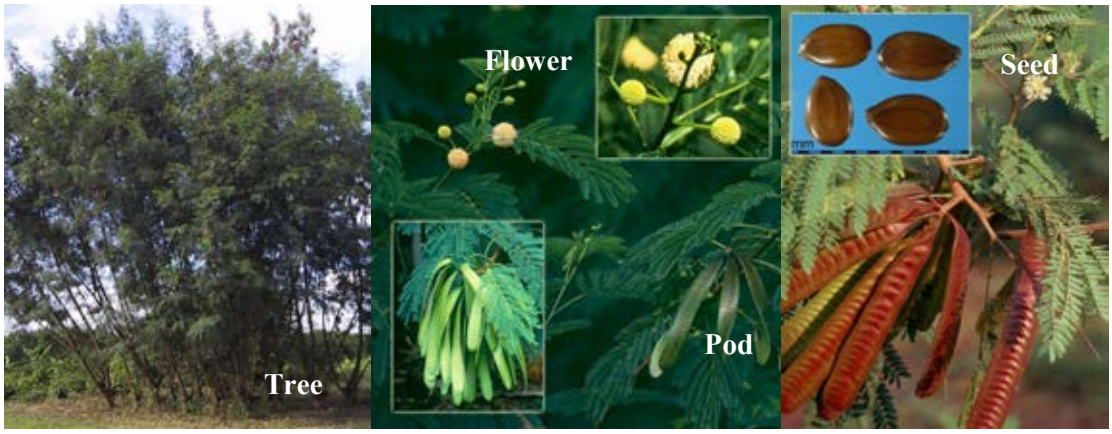


Figure 2.12 *L. leucocephala* de Wit

Source of photos: http://www.tropicalforages.info/key/Forages/Media/Html/Leucaena_leucocephala.htm

L. leucocephala (Family Mimosaceae, subfamily of Leguminosae) is locally known as "Krathinthai". *L. leucocephala* was reported to have few medicinal properties ranging from controlling of stomach diseases to contraception (Jagan Mohan and Azeemoddin, 1988). *L. leucocephala* contains different classes of chemical constituents listed in Table 2.7.

2.1.12.1 Chemical constituents

Table 2.7 The classes of chemical constituents reported in *L. leucocephala*

Class	Chemical constituents	Reference
diterpenoid acids	gibberellin (phytohormone)	Arigayo *et al*., 1983
sterol	ß-sitosterol	Verma and Chandra, 1979
flovanoid	guaijaverin, kaempferol, hyperoside	Morita *et al*., 1977
	quercetagetin and quercetin	Ranganathan and Nagarajan, 1980

2.1.12.2 Anticancer activity

There was no report on anticancer activity of *L. leucocephala*

2.1.13 *Butea superba* Roxb.

B. superba (Family Papilionaceae-Leguminosae) is locally known as "Kwaokrua daeng". The tuber and stem of *B. superba* are used as Thai traditional medicine for tonic and rejuvenile. The plant is enriched with flavonoid and flavonoid glycoside. The bioactivity of each constituent was tested for an inhibitory effect towards cAMP phosphodiesterase, which has been shown to be important in controlling bodily function and involved a wide number of diseases (Roengsumran *et al*., 2000). *B. superba* contains the different classes of chemical constituents listed in Table 2.8.

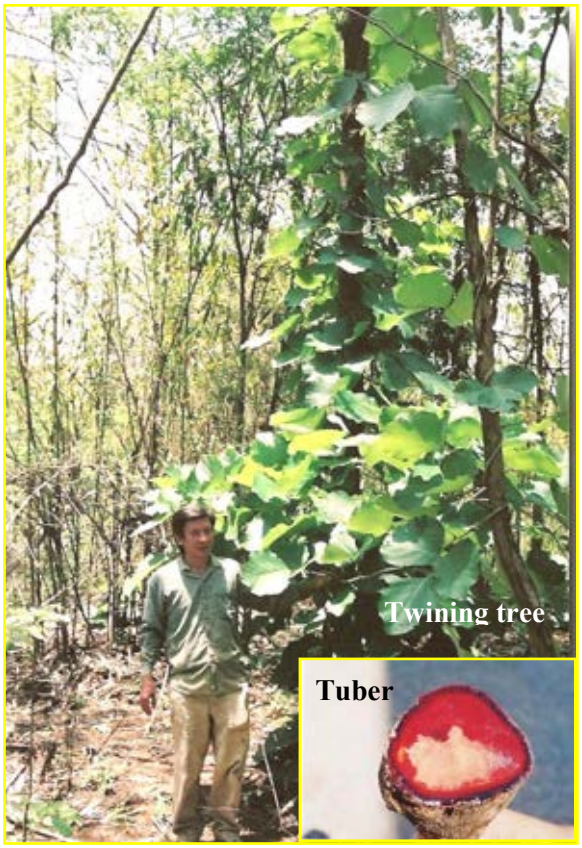


Figure 2.13 *B. superba*

2.1.13.1 Chemical constituents

Table 2.8 The classes of chemical constituents reported in *B. superba* (Sangkapong, 2005)

Class	Chemical constituents	Reference
carboxylic acid	Straight acid carboxylic acid (C_{22}-C_{26}), 3-hexacosanoloxy-propane-1,2-diol	Rakslip, 1995
steroid	Campesterol	Rakslip, 1995
steroid glycoside	β-sitosteryl 1-3-O-β-D-glucopyranside, Stigmasteryl 1-3-O-β-D-glucopyranside	Rakslip, 1995
flavonoid	3-hydroxy-9-methoxypterocarpan (Medicarpin), 7-hydroxy-4_-methoxy-isoflavone (Formononetin), 5,4'-dihydroxy-7-methoxy-isoflavone (Prunetin)	Ngamrojanavanich *et al.,* 2007
flavonoid glycoside	7-hydroxy-6,4'-dimethoxyisoflavone	Roengsamran *et al.,* 2000 Ngamrojanavanich *et al.,* 2007
isoflavone	7,4'-dimethoxyisoflavone	Ngamrojanavanich *et al.,* 2007

2.1.13.2 Anticancer activity

Formononetin and prunetin showed moderate cytotoxic activity on KB (human epidermoid carcinoma of cavity) cell lines with IC_{50} values 37.3 and 71.1 μM and on BC (breast cancer) cell lines with IC_{50} values 32.7 and 47.3 μM, respectively (Ngamrojanavanich *et al*., 2007).

2.1.14 *Pueraria mirifica* Airy Shaw et Suvatabhandu

P. mirifica (Family Papilionaceae-Leguminosae) is locally known as "Kwaokrua khao".

Figure 2.14 *P. mirifica*

The tuberous roots have been consumed effectively as "rejuvenating" folk medicine for both aged men and women. *P. mirifica* contains phytoestrogens with estrogenic activity (Cherdshewasart et al., 2004[a]). *P. mirifica* contains different classes of chemical constituents listed in Table 2.9.

2.1.14.1 Chemical constituents

Table 2.9 The classes of chemical constituents reported in *P. mirifica* (modified from Cheewasopit, 2001)

Class	Chemical constituents	Reference
isoflavonoids	daidzein, genistein, kwakhurin and kwakhurin hydrate	Ingham *et al.*, 1986[a] Ingham *et al.*, 1986[b] Ingham *et al.*, 1989
isoflavone glycoside	daidzin, genistin, mirificin, puerain 6'-monoacetate and puerarin	Ingham *et al.*, 1986[b] Ingham *et al.*, 1989
chromene	miroestrol, deoxymiroestrol and isomiroestrol	Schoeller *et al.,* 1940 Chansakaew *et al.,* 2000[a]
coumestans	coumestrol, mirificoumestan, miricoumestan glycol and	Ingham *et al.*, 1986 Ingham *et al.*, 1988

	miricoumestan hydrate	
sterol	β-sitosterol and stigmasterol	Hoyodom, 1971
pterocapans	pueeriicapene and tuberosin	Chansakaew *et al.*, 2000[a]
acid	tetracosanoic acid	Chansakaew *et al.*, 2000[b]

2.1.14.2 Anticancer activity

P. mirifica extract exhibited antiproliferation activity to MCF-7 cells (Cherdshewasart *et al.*, 2004[a]). *P. mirifica* powder decreased the evidence and virulence of the induced mammary breast tumor in female rats (Cherdshewasart *et al.*, 2007[b]).

2.1.15 *Mucuna collettii* Lace**,** Synonym; *Mucuna macrocarpa* Wallich

M. collettii (Family Papilionaceae-Leguminosae) is locally known as "Kwaokrua dum**"**.

Figure 2.15 *M. collettii*

2.1.15.1 Chemical constituents

The whole stem of *M. collettii* contains the flavonoids namely; kaempferrol, quercetin and hopeaphenol (Roengsamran *et al.,* 2001).

2.1.15.2 Anticancer activity

Hopeaphenol the chemical that was also present in the stem of *M. collettii* exhibited cytotoxicity against KB cell line (Ohyama *et al.*, 1999). The plant crude extract exhibited cytotoxicity to MCF-7 (Cherdshewasart *et al.*, 2004[a]) and HeLa (Cherdshewasart *et al*., 2004[b]).

2.1.16 *Fagraea fragrans* Roxb.

F. fragrans (Family Potaliaceae) is locally known as "Kankrao".

Figure 2.16 *F. fragrans* Roxb.

Source of photos: http://www.rspg.thaigov.net/plants_data/plantdat/loganiac/ffragr_1.htm
http://www.agri.ubu.ac.th/publish/Movie1.html

2.1.16.1 Chemical constituents

The plant known chemical compounds are gentianine (Natarajan *et al.*, 1974), secoiridoid glycoside namely, swertiamarin (Kun-Anake and Rajvatin, 1976). A secoiridoid, named fagraldehyde, gentiopicroside, sweroside and swertiamarin were isolated from the bark and leaves of *F. fragrans* (Marie *et al.*, 2008).

2.1.16.2 Anticancer activity

There was no report on anticancer activity of *F. fragrans*

2.1.17 ***Anaxagorea luzonensis*** Gray

A. luzonensis (Family Annonaceae) is locally known as "Kamlang Wua Thaloeng". The plant is indigenous to Thailand and is used as a traditional medicinal plant as a blood tonic, stomachic, antipyretic and for treatment of muscular pain. The fresh leaves are used as topicals for articular rheumatism (Gonda *et al.*, 2000). *A. luzonensis* contains different classes of chemical constituents listed in Table 2.10.

Figure 2.17 *A. luzonensis* Gray

Source of photos: http://www.dnp.go.th
http://www.bloggang.com/viewdiary.php?id=endless9&group=11

2.1.17.1 Chemical constituents

Table 2.10 The classes of chemical constituents reported in *A. luzonensis*

Class	Chemical constituents	Reference
phenolic (xanthone)	1,3,6-trihydroxy-5-methoxy-4-prenylxanthone, 1,3,6-trihydroxy-4-prenylxanthone,	Gonda *et al*., 2000
	1,3,5-trihydroxy-6-methoxy-2-prenylxanthone, 3,6-dihydroxy-1,5-dimethoxyxanthone	Gonda *et al*., 2000
	methylbutyl)xanthone, 6-deoxy-isojacareubin	Sabphon, 2008
flavonoid	flavone, biochanin A, chrysin, 39-	Gonda *et al*., 2000

	methylorobol, orobol, taxifolin, kaempferol**,** quercetin, naringenin, aromadendrin	Sabphon, 2008
isoflavones	daidzein, genistein	Sabphon, 2008
sterols	stigmasterol	Sabphon, 2008

2.1.17.2 Anticancer activity

There was no report on anticancer activity of *A. luzonensis*

2.1.18 *Dracaena conferta* Ridl.

D. conferta (Family Agavaceae) is locally known as "Kamlang Hanuman". *D. conferta* has been used by Thai traditional medicines for the treatment of bile disorder, analeptic muscle and sinew (วุฒิ, 2540).

Figure 2.18 *D.* species; **Source of photo** http://www.arbolesornamentales.com/Dracenas.htm

2.1.18.1 Chemical constituents

There was no report on the chemical constituents of *D. conferta*

2.1.18.2 Anticancer activity

There was no report on anticancer activity of *D. conferta*

2.1.19 *Vitex trifolia* Linn.

V. trifolia (Family Verbenaceae) is locally known as "Khonthiso". Leaves are commonly used as poultice for inflammation, rheumatic pains, sprains and fever. Roots are used to treat painful inflammations, febrifuge, cough and fever. Flowers are used in treating fever and fruit are used in amenorrhoea (Herna´ndez *et al*., 1999). There have been various studies on the chemical structures of compounds isolated from both *V. trifolia* leaves and fruit (Pan *et al.*, 1989). The *V. trifolia* contains the different classes of chemical constituents listed in Table 2.11.

Figure 2.19 *V. trifolia* Linn.

Source of photos: http://bot.swu.ac.th/upload/meattree_document/1229050283.pdf
http://thaiherb.most.go.th/plantdetail.phpid=517

2.1.19.1 Chemical constituents

Table 2.11 The classes of chemical constituents reported in *V. trifolia*

Class	Chemical constituents	Reference
terpenoid	caryophyllene, friedelin, sabinene and α̃-pinene,	Suksamrarn *et al*., 1991
sterol	daucosterol, β-sitosterol and β-sitosterol-β-D-glucoside	Vedantham and Subramanian, 1976 Zeng *et al*., 1996
flavonoid	casticin, agnuside, luteolin,	Nair *et al*., 1975

	isoorientin, persicogenin, penduletin, artemetin, chrysosplenol-D, vitexin, vitexicarpin 5-methyl artemetin, 7-desmethyl artemetin, and 3, 6, 7-trimethyl quercetagetin	Ramesh *et al.,* 1986 Zeng *et al.,* 1996 Li *et al.,* 2005
flavonoid glycoside	luteolin-7-*O*-β-D-glucuronide, luteolin-3-*O*-β-D-glucuronide	Ramesh *et al.,* 1986
terpenoid	caryophyllene, friedelin, sabinene and α̃-pinene,	Suksamrarn *et al*., 1991
sterol	daucosterol, β-sitosterol and β-sitosterol-β-D-glucoside	Vedantham and Subramanian, 1976 Zeng *et al.,* 1996
fatty acid	linoleeic acid, myristic acid, palmitic acid, palmitoleic acid, γ-tocopherol and steric acid	Prasad and Nigam, 1982

2.1.19.2 Anticancer activity

Extracts from dried fruit of *V. trifolia* has persicogenin and penduletin with anticancer activity (Li *et al.*, 2005[a]), inducing apoptosis and inhibiting cell cycle at the G0/G1 and G2/M phases in mammalian cancer (tsFT210 cells) (Li *et al.*, 2005[b]).

2.1.20 *Phyllanthus emblica* Linn.

P. emblica (syn. *Emblica officinalis*, Family Euphorbiaceae) is locally known as "Makhampom". The fresh (or) the dry fruit is used for the treatment of diarrhea, jaundice and inflammations (Deokar, 1998). The pulp of the fruit is used to treat headache and dizziness (Perry, 1980). *P. emblica* leaves and fruit have been used for fever and inflammatory treatment (Perianayagama *et al*., 2004). *P. emblica* contains different classes of chemical constituents listed in Table 2.12.

Figure 2.20 *P. emblica* Linn.

Source of photos: http://www.agronavigator.cz
http://www.nationaalherbarium.nl/thaieuph/ThPspecies/ThPhyllanthus.htm

2.1.20.1 Chemical constituents

Table 2.12 The classes of chemical constituents reported in *P. emblica*. (modified from Summanen, 1999)

Class	Chemical constituents	Reference
alkaloid	phyllantine, phyllantidine, zeatin, zeatin, nucleotide, zeatin and riboside	Khanna and Bansal, 1975 Ram and Rao, 1976
benzenoid	chebulic acid, chebulinic acid and chebulagic acid	Theresa *et al*., 1965, 1967
	gallic acid	Theresa *et al*., 1965, 1967 Basa and Srinivasulu, 1987
	ellagic acid	Theresa *et al*., 1965, Hui and Sung 1968, Subramanian *et al.*, 1971 Desai *et al.*, 1977

	amlaic acid, corilagin,	Theresa *et al*., 1967
	3-6-di-O-galloyl-glucose and	Srivastava and Ranjan, 1967
	ethyl gallate	
	ß-glucogallin	Theresa *et al*., 1967
		Srivastava and Ranjan, 1967
	1,6-di-O-galloyl-ß-D-glucose,	El-Mekkawy *et al*., 1995
	1-di-O-galloyl-ß-D-glucose,	
	putranjivain A and digallic acid	
	phyllemblic acid, emblicol and	Pillay and Iyer, 1958
	music (=galactaric) acid	Basa and Srinivasulu, 1987
triterpene	lupeol	Desai *et al.*, 1977
		Hui and Sung, 1968
furanolactone	ascorbic acid	Damoradan and Srinivasan, 1935
		Quadry *et al.*, 1962
		Shah and Hamid, 1968
		Basa and Srinivasulu, 1987
diterpene	gibberellin	Ram and Raja, 1978
flavonoid	leucodelphinidin,	Laumas and Seshardi, 1958
	kaempherol, quercetin and	Subramanian *et al.*, 1971
	kaempherol-3-glucoside rutin	Yrjönen *et al.*,
		unpublished results
	kaempherol-3-O-ß-D- glucoside	El-Mekkawy *et al*., 1995
	and quercetin-3-O-ß-D-glucoside	
sterol	ß-sitosterol	Hui and Sung, 1968
carbohydrate	acidic, neutral polysaccharides	Nizzamuddin *et al.*, 1982
	and glucose	Theresa *et al*., 1967

2.1.20.2 Anticancer activity

Eighteen main compounds, including phenolic compounds, proanthocyanidin polymers and norsesquiterpenoids isolated from *P. emblica* roots were estimated for their antiproliferative activities against MK-1 (human gastric

adenocarcinoma), HeLa (human uterine carcinoma), and B16F10 (murine melanoma) cells (Zhang *et al.,* 2004).

2.1.21 *Melia azedarach* Linn.

M. azedarach (Family Meliaceae) is locally known as "Lian". The plant has long been recognized for traditional medicinal and insecticidal properties (Cabral *et al.*, 1995 and Bohnenstengel *et al.*, 1999). *M. azedarach* extracts inhibited vesicular stomatitis (VSV), polio and herpes simplex (HSV) viruses in cell cultures (Wachsman *et al.*, 1982). The fruit extracts possess ovicidal and larvicidal activity (Wandscheer *et al.*, 2004 and Corpinella *et al.*, 2007). The leaf extracts also possess antiviral and antifertility activity (Choudhary *et al.*, 1990).

Figure 2.21 *M. azedarach* Linn.

Source of photos: http://www.swsbm.com/Images/New2-2001/Melia_azedarach.jpg
http://www.dnp.go.th/MFCD1/saraburisite/webpage/tree19.htm

2.1.21.1 Chemical constituents

Different phytochemicals including melianoninol, melianol, melianone, meliandiol, vanillin and vanillic acid have been isolated from fruits (Han *et al.*, 1991). Limonoids and triterpenoids have been isolated from fruits and bark (Lee *et al.*, 1999). Euphane triterpene (Kelecom *et al.,* 1996) and four lignans (Cabral *et al.,* 1995) were isolated from the methanol extract of the seeds of *M. azedarach*.

2.1.21.2 Anticancer activity

There was no report on anticancer activity of *M. azedarach*

2.1.22 *Cyperus rotundus* Linn.

C. rotundus (Family Cyperaceae) is locally known as "Ya haewmoo". *C. rotundus* is a traditional medicinal plant appearing among Indian, Chinese and Japanese natural drugs. *C. rotundus* rhizomes have been used to treat inflammatory bowel disease, fever, pain, and various blood disorders (Raut and Gaikwad 2006, Soumaya *et al*., 2009). The plant has been reported to contain oils, alkaloids, glycosides, tannins, starch and carbohydrates. *C. rotundus* contains different classes of chemical constituents listed in Table 2.13.

Figure 2.22 *C. rotundus* Linn.

Source of photos: http://en.wikipedia.org/wiki/Cyperus_rotundus
http://luirig.altervista.org/photos-int/cyperus-rotundus-tiririca.htm
http://www.cdfa.ca.gov/PHPPS/IPC/weedinfo/cyperus-rotundus.htm

2.1.22.1 Chemical constituents

Table 2.13 The classes of chemical constituents reported in *C. rotundus*

Class	Chemical constituents	Reference
flavonoid	quercetin, luteolin, afzelechin, catechin and auresidin	Cai *et al*., 1991 Masahiko and Kimiye, 1996 Tamura *et al*., 2002 Kiendrebeogo *et al*., 2005
coumarin	6,7-dimethoxycoumarin	Uzi *et al*., 1986
phenolic acids	galloyl quinic acid, ferulic acid and 3-hydroxy- 4-methoxy-benzoic acid	Chen *et al*., 2002 Romani *et al*., 2002

		Kuete *et al*., 2007
sterols	α-cyperone, sitosterol	Thebtaranonth *et al*., 1995
		Kapadia *et al*., 1967
saponin	saponon-olenolic acid	Hikino *et al*., 1967
sesquiterpenes	cyperene, cyperenone, rotunol and selinene	Soumaya *et al*., 2009

2.1.22.2 Anticancer activity

Luteolin exhibited anti-proliferation to K-562 (chronic myelogenous leukemia) cells at IC_{50} of 25 μ/ml (Soumaya *et al*., 2009).

2.2 The cancer

Cancer is the uncontrolled growth and spread of cells that may affect almost any tissue of the body. Activation of ontogenesis or inactivation of tumor suppressor genes is the main molecular causes of cancer. The cells could escape normal control mechanisms in cell proliferation, differentiation, migration and death which collectively maintain the normal cellular architecture and functions in an organized tissue. The chemicals in the cellular microenvironment and the heritable genetic predisposition could initiate somatic mutations leading to cancer (Jongsomboonkusol, 2005).

More than 10 million people are diagnosed with cancer every year. Among the at least 200 different kinds of cancer, lung, liver, colon breast and prostate cancer cause the most cancer deaths each year worldwide. Cancer causes 7.9 million deaths or 13% of deaths worldwide in 2007. It is estimated that there will be 12 million deaths in 2030 (WHO).

In Thailand, the statistical report ten leading sites of cancer from Siriraj Cancer Center in the year 2007 that is shown in Table 2.14. Prostate cancer was the second ranking cause of cancer in males, behind only liver cancer, followed by lung, colon and lymphoma cancer. In women, breast cancer was the most important, followed by cervix, colon, thyroid and lung cancer.

Table 2.14 The ten leading sites of cancer in Thailand (2007) from Siriraj Cancer Center (Ratanawichitrasin, 2007)

Sites	Male	Female	Population	Percentage
liver	477	178	655	9.10
breast	6	1,004	1,010	14.04
prostate	425	0	425	7.30
colon and rectum	304	290	594	8.26
lung	395	189	584	8.12
cervix	0	546	546	7.59
lymphoma	178	124	302	4.20
thyroid	60	196	256	3.56
leukemia	151	104	255	3.54
oral cavity	143	100	243	3.38
all sites	3,370	3,825	7,195	100.00

Prostate cancer is the common cancer found in Western countries and the second leading cause of cancer related deaths in men (American cancer society, 2007). In Thailand, the estimated incidence of prostate cancer is increasing every year. The register data of cancer patients in Siriraj Hospital shows that prostate cancer is the second leading cause of cancer registered in male (Sangruchi, 2004).

The risk of developing prostate cancer increases as age. The disease is more common for men over the age of 50. More than 70% of men diagnosed with prostate cancer are over the age of 65. Other risk factor, the association of race, familial and geographic patterns with mortality directs attention to a significant role for genetic-environmental interactions as determining patterns of disease. Dietary patterns suggest that saturated fat is a significant risk factor (World Cancer Report, 2003).

2.3 Anti-cancer potential base on cytotoxicity assay

The cytotoxicity test method is to measure the effect of test substances on living cells, especially cancer cells. Among the most often used assays are those measuring the metabolic activity of viable cells using colorimetric changes based on tetrazolium salt reduction. This assay presents the changing of colorimetric formation in wells and measuring by automatic microplate reader (Mosmann, 1983). Only the living cells could

reduce the soluble yellow tetrazolium salt, 3-4,5 dimethylthiazol-2,5 diphenyl tetrazolium bromide (MTT), into an insoluble blue formazan crystal by intracellular succinate dehydrogenase. The assay is practically done in a multi-well plate, and MTT formazan production could be analyzed using a scanning multi-well spectrophotometer. This would enable many samples and anti-cancer drugs to be analyzed simply and rapidly (Rocha and Lopes, 2001).

2.4 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 2.23) that have been defined on the basis of morphological criteria (Kanduc *et al.,* 2002).

2.4.1 Necrosis

Necrosis occurs when cells are exposed to extreme variance from physical and chemical trauma which may result in damage to the cell. It begins with an impairment of the cell's ability to maintain homeostasis, leading to an influx of water and extracellular ions. Intracellular organelles including mitochondria and the entire cell swell and rupture (cell lysis). Due to the ultimate breakdown of the plasma membrane, 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).

2.4.2 Apoptosis

Apoptosis is a mode of cell death that occurs under normal physiological conditions. The processes include 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. The apoptotic bodies are rapidly recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill *et al.*, 1989). The apoptotic bodies and the remaining cell fragments will undergo swelling and lyse. The terminal phase of cell death is secondary necrosis (Krähenbühl and Tschopp, 1991).

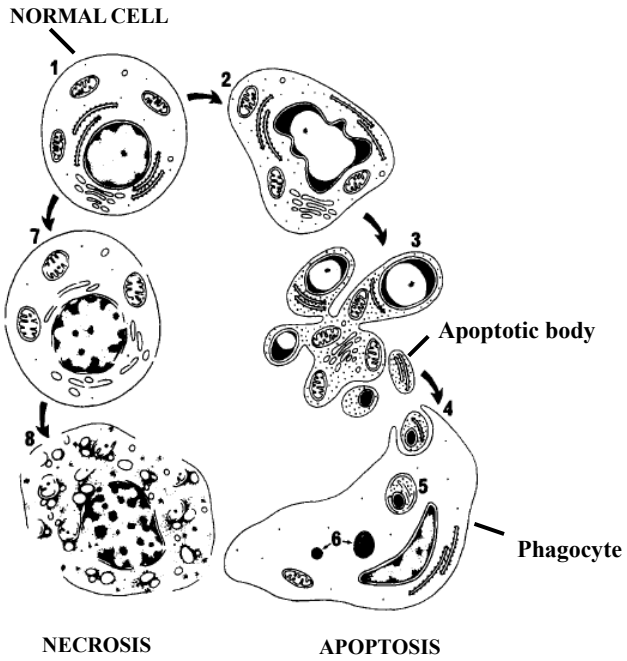


Figure 2.23 The different cell death pathway between necrosis and apoptosis (Kerr *et al.,* 1994)

The diagram (Figure 2.23) shows the sequence of ultra structural changes in apoptosis and necrosis: (2-6 show pathways of apoptosis, 7 and 8 show pathway of necrosis)

(1) Normal cell (early apoptosis)

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

(3) Late apoptosis: nucleus fragmentation and protuberances on the cell surface separate to produce apoptotic bodies.

(4) Phagocytosed of apoptotic bodies by nearby cells.

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

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

(8) Disintegration of membrane, the cell usually retains its overall shape until removed by mononuclear phagocytes.

Some gene products can act as enhancer/inhibitor for apoptosis (Table 2.15). The appearance of these gene products could be used as indicator of the potential of the anti-cancer drugs.

Table 2.15 Gene products influencing apoptosis (modified from Sangkapong, 2005)

Enhancers of apoptosis	Inhibitors of apoptosis
Bcl-x	*Bcl*-2
Bax	*Bcl*-x_L
Bak	*Bcl*-w
Bad	*Mcl*-1
Nbk	*p53*
Bik 1	Colony-stimulating factors
TNF-α	
Fas/Apo1/CD95	
Interleukin-1β-converting enzyme (ICE)	
c-myc	

2.4.3 The differences between necrosis and apoptosis

There are differences in morphological characteristic, physiological impact, biochemical features between necrosis and apoptosis (Table 2.16-2.18) (modified from Rode *et al.,* 2008).

Table 2.16 The differences morphological characteristic between apoptosis and necrosis

Characteristic	Necrosis	Apoptosis
outset	swelling of cytoplasm and mitochondria.	shrinking of cytoplasm, condensation of nucleus.
plasma membrane	loss of membrane integrity	blebbing of plasma membrane without loss of integrity
chromatin	-	aggregation of chromatin at the nuclear membrane.
organelles	disintegration (swelling) of organelles	mitochondria become leaky due to pore formation involving proteins of the *bcl-2* family.

vesicles	no vesicle formation, complete lysis	formation of membrane bound vesicles (apoptotic bodies)
terminal	total cell lysis	fragmentation of cell into smaller bodies

Table 2.17 The differences physiological impact between apoptosis and necrosis

Characteristic	Necrosis	Apoptosis
extent	affects groups of contiguous cells.	localized effect that destroys individual cells.
phagocytosis	phagocytosis by macrophages	phagocytosis by adjacent cells or macrophages.
immune system	significant inflammatory response.	no inflammatory response.
induction	appeared by non-physiological disturbances (complement attack, lytic viruses, hypothermia, hypoxia, ischemica, metabolic poisons)	induced by physiological stimuli (lack of growth factors, changes in hormonal environment).

Table 2.18 The differences biochemical features between apoptosis and necrosis

Features	Necrosis	Apoptosis
regulation	loss of regulation of ion homeostasis.	tightly regulated process involving activation and enzymatic steps.
energy input	no energy requirement (passive process, also occurs at 4°C)	energy (ATP)-dependent (active process, does not occur at 4°C)
DNA	random digestion of DNA (smear of DNA after agarose gel electrophoresis)	non-random mono- and oligonucleosomal length fragmentation of DNA (ladder pattern after agarose gel electrophoresis)
biochemical events	-	• release of various factorsrious factors

		(cytochrome C, AIF) into cytoplasm by mitochondria. • activation of caspase cascade. • alterations in membrane asymmetry (translocation of phosphatidylserine from the cytoplasmic to the extracellular side of the membrane)

2.5 Flow cytometry and apoptosis assay

Apoptosis and necrosis can be distinguished on the basis of differences in morphological, biochemical and molecular changes occurring in the dying cells. Cells undergoing apoptosis display morphologic changes (cell shrinkage, condensation of chromatin and cytoplasm) which are well measurable by flow cytometry (Duvall *et al.*, 1986).

Flow cytometric evaluation of annexin V/propridium iodide (PI) staining was used to determine the form of cell death by measuring the externalization of phosphatidylserine (PS). Induction of apoptosis is associated with plasma membrane changes where PS is translocated from the inner layer of plasma membrane to the outer leaflet (Savill *et al.*, 2000) which can be assessed by measuring the binding of annexin V-conjugated to fluorescein isothiocyanate (FITC) to cells by flow cytometry which will specific bind to apoptotic cells with externalized PS and necrotic cells with leaky membranes. Counterstaining with PI allows differentiation of necrotic and apoptotic cells. Living cells 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. AnnexinV-positive and PI-positive (An+/PI+) cells were found in the late stage of apoptosis. Necrotic cells are PI positive and annexinV-negative (An-/PI+) stained cells (Vermes *et al*., 1995).

2.6 Phytotherapy of prostate cancer

Green tea, pomegranate, lupeol, fisetin, and delphinidin initiate phytotherapic potential for prostate cancer (Syed *et al.*, 2008). Flavonoid vicenin-2 (VCN-2) is active compound of the medicinal herb, can induced antiproliferative and pro-apoptotic effect in prostate cancer (Nagaprashantha *et al.*, 2011).

Dietary nutrients including carotenoids, retinoids, vitamin E, vitamin C, selenium and polyphenols showed the effect in preventing of prostate cancer cell proliferation (Willis *et al.*, 2003). Licochalcone-A, had been reported to exhibit anticancer activity in PC-3 prostate cancer cells (Fu *et al.*, 2004). Neoxanthin and fucoxanthin induce apoptosis in PC-3 prostate cancer cells (Nara *et al.*, 2005). Isopropanolic extract of black cohosh inhibit cell proliferation, induce apoptosis and activation of caspases in prostate cancer cells (Hostanska *et al.*, 2005). Extracts from neem leaf exhibited against PC-3 prostate cancer cell line (Kumar *et al.*, 2006). Baicalin and oridonin, are compounds found in PC-SPES with potential to induce apoptosis in prostate cancer cells (Marks *et al.*, 2002). Gossypol induces apoptosis in human PC-3 prostate cancer cells (Zhang *et al.*, 2007). Tocotrienol extracted from palm oil induce apoptosis in prostate cancer cells (Srivastava *et al*., 2006). β-Lapachone isolated from *Tabebuia avellanedae* induced apoptosis and arrested the cell cycle progression at the G1 phase of the prostate cells (Choi *et al.*, 2003). β-sitosterol and stigmasterol isolated from Saw Palmetto Berry Extract can inhibit the growth of prostate cancer cells (Scholtysek *et al.*, 2009).

2.7 Applications of proteomic techniques in cancer research

Proteome was introduced to describe “all proteins expressed by a genome or tissue” (Wilkins *et al.*, 1997). Proteomics is the study of all expressed proteins in a cell, tissue or organism (Pennington *et al*., 1997). Proteomics, indeed, is the link between genes, proteins and disease (Lohr and Faissner, 2004).

Applications for proteomics in the drug discovery process include the identification of biomarkers of human and animal diseases, searching of proteins involved in carcinogenesis (Fredolini *et al.*, 2010). It is possible to identify novel biomarkers that indicate a respond of cancer cell to specific inhibitor. In this study proteomic approach was applied to aid identification of certain protein that respond to the herbal plant extract treatment to PC-3 cells.

CHAPTER III

MATERIALS AND METHODS

3.1 Plant materials

3.1.1 Plant sample collection

Twenty two Thai herbal plants were selected for this study based on their appearance in the rejuvenating Thai remedies. The plants were identified and authenticated by the domestic traditional medicinal experts, the medicinal expert of the largest traditional herbal plants in Bangkok and the expert researcher. The plant domestic names (Table 3.1) were subsequently adapted to scientific name based on the reference book (วงศ์สถิตย์ ฉั่วสกุล และคณะ , 2543). The plant samples are also being confirmed with the national herbarium**.**

3.1.2 Preparation of crude plant extracts

The plant materials were sliced into pieces and dried in a hot air oven at 70ºC. The dried materials were ground into powder. Fifty grams of plant powders were extracted with 500 ml 95% ethanol for 7 days under darkness. The supernatants were filtered through Whatman No.1 filter paper. The pellet was re-extracted twice with 500 ml 95% ethanol for 3 days. The total supernatants were pooled and evaporated in a rotary evaporator until completely dried. The crude ethanolic plant extracts were collected and stored in light-protect bottles at 4ºC (Figure 3.1).

3.1.3 Preparation of stock solution of plant crude extracts

The crude ethanolic plant extracts were freshly dissolved in 100% dimethyl sulfoxide (DMSO) at the concentration of 100 mg/ml as a stock solution. The stock solutions were diluted to establish the test concentrations of 0.1, 1, 10, 100 and 1,000 µg/ml with 100% DMSO with a criterion of 1% final concentration.

Table 3.1 The plant scientific name, domestic name**,** family, part used and source

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

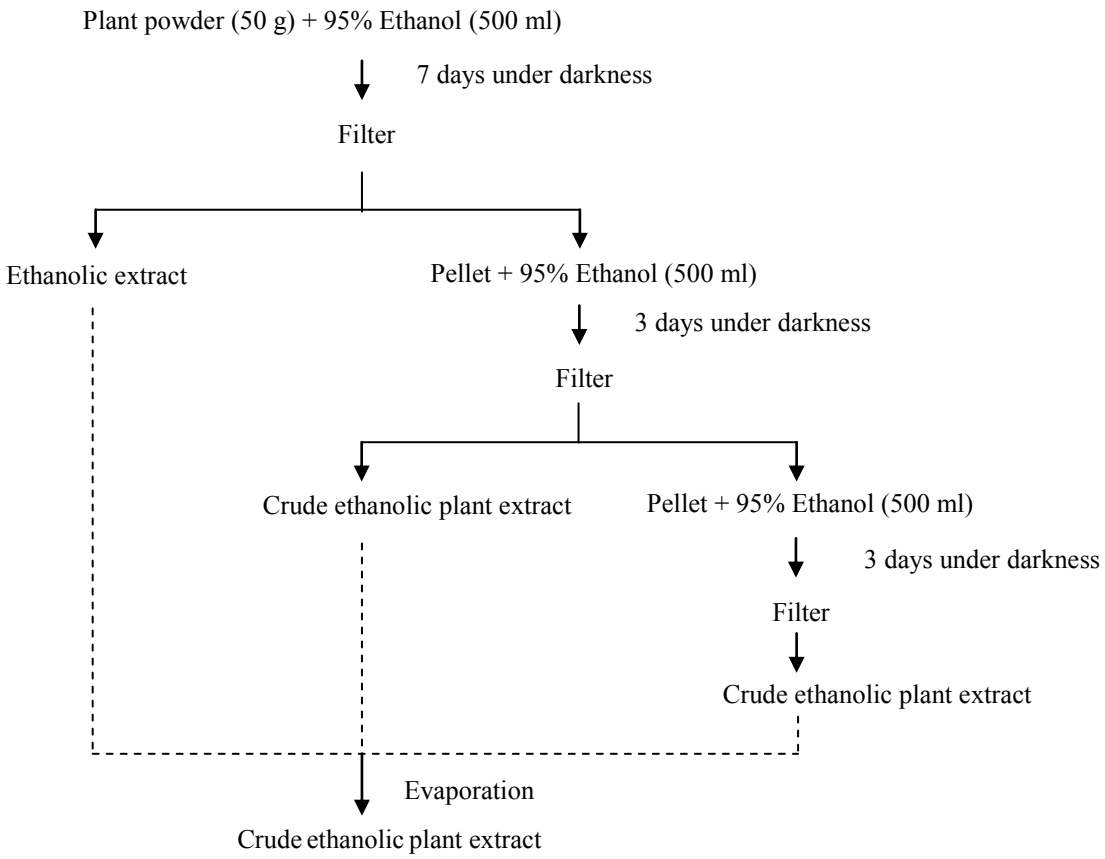


Figure 3.1 The preparation of crude ethanolic plant extract

3.2 Cell line and cell culture

3.2.1 Cell line

PC-3 (ATCC No. CRL-1435), a classical human androgen independent prostate cancer cell line was isolated from bone metastasis of a grade IV prostatic adenocarcinoma tumor of a Caucasian male 62 years old patient. The cells is near-triploid with 62 chromosomes. PC-3 has characteristics of low acid phosphatase and testosterone-5-alpha reductase activity. The cell was purchased from American Type Culture Collection (ATCC) in February 2010.

3.2.2 Culture of PC-3 cells

PC-3 cells were cultured in a culture medium (complete medium; RPMI 1640 medium containing 1 % penicillin/ streptomycin and 10 % fetal bovine serum (FBS)) in a 75 cm^2 tissue culture flask. The cells were maintained at 37ºC under the 95% humidity

atmosphere in a 5% CO_2 incubator. The cells were subcultured when they reached 80% confluence (3-4 days) with the aid of trypsinization.

3.2.3 Subculture of PC-3 cells

The culture medium was removed from the 75 cm^2 culture flask and washed with 4 ml phosphate buffer saline (PBS). 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 min. The solution 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 15 ml and cultured in the 37ºC, 5% CO_2 incubator.

3.2.4 Cell suspension preparation for assay

PC-3 cells were propagated 3-4 days before starting of the experiment. The cells were rinsed with 4 ml PBS followed by removal of the solution. The cells were detached from surface of the flask with the aid of 2.5 ml 0.05% trypsin in 0.01% EDTA for 1-2 min. 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 100 g_{max} for 10 min. The supernatant was discarded and the pellet was resuspended with a fresh culture medium.

3.2.5 Cell count and dilution

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 haemocytometer 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 3.2).

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

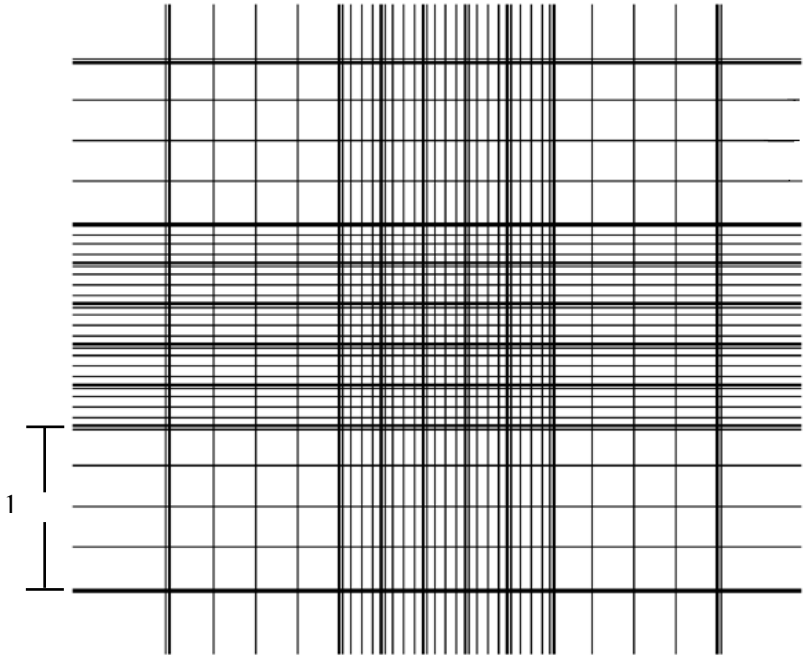


Figure 3.2 Magnified view of the cell counting chamber grid. The central 1 mm^2 area is divided into 25 smaller squares, 1/25 mm^2 each and are further subdivided into 16 squares, each 1/400 mm^2

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

Cell per 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 dept 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)

3.3 Antiproliferative assay

The cell viability was assessed with MTT (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl tetrazolium bromide) assay based on the cleavage of tetrazolium salt by viable cells to form an insoluble purple formazan crystal. The amount of formazan product is directly proportional to the number of viable cells (Carmichael *et al.,* 1987 and Twentyman, 1987). Therefore, this procedure could determine the inhibitory dose of plant crude extracts on cancer cells (Figure 3.3).

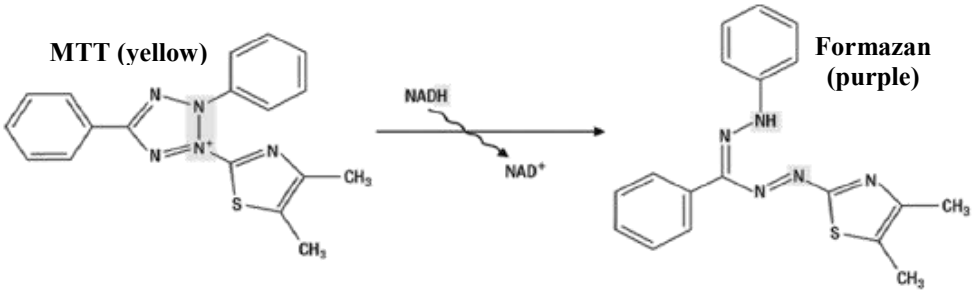


Figure 3.3 Molecular structure of MTT and its corresponding reaction product

The cells were seeded into a 96-well culture plate at a density of 5×10^3 cells/100 µl culture medium/well and incubated at 37ºC, 5% CO_2 incubator for 24 h to enable cell attachment. The medium was removed and replaced with a fresh culture medium containing the tested concentrations of the plant crude extracts. The cells were incubated at 37ºC, 5% CO_2, for 72 h. The culture medium was discarded and 10 µl of normal saline containing 5 mg/ml MTT solution was added to the well. The plate was incubated under darkness at 37ºC for 4 h in order to allow MTT to be metabolized and formed crystals. The MTT solutions were discarded and the MTT-formazan crystals were dissolved in 150 µl DMSO. The plate was gently shaken with the plate shaker (IKA, Werk Janke & Kunkel) at 25ºC for 5 min to aid complete solubilization. The absorbance of the solutions

was determined at 540 nm using a microplate reader (Tecan, Durham, NC, USA). The results were shown in a line graph of the percentage of cell viability (Y-axis) against the concentrations of the sample (X-axis). The concentration of 50% cytotoxicity (IC_{50}) was subsequently calculated.

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 was calculated from the antiproliferation plot. It was defined as the 50% reduction of the absorbance or 50% of the percentage of cell viability compared with cells that were treated with DMSO as a negative control in the MTT assay.

3.4 Flow cytometry analysis

The cells were plated into the 6-well culture plates at a density of 2×10^5 cells per 1 ml culture medium per well and incubated for 24 h at 37ºC, 5% CO_2 incubator which enable cell attachment. The culture medium was discarded and replaced with a fresh culture medium containing the crude plant extract at the concentration of IC_{50} for 72 h. The adherent cells were trypsinized, mixed with the floating cells from the supernatant collected from the wells, transferred to a 15 ml centrifuge tube and centrifuged at 520 g_{max} at 4°C for 10 min. The pellet was dissociate and washed with cold PBS and centrifuged twice. The pellet was dissolved with 0.5 ml cold PBS, mixed and transferred to a 1.5 ml Eppendorf tube. The cell suspension was mixed with 50 µl binding buffer containing 5 µl of annexin V-conjugated to fluorescein isothiocyanate (FITC) and 5 µl of propidium iodide (PI; 50 µg/ml). The cells were gently vortexed and incubated for 15-20 min at room temperature under darkness and additional 150 µl binding buffer was added before analysis with a flow cytometer.

3.5 Proteomic analysis

3.5.1 Protein preparation

The cells were seeded in 75 cm^2 culture flasks (0.8 x 10^6 cells/flask) and were cultured in a culture medium at 37ºC, 5% CO_2, for 72 h before treatment. To investigate

the differential protein expressions in the treated cells prior to apoptosis, the cells were treated with the plant crude ethanolic extracts at the concentration lower than IC_{50} and also a shorter treatment period, Hence, the cells were treated with the plant ethanolic crude extract at IC_{20} for only 48 h. The attached cells were triplicate washed with 3 ml 0.25 M sucrose. The attach cells were imbedded in 450 µl 0.25 M sucrose containing protease inhibitor cocktail (500:1, v/v) before harvested by scraping. The treatments were triplicate repeated, followed by centrifugation at 520 g_{max}, 4°C for 15 min. The supernatants were discarded. The pellets were re-suspended in 50-100 µl lysis buffer containing 2% dithiothreitol (DTT), 4% 3-[3-(cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 M thiourea, 7 M urea, 2% ampholytes (pH 3-10) and 1% protease inhibitor cocktail, vortexed and incubated at 4°C for 1 h. The cells were sonicated on ice for 5-10 times or until cells broke (the clear lysis buffer appeared) to avoid protein to be denatured from heat during sonication. The cellular debris were removed by centrifugation at 13,200 g_{max}, 4°C for 10 min. The protein concentration in the supernatant was determined using the Bradford assay.

The 150 µg protein solution was aliquot and mixed with the lysis buffer and set a total volume to 100 µl. The solutions were mixed with 150 µl rehydration buffer (containing 0.28% DTT, 4% CHAPS, 8 M urea, 2% IPG buffer) and 2 µl 1% bromophenol blue.

3.5.2 Two dimensional electrophoresis (2-DE)

The protein separation with the aid of electrophoresis was applied to the isolated protein solution harvested from the treated cells. The first dimension of electrophorsis is to separate proteins by isoelectric focusing (IEF) based on their charges. IEF was conducted using the commercially available IEF Immobiline Drystrips (7-13 cm, non-linear, pH 3-10 gradient) (GE Healthcare). The protein solutions were soaked to be absorbed into IEF Drystrips for 24 h at room temperature. The first dimension of electrophoresis was run under the conditions of 20°C, 20,001 V/hrs with the limited current of 55 µA per gel strip. The IEF strips were incubated in equilibration buffers A (containing DTT, urea, sodium dodecyl sulfate (SDS), 0.5% Tris-HCl pH 6.8 and glycerol) and B (containing iodoacetamide, urea, SDS, 0.5% Tris-HCL pH 6.8 and glycerol) for 10 min each, under the gentle shaking at room temperature. The equilibrated IEF strips were applied to the top of the separating gel and covered with 0.5 % agarose.

The second dimension of electrophoresis to separate proteins according to their molecular weights was performed in 14% SDS-polyacrylamide electrophoresis gels at the constant current of 25 mA/gel. The protein separations were stopped when the bromophenol blue reached the bottom of the gel.

3.5.3 Gel staining, image analysis and data interpretation

Protein spots on the gel were visualized with the aid of Coomassie blue R-250 staining overnight with gently shaken at room temperature. The gels were destained with the destain I solution (40 % methanol, 10 % acetic acid) for 1 h, followed by destain II solution (10 % methanol, 5 % acetic acid) for 1 h or until the clear backgrounds were appeared. The destained gels were scanned with the aid of Amersham Biosciences image scanner. The experiments were triplicated performed including set of controls and treated cells, which were to be aligned, match and analyzed with the aid of the Image Master 2-DE platinum 7.0 software. The parameters used for the spot detection were including the minimal area of 45 pixels; smooth factor of 4.0 and saliency of 10. The significantly different protein spots in terms of the percentage of volume ($p < 0.05$) were selected for further analysis for amino acid sequence by mass spectrometry.

3.5.4 In-gel protein digestion

The interesting protein spots (in 3.5.3) were cut and transferred into an Eppendorf tube and washed twice with deionized water (100 µl). The gel cubes were sliced into pieces, followed by destaining with 0.1 mM NH_4HCO_3 and 50 % acetonitrile (ACN) for 20 min in the shaking incubator at 30°C (repeated two or three times or until the gels were clear). The supernatants were removed and the pieces of gel were dried under a speed vacuum for 10 min. Gels were swelled in 0.1 mM NH_4HCO_3, 10 mM DTT and 1 mM EDTA in the shaking incubator at 60°C for 45 min in order to reduce the disulfide bonds. The supernatants were discarded. The 100 mM iodoacetamide and 0.1 mM NH_4HCO_3 were added and incubation at 25°C for 30 min under darkness to promote alkylation of the proteins. The supernatants were discarded and the pieces of gel were washed 3 times with 50 µl of 0.05 M Tris-HCl, pH 8.5 and 50 % ACN. The pieces of gel were dried under a speed vacuum for 10 min. The pieces of gel were added with digestion buffer 30 µl; buffer (0.05 M Tris-HCl, pH 8.5, 10 % ACN and 1 mM $CaCl_2$) and trypsin (10 % acetic acid and 0.2 mg/ml trypsin) (9:1, v/v). The reaction was incubated at 37°C

overnight. The supernatants containing proteins were transferred into a new master tube. The reaction from trypsin was terminated with 2 % trifluoacetic acid (2% TFA) before keeping in the shaking incubator for 30 min at 30°C. The supernatants containing proteins were collected in the master tube. The 0.05 M Tris-HCl, pH 8.5 and 1 mM $CaCl_2$ were added into the original tube, followed by keeping in the shaking incubator at 30°C for 10 min. The protein solutions were sonicated for 5 min. The supernatants containing proteins were transferred to the master tube. The 5 % formic acid and 100 % ACN were added into the original tube in the shaking incubator at 30°C for 10 min followed by sonication for 5 min. The supernatants containing proteins were collected in the master tubes. The master tubes were completely dried under the speed vacuum. The dried protein samples were analyzed by mass spectrometer.

3.5.5 Identification of proteins by LC/MS/MS

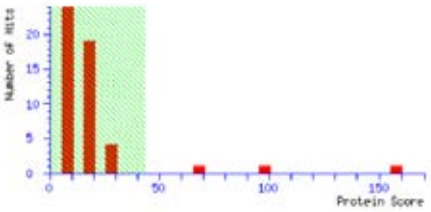
Nanoflow liquid chromatography coupled with electrospray ionization (nano ESI MS/MS) quadrupole-time of flight tandem mass spectrometry (Q-ToF micro; Micromass, UK) was used to identify the trypsinized protein spots. A 75 μm id x 150 mm C_{18} PepMap column was used to concentrate and desalt the trypsinized peptides. To elute peptides, 0.1% formic acid in 97% water, 3% ACN (solution A) and 0.1% formic acid in 3% water, 97% ACN (solution B) were used, respectively. The protein samples were injected into the nano-LC system for separation. The Nano ESI MS/MS was used to produce MS/MS spectra under the automatically processes. The trypsinized proteins were sought against a non-redundant database named ProteinLynx Global SERVER (www.micromass.co.uk). Additionally, the trypsinized proteins were identified by Mascot searching tool (www.matrixscience.com) (Figure 3.4).

Mascot Search Results

User : tuk
Email : tuk_chu@hotmail.com
Search title :
MS data file : CU_Tuk_E18-138D_11_1_12R1.pkl
Database : NCBInr 20120212 (17258491 sequences; 5919220959 residues)
Taxonomy : Homo sapiens (human) (244004 sequences)
Timestamp : 17 Feb 2012 at 07:27:20 GMT
Protein hits : gi|4507953 14-3-3 protein zeta/delta [Homo sapiens]
gi|9507245 14-3-3 protein gamma [Rattus norvegicus]
gi|4507949 14-3-3 protein beta/alpha [Homo sapiens]

Mascot Score Histogram

Ions score is -10*Log(P), where P is the probability that the observed match is a random event.
Individual ions scores > 43 indicate identity or extensive homology (p<0.05).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



Peptide Summary Report

Format As: Peptide Summary — Help
Significance threshold p< 0.05 — Max. number of hits AUTO
Standard scoring / MudPIT scoring — Ions score or expect cut-off 0 — Show sub-sets 0
Show pop-ups / Suppress pop-ups — Sort unassigned Decreasing Score — Require bold red
Select All | Select None | Search Selected | Error tolerant

1. gi|4507953 Mass: 27728 Score: 157 Matches: 5(1) Sequences: 5(1) emPAI: 0.47
14-3-3 protein zeta/delta [Homo sapiens]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
75	669.4410	668.4337	668.3897	0.0440	0	35	0.3	1		K.VFYLK.M
113	907.4886	906.4813	906.5174	-0.0361	0	20	14	1		R.NLLSVAYK.N
137	640.3196	1278.6246	1278.6456	-0.0209	1	19	17	1	U	R.YLAEVAAGDDKK.G
139	652.8284	1303.6422	1303.6772	-0.0349	0	28	2.4	1	U	K.FLIPNASQAESK.V
144	774.8248	1547.6350	1547.7063	-0.0713	0	55	0.0034	1	U	K.SVTEQGAELSNEER.N

Proteins matching the same set of peptides:
gi|30354619 Mass: 35314 Score: 157 Matches: 5(1) Sequences: 5(1)
YWHAZ protein [Homo sapiens]
gi|49119653 Mass: 29929 Score: 157 Matches: 5(1) Sequences: 5(1)
YWHAZ protein [Homo sapiens]
gi|68085578 Mass: 27696 Score: 157 Matches: 5(1) Sequences: 5(1)
Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide [Homo sapiens]
gi|83754467 Mass: 29242 Score: 157 Matches: 5(1) Sequences: 5(1)
Chain A, Molecular Basis For The Recognition Of Phosphorylated And Phosphoacetylated Histone H3 By 14-3-3
gi|161172138 Mass: 26268 Score: 157 Matches: 5(1) Sequences: 5(1)
Chain A, Phosphorylation Independent Interactions Between 14-3-3 And Exoenzyme S: From Structure To Pathogenesis
gi|347948616 Mass: 28009 Score: 157 Matches: 5(1) Sequences: 5(1)
Chain A, X-Ray Induced Covalent Inhibition Of 14-3-3

2. gi|9507245 Mass: 28285 Score: 101 Matches: 4(0) Sequences: 4(0) emPAI: 0.29
14-3-3 protein gamma [Rattus norvegicus]
Check to include this hit in error tolerant search

Query	Observed	Mr(expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Unique	Peptide
75	669.4410	668.4337	668.3897	0.0440	0	35	0.3	1		K.VFYLK.M
113	907.4886	906.4813	906.5174	-0.0361	0	20	14	1		R.NLLSVAYK.N
122	540.7783	1079.5420	1079.5499	-0.0078	0	34	0.49	1	U	R.YLAEVATGEK.R
148	822.3933	1642.7720	1642.7798	-0.0078	0	11	96	1	U	K.NVTELNEPLSNEER.N

Figure 3.4 Mascot searching tool (www.matrixscience.com) was used to identify proteins after analysis with LC/MS/MS.

3.6 Immunoblotting analysis

The cells were plated in a 25 cm^2 culture flask (1 x 10^5 cells/flask) and cultured in culture medium and incubated at 37ºC, 5% CO_2, for 48 h prior to the test. The refreshed cells were treated with the ethanolic crude plant extracts at the concentration of IC_{20} in a culture medium for 48 h. The cells were triplicate washed with 2 ml 0.25 M sucrose. The cells were imbedded in 150 µl of Radio Immune Precipitation Assay (RIPA) buffer containing protease inhibitor cocktail (500:1, V/V) before harvest by scraping. The cells were vortexed for 30 s and sonicated on ice to aid extraction of proteins. The cells were centrifuged at 13,200 g_{max}, 4°C for 15 min and the supernatants were collected. The protein concentration was determined by using Bradford assay. The 15-20 µg of proteins were mixed with sample running buffer and boiled for 5 min. The protein samples could be kept at -80°C. Proteins were loaded onto a 10 % SDS-PAGE at the constant 10 mA/gel for 2-2.5 h. The separated proteins were electrotransfered onto a polyvinylidene fluoride (PVDF) membrane at 100 V for 1 h at 4°C. The membranes were incubated at 4°C with a blocking buffer consisted of TBS/T (Tris-buffered saline, 0.1% Tween-20) and 10% non-fat dry milk overnight. The membranes were washed and incubated with the appropriate dilution of specific primary antibody to proteins of interest for overnight at 4°C (The dilution of the first and second antibody was shown in Table 3.2). The membranes were washed with 10% non-fat dry milk and incubated with secondary antibody for 1 h, followed by washing and incubating for 5 min with enhanced chemiluminescence reagent. The positive signals on the membranes were detected with the high-performance film.

Table 3.2 The dilution of first and second antibody in immunoblotting

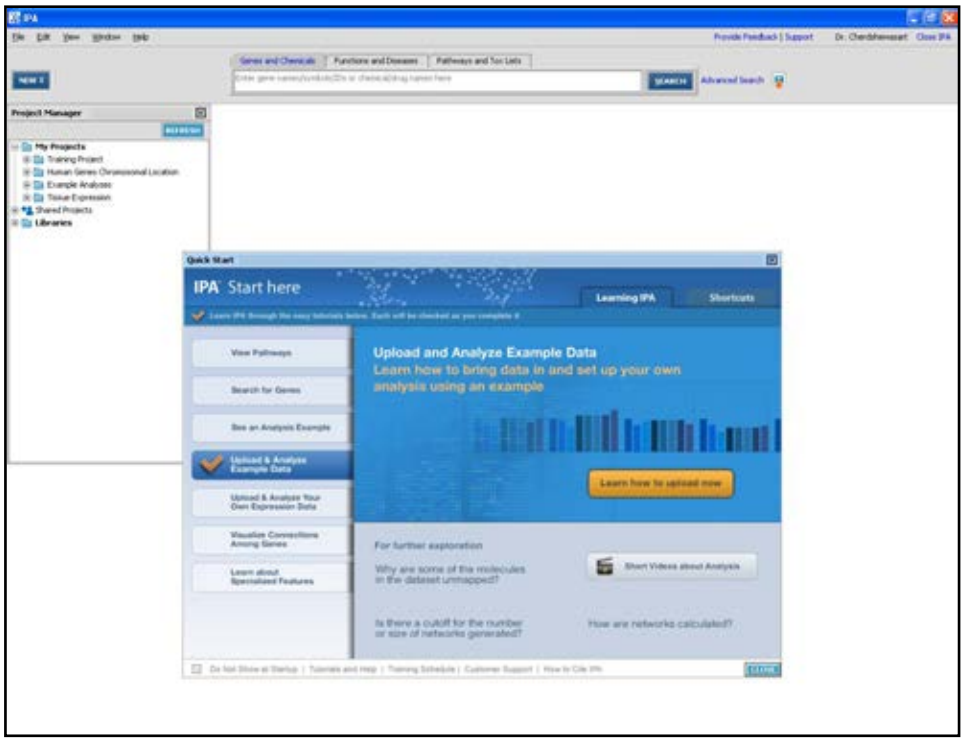
Protein	MW (kDa)	First antibody Dilution	Secondary antibody	Secondary antibody dilution
Bcl-2	26	1 : 1,000	Rabbit	1 : 2,000
Bax	20	1 : 1,000	Rabbit	1 : 2,000
Caspase-3	17, 19, 35	1 : 500	Rabbit	1 : 1,000
PARP	24, 89, 116	1 : 1,000	Rabbit	1 : 2,000
Pan 14-3-3	30	1 : 2,000	Rabbit	1 : 4,000
14-3-3sigma	26-30	1 : 500	Mouse	1 : 1,000
GAPDH	36	1 : 1,000	Rabbit	1 : 2,000
PKM2	58	1 : 2,000	Mouse	1 : 4,000
α-tubulin	52	1 : 5,000	Mouse	1 : 10,000

3.7 Global network analysis

The change in expression level of proteins by 2-fold or more related to the biological pathways were uploaded (accession number and fold-change) to global protein network analysis using Ingenuity Pathways Analysis (IPA) tool, version 7.0 by Ingenuity® systems (http://www.ingenuity.com). The generating set of networks was done by mapping of each protein and its corresponding gene using the Ingenuity Pathway knowledge data base (Figure 3.5). The scores for each network according to the significant genes were computed by IPA. The p-values of candidate genes in a network were calculated and their biological functions were referenced from the relating genes, cells, diseases, drugs and other biological entities.

3.8 Statistical analysis

For MTT analysis, the data were shown as mean ± standard error of mean (S.E.M.) of three replicated experiments (n = 3). 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 $p < 0.05$ with the aids of SPSS® version 14.0. For two-dimension electrophoresis, the protein spots were compared with the percentage of volume of total protein spots and reported as percentage of volume using inter-class statistical analysis (ANOVA).



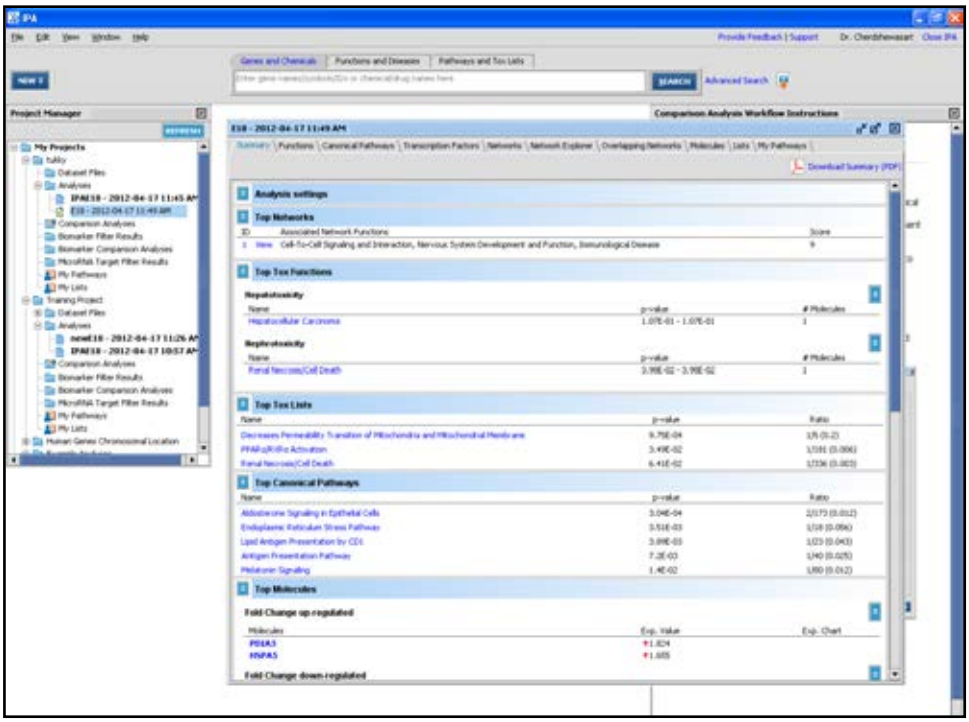


Figure 3.5 Ingenuity Pathways Analysis (IPA) tool, version 7.0 by Ingenuity® systems (http://www.ingenuity.com) was used to generate biological pathways and also interrelationship between networks.

CHAPTER IV

RESULTS

4.1 Characteristics of the plant crude extracts

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

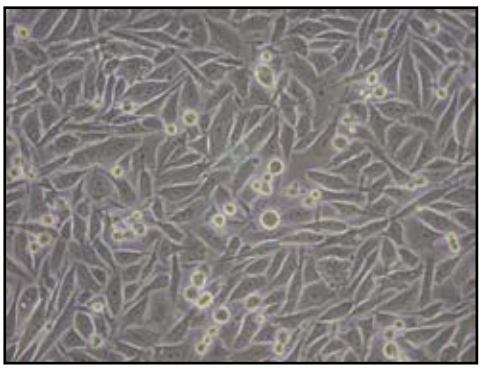
Table 4.1 The percentage yields and characteristics of plant extracts

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 gum
Betula alnoides	23.22	red-brown solid
Butea superba	15.14	brown sticky gum
Cyperus rotundus	4.9	red-brown viscous gum
Diospyros rhodocalyx	4.74	black sticky gum
Dracaena conferta	4.52	red powder
Fagraea fragrans	11.64	brown sticky gum
Kaempferia parviflora	2.64	green-yellow viscous gum
Leucaena leucocephala	4.9	red-brown sticky gum
Melia azedarach	18.84	black-brown powder
Mucuna collettii	4.76	red-brown viscous gum
Phyllanthus emblica	9.96	brown viscous gum
Piper nigrum (seed)	11.08	black-brown viscous gum
Piper nigrum (fruit)	9.63	yellow viscous gum
Pueraria mirifica	14.72	light yellow solid
Stephania erecta	10.82	red-brown powder
Stephania venosa	5.34	red-brown solid

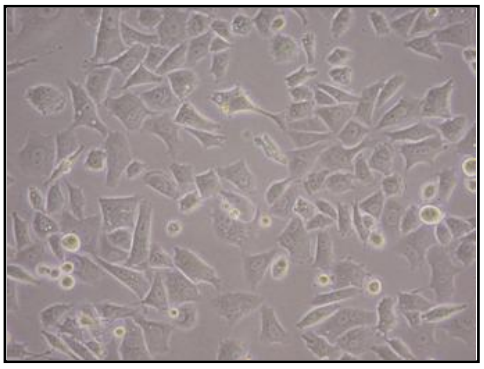
Streblus asper	4.18	green-brown viscous gum
Suregada multiflorum (stem)	10.56	black sticky gum
Suregada multiflorum (leaf)	12.42	yellow viscous gum
Tinospora crispa	4.52	brown viscous gum
Vitex trifolia	5.26	yellow-brown solid

4.2 Changes in cell morphology

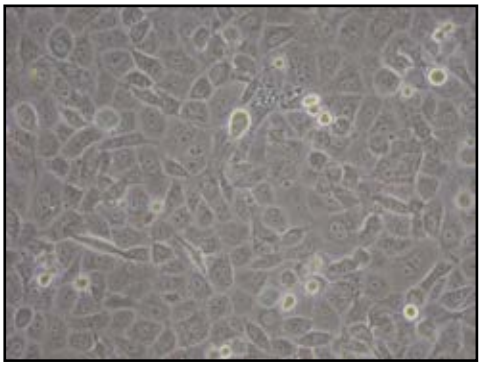
The morphology of PC-3 cells treated with the plant ethanolic crude extract were compared with the DMSO control cells under a visualization with the aid of a phase-contrast inverted microscope. There were differences in morphologic characteristics among the control and the treated cells. The shrink, round and float cells that had lost their anchorage ability were found more in the plant ethanolic crude extract treated groups than in the DMSO control group (Figure 4.1).



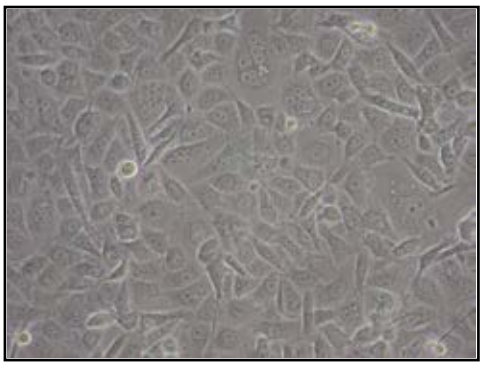
(a) PC-3 cells treated with DMSO



(b) PC-3 cells treated with *S. erecta*



(c) PC-3 cells treated with *S. venosa*



(d) PC-3 cells treated with *S. asper*

Figure 4.1 Morphological changes in PC-3 cells treated with plant ethanolic crude extract at the IC_{50} value for 72 h (a) DMSO control, (b) cells treated with *S. erecta* ethanolic crude extract, (c) cells treated with *S. venosa* ethanolic crude extract and (d) cells treated with *S. asper* ethanolic crude extract

4.3 Antiproliferation of PC-3 cells

The results of antiproliferation of the plant ethanolic crude extracts against PC-3 cells from the triplicated experiments determined with MTT assay are presented as IC_{50} values (Table 4.2).

There were 3 out of 24 plant ethanolic crude extracts namely *S. erecta*, *S. venosa* and *S. asper* exhibited antiproliferation against PC-3 cells with IC_{50} within the range of 1-100 μg/ml. The IC_{50} values were of 4.30, 4.47 and 10.78 μg/ml, respectively.

There were 9 out of 24 plant ethanolic crude extracts, *P. nigrum* (seed), *S. multiflorum* (leaf), *T. crispa*, *A. procera, S. multiflorum* (stem), *P. nigrum* (fruit), *K. parviflora*, *D. rhodocalyx* and *B. alnoides* showed antiproliferative activity against PC-3 cells with IC_{50} within the range of 100-1,000 μg/ml. The IC_{50} values were 124.37, 127.36, 331.01, 339.18, 508.54, 529.50, 692.65, 911.22 and 985.33 μg/ml, respectively.

There were 12 out of 24 plant ethanolic crude extracts, *A. farnesiana, L. leucocephala, B. superba, P. mirifica*, *M. collettii, F. fragrans, A. luzonensis, D. conferta*, *V. trifolia*, *P. emblica*, *M. azedarach* and *C. rotundus*, showed no antiproliferation activity against PC-3 cells. The IC_{50} values were greater than 1,000 μg/ml.

The growth curves of the PC-3 cells treated with the plant extracts with the top three of IC_{50} values from Table 4.2 are demonstrated in Figure 4.2-4.4.

Genistein was used as a positive control in the present study. The antiproliferation effect of genistein against PC-3 cells was tested at the concentration of 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5} and 10^{-4} M. The percent of cell viability slowly decreased as the concentration of genistein increased. Therefore, genistein showed dose-dependent antiproliferation activity on PC-3 cells (Figure 4.5).

Table 4.2 Antiproliferation of the plant ethanolic crude extracts against PC-3 cells determined with MTT assay presented as IC_{50} values (mean ± S.E.M.) in comparison with genistein (positive control).

Ranking no.	Rejuvenating herbal plant	IC_{50} (µg/ml)
1	*Stephania erecta*	4.30 ± 0.02^{a}
2	*Stephania venosa*	4.47 ± 0.03^{a}
3	*Streblus asper*	10.78 ± 0.02^{a}
4	*Piper nigrum* (seed)	124.37 ± 0.03^{b}
5	*Suregada multiflorum* (leaf)	127.36 ± 0.02^{b}
6	*Tinospora crispa*	331.01 ± 0.04^{c}
7	*Albizia procera*	339.18 ± 0.03^{c}
8	*Suregada multiflorum* (stem)	$508.54 \pm 0.02^{c,d}$
9	*Piper nigrum* (fruit)	$529.50 \pm 0.02^{c,d}$
10	*Kaempferia parviflora*	692.65 ± 0.02^{d}
11	*Diospyros rhodocalyx*	911.22 ± 0.03^{e}
12	*Betula alnoides*	985.33 ± 0.01^{f}
13	*Acacia farnesiana*	$>1{,}000^{g}$
14	*Leucaena leucocephala*	$>1{,}000^{g}$
15	*Butea superba*	$>1{,}000^{g}$
16	*Pueraria mirifica*	$>1{,}000^{g}$
17	*Mucuna collettii*	$>1{,}000^{g}$
18	*Fagraea fragrans*	$>1{,}000^{g}$
19	*Anaxagorea luzonensis*	$>1{,}000^{g}$
20	*Dracaena conferta*	$>1{,}000^{g}$
21	*Vitex trifolia*	$>1{,}000^{g}$
22	*Phyllanthus emblica*	$>1{,}000^{g}$
23	*Melia azedarach*	$>1{,}000^{g}$
24	*Cyperus rotundus*	$>1{,}000^{g}$
Control	Genistein	4×10^{-5} M

*The difference at the level of ^{a,b,c,d,e,f,g} is verified by Duncan's test.

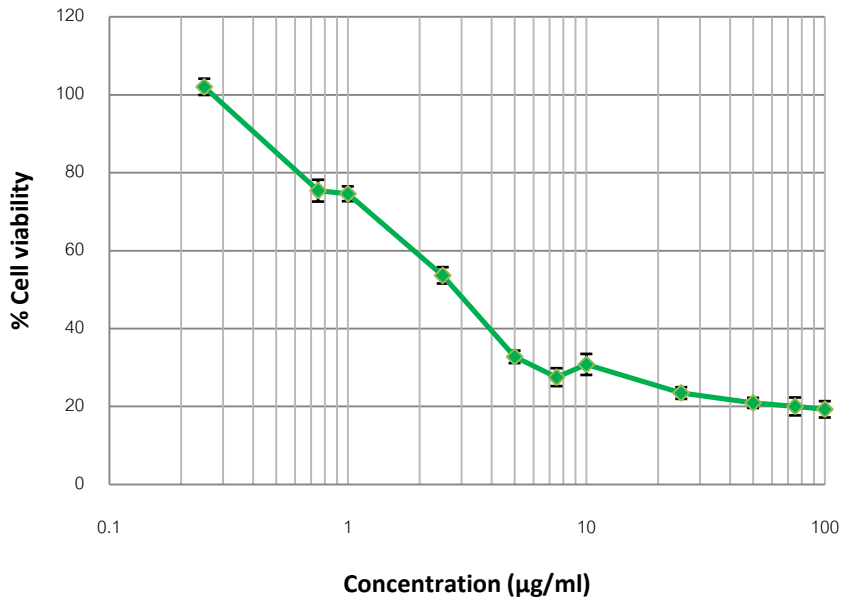


Figure 4.2 The antiproliferation effect of *S. erecta* ethanolic crude extract against PC-3 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of the plant ethanolic crude extract.

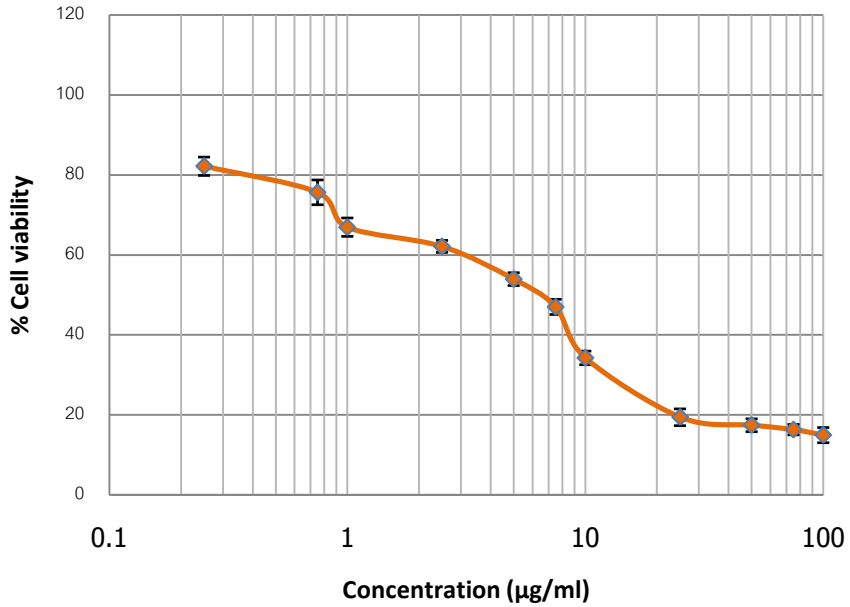


Figure 4.3 The antiproliferation effect of *S. venosa* ethanolic crude extract against PC-3 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of the plant ethanolic crude extract.

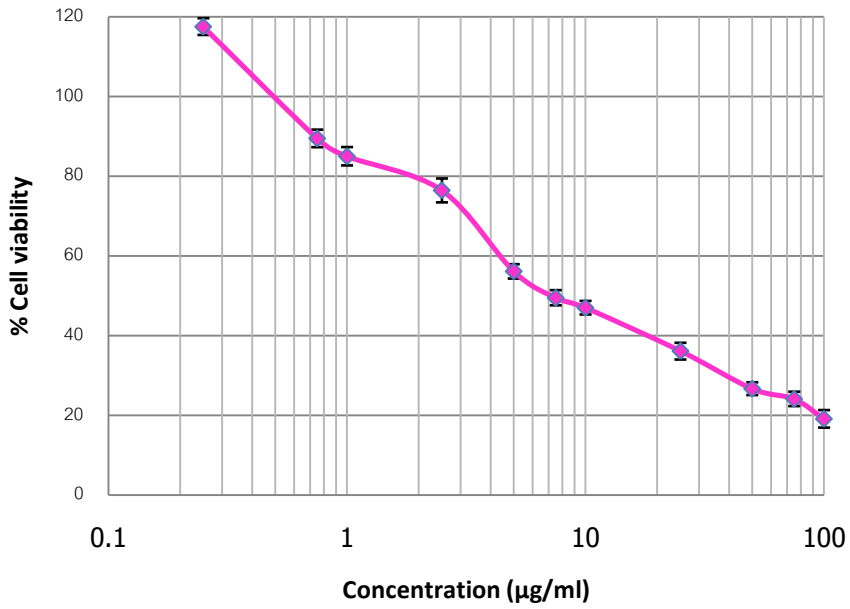


Figure 4.4 The antiproliferation effect of *S. erecta* ethanolic crude extract against PC-3 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations of the plant ethanolic crude extract.

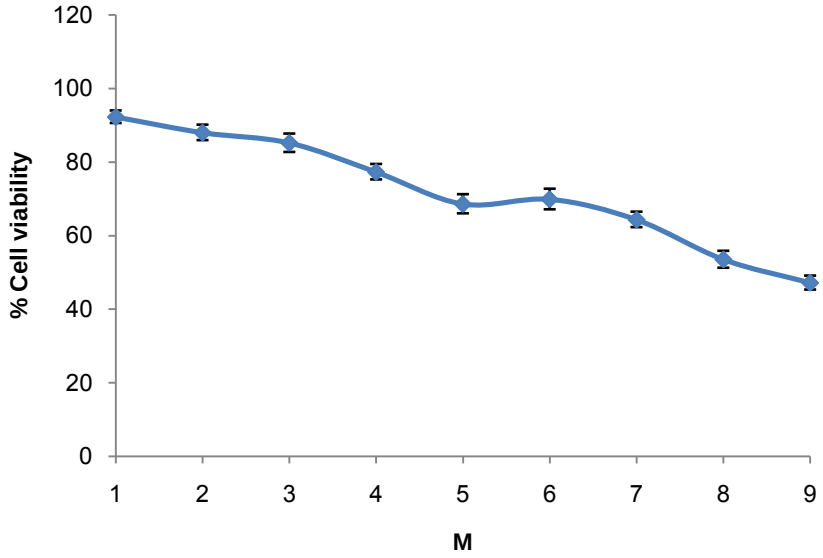
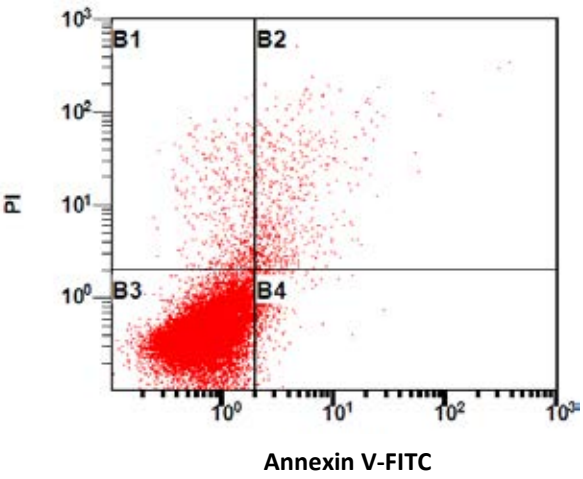


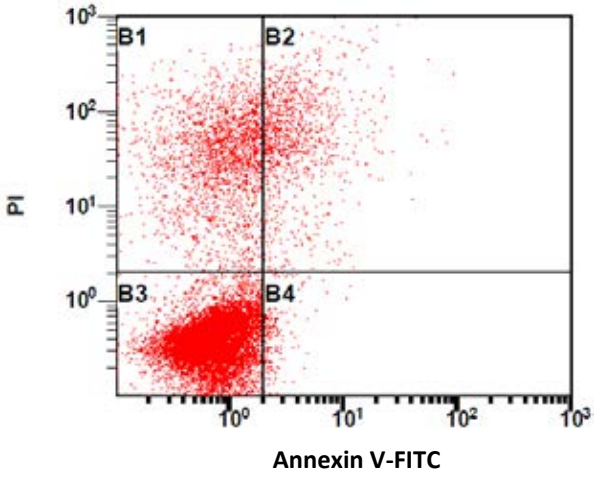
Figure4.5 The antiproliferation effect of genistein against PC-3 cells determined with MTT assay based on a treatment at IC_{50} for 72 h. The percentages of cell viability were plotted against the log dose concentrations (10^{-12}-10^{-4} M) of genistein.

4.4 Flow cytometric analysis

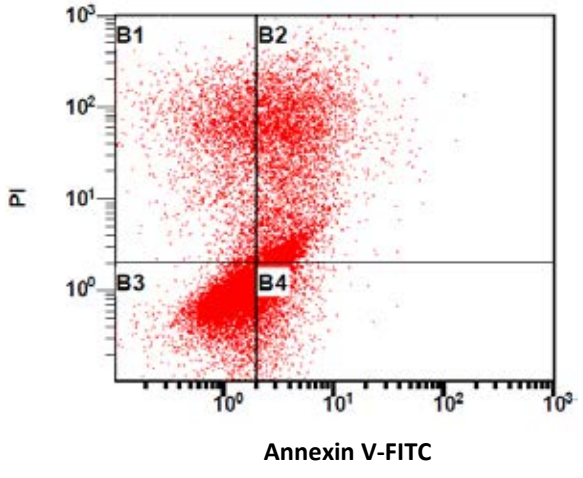
The application of the double stain of annexin V-FITC and propidium iodide allows separation of living cells, cells entering early apoptosis and cells in the late stage apoptosis/necrosis. Induction of apoptosis of PC-3 cells was verified by flow cytometric analysis of annexin V-FITC/propridium iodide (PI)-labeled cells. Cells were determined after exposure to genistein, *S. erecta, S. venosa* and *S. asper* extract at the IC_{50} concentration for 72 h (Figure 4.6).



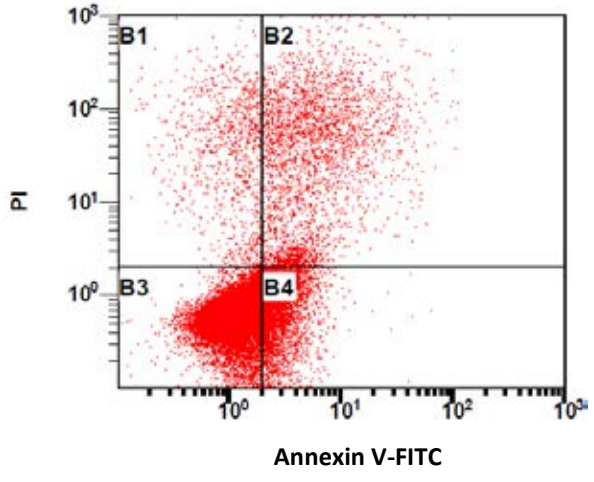
(a) DMSO control



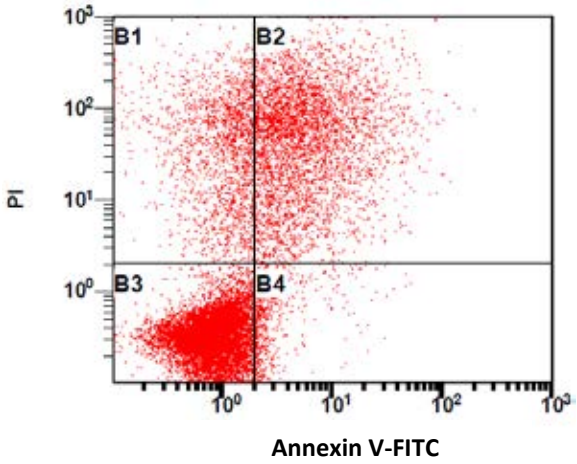
(b) PC-3 treated with Genistein



(c) PC-3 treated with *S. erecta*



(d) PC-3 treated with *S. venosa*



(e) PC-3 treated with *S. asper*

Figure 4.6 Flow cytograms of (a) PC-3 cells treated with DMSO for 72 h, (b) PC-3 cells treated with genistein at the IC_{50} value for 72 h, (c) PC-3 cells treated with *S. erecta* ethanolic crude extract at the IC_{50} value for 72 h, (d) PC-3 cells treated with *S. venosa* ethanolic crude extract at the IC_{50} value for 72 h and (e) PC-3 cells treated with *S. asper* ethanolic crude extract at the IC_{50} value for 72 h. Cells in the B1, B2, B3 and B4 quadrant were identified as necrotic cells, late stage apoptotic cells, living cells and early apoptotic cells, respectively.

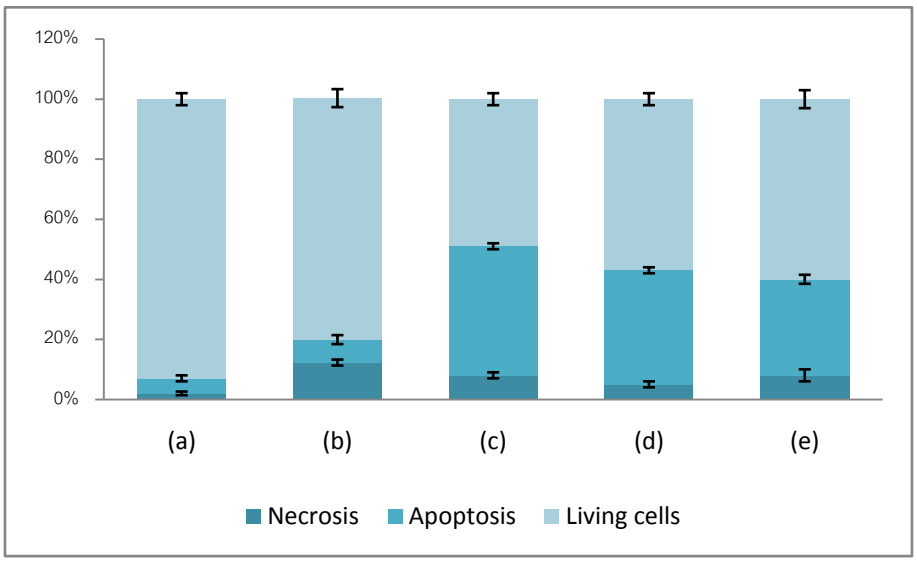


Figure 4.7 The percentage bar graphs represents the percentage of living, necrosis and apoptotic cells derived from the flow cytometric analysis of (a) PC-3 cells treated with DMSO for 72 h, (b) PC-3 cells treated with genistein at the IC_{50} value for 72 h, (c) PC-3 cells treated with *S. erecta* ethanolic crude extract at the IC_{50} value for 72 h, (d) PC-3 cells treated with *S. venosa* ethanolic crude extract at the IC_{50} value for 72 h and (e) PC-3 cells treated with *S. asper* ethanolic crude extract at the IC_{50} value for 72 h.

Table 4.3 The percentage of apoptotic PC-3 cells after treatment with plant ethanolic crude extract in quadrant analysis derived from the flow cytometric analysis

Samples	IC_{50}(µg/ml)	Viable cells (%)	Early apoptotic cells (%)	Early apoptotic/ viable cells	Late apoptotic cells (%)	Late apoptotic/ viable cells	Early and late apoptotic/ viable cells	Necrosis cells (%)	Necrosis/ viable cells	Early and late apoptotic/ necrosis cells
DMSO control	-	92.98 ± 0.02^{b}	2.83 ± 0.03^{a}	0.03	2.21 ± 0.02^{a}	0.02	0.05	1.98 ± 0.01^{a}	0.02	2.55^{b}
Genistein	4×10^{-5} M	80.45 ± 0.01^{b}	3.04 ± 0.01^{a}	0.04	4.61 ± 0.02^{a}	0.06	0.10	12.25 ± 0.01^{c}	0.15	0.62^{a}
S. erecta	4.30 ± 0.02^{a}	44.95 ± 0.04^{a}	18.56 ± 0.03^{c}	0.41	21.16 ± 0.04^{d}	0.47	0.88	7.61 ± 0.02^{b}	0.17	5.22^{c}
S. venosa	4.47 ± 0.03^{a}	56.61 ± 0.03^{a}	24.95 ± 0.02^{c}	0.44	8.89 ± 0.03^{b}	0.16	0.60	3.08 ± 0.02^{a}	0.05	10.99^{d}
S. asper	10.78 ± 0.02^{a}	58.37 ± 0.01^{a}	10.45 ± 0.01^{b}	0.18	14.36 ± 0.02^{c}	0.25	0.43	7.68 ± 0.02^{b}	0.13	3.23^{b}

*The difference at the level of ^{a,b,c,d} is verified by Duncan's test.

4.5 Immunoblotting of apoptosis-related proteins

To confirm the effect of the ethanolic crude plant extract on apoptotic protein expression of PC-3 cells, immunoblotting (Western blot) analysis was performed for apoptosis-related proteins to confirm for the expression patterns derived from apoptosis analysis. Four antibodies including Bcl-2, Bax, caspase-3, PARP, were used against the internal control protein (α-tubulin).

Immunoblotting analyses were performed with the protein extracts from PC-3 cells. The cells were studied after exposure to the plant ethanolic crude extracts at IC_{20} for 48 h.

4.5.1 Immunoblotting analysis (Western blot) of α-tubulin

α-tubulin protein was used as an internal loading control. There was no significant different in expression level of α-tubulin followed the plant ethanolic crude extract treatments (Figure 4.8).

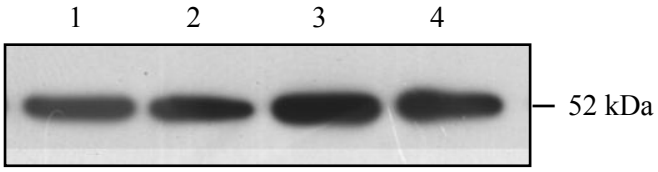


Figure 4.8 Immunoblotting analysis of the expression of α-tubulin protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated

4.5.2 Immunoblotting analysis (Western blot) of Bcl-2

The immunoblotting analysis showed the underexpression of Bcl-2 after treatment with *S. asper* (the least decreased), *S. venosa* (the most decreased) and *S. erecta* (the moderate decreased) (Figure 4.9).

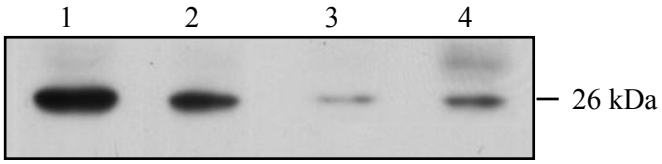


Figure 4.9 Immunoblotting analysis of the expression of Bcl-2 protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated

4.5.3 Immunoblotting analysis (Western blot) of Bax

The immunoblotting analysis showed the overexpression of Bax after treatment with *S. asper S. venosa* and *S. erecta* in a similar level (Figure 4.10).

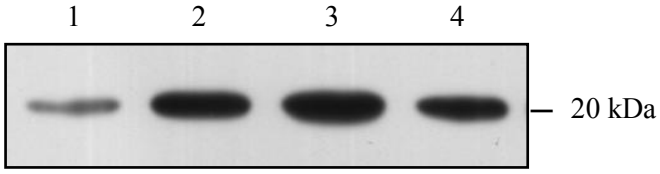


Figure 4.10 Immunoblotting analysis of the expression of Bax protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated

4.5.4 Immunoblotting analysis (Western blot) of caspase-3

The expression and activation of procaspase-3 was decreased by the plant ethanolic crude extract treatment. The 34 kDa 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 (Figure 4.11).

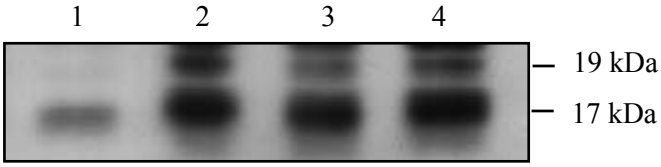


Figure 4.11 Immunoblotting analysis of the expression of caspase-3 protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated.

4.5.5 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 (Figure 4.12).

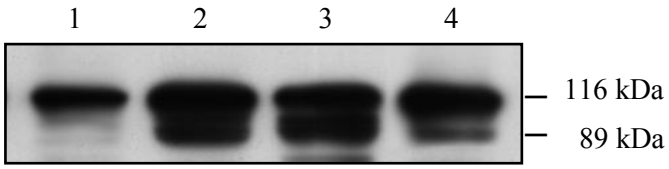


Figure 4.12 Immunoblotting analysis of the expression of poly (ADP-ribose) polymerase (PARP) protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated

4.6 Changes in proteomics

In order to study the effects of plant ethanolic crude extract on PC-3 cells at the protein expression level, proteomic studies were performed in both untreated and treated PC-3 cells. The extracted cellular proteins of the untreated and treated PC-3 cells were compared to verify for the differential protein expression.

4.6.1 Determination of IC_{20} of PC-3 cells

The three plant ethanolic crude extracts harbouring the highest antiproliferation activity including *S. erecta*, *S. venosa* and *S. asper* were selected for the proteomic study. The ethanolic crude plant extracts at the IC_{20} were used to treat the PC-3 cells for 48 h. The IC_{20} values were calculated from the triplicated experiments of the antiproliferation assay. The IC_{20} of *S. erecta*, *S. venosa* and *S. asper* were 2.33 ± 0.02, 2.84 ± 0.03 and 4.76 ± 0.12 μg/ml, respectively (Table 4.4).

Table 4.4 The IC_{20} values of the top three ethanolic crude plant extracts derived from the treatment of PC-3 cells

Plants	IC_{20} (µg/ml)
S. erecta	2.33 ± 0.02
S. venosa	2.84 ± 0.03
S. asper	4.76 ± 0.12

4.6.2 Proteomic analysis of the effects of *S. erecta* extract on PC-3 cells

In the quantitative proteomic assay using the Image Master 2-DE platinum software to analyze the number of the spot proteins of the untreated and treated PC-3 cells, approximately 613 and 599 protein spots were detected in the 2-DE gel of the untreated and *S. erecta* extract treated PC-3 cells, respectively.

In the qualitative proteomic assay using the Image Master 2-DE platinum software to differential the intensity of the spot proteins between the untreated and treated PC-3 cells, there were statistically different ($p<0.05$) in expression of 6 protein spots after treatment with *S. erecta* extract (Figure 4.13). There were three proteins including protein disulfide-isomerase A3, sequestosome-1 and 78 kDa glucose-regulated protein had increased expression. There were three proteins including peroxiredoxin-6, 14-3-3 protein zeta/delta and tropomyosin alpha-3 chain had decreased expression (Table 4.5).

4.6.3 Proteomic analysis of the effects of *S. venosa* extract on PC-3 cells

In the quantitative proteomic assay using the Image Master 2-DE platinum software to analyze the number of the spot proteins between the untreated and treated PC-3 cells, approximately 613 and 615 protein spots were detected in the 2-DE gel of the untreated and *S. venosa* extract treated PC-3 cells, respectively.

In the qualitative proteomic assay using the Image Master 2-DE platinum software to differential the intensity of the spot proteins between the untreated and treated PC-3 cells, there were 6 protein spots with significantly different expression after treatment with *S. venosa* extract (Figure 4.14). There were increasing in expression of four proteins namely heterogeneous nuclear ribonucleoprotein H, pyruvate kinase

isozymes M1/M2, moesin and Ran GTPase-activating protein 1. There were two proteins with decreased expression include 14-3-3 protein zeta/delta and lamin-B1 isoform 1 (Table 4.6).

4.6.4 Proteomic analysis of the effects of *S. asper* extract on PC-3 cells

In the quantitative proteomic assay using the Image Master 2-DE platinum software to analyze the number of the spot proteins between the untreated and treated PC-3 cells, approximately 613 and 602 protein spots were detected in the 2-DE gel of the untreated and *S. asper* extract treated PC-3 cells, respectively.

In the qualitative proteomic assay using the Image Master 2-DE platinum software to differential the intensity of the spot proteins between the untreated and treated PC-3 cells, there were 7 protein spots with significantly different expression after treatment with *S. asper* extract (Figure 4.15). There were three proteins including cofilin-1, annexin A5 and moesin had increased expression. There were four proteins including DNL-type zinc finger protein, 14-3-3 protein zeta/delta, endoplasmin and voltage-dependent anion-selective channel protein 2 had decreased expression (Table 4.7).

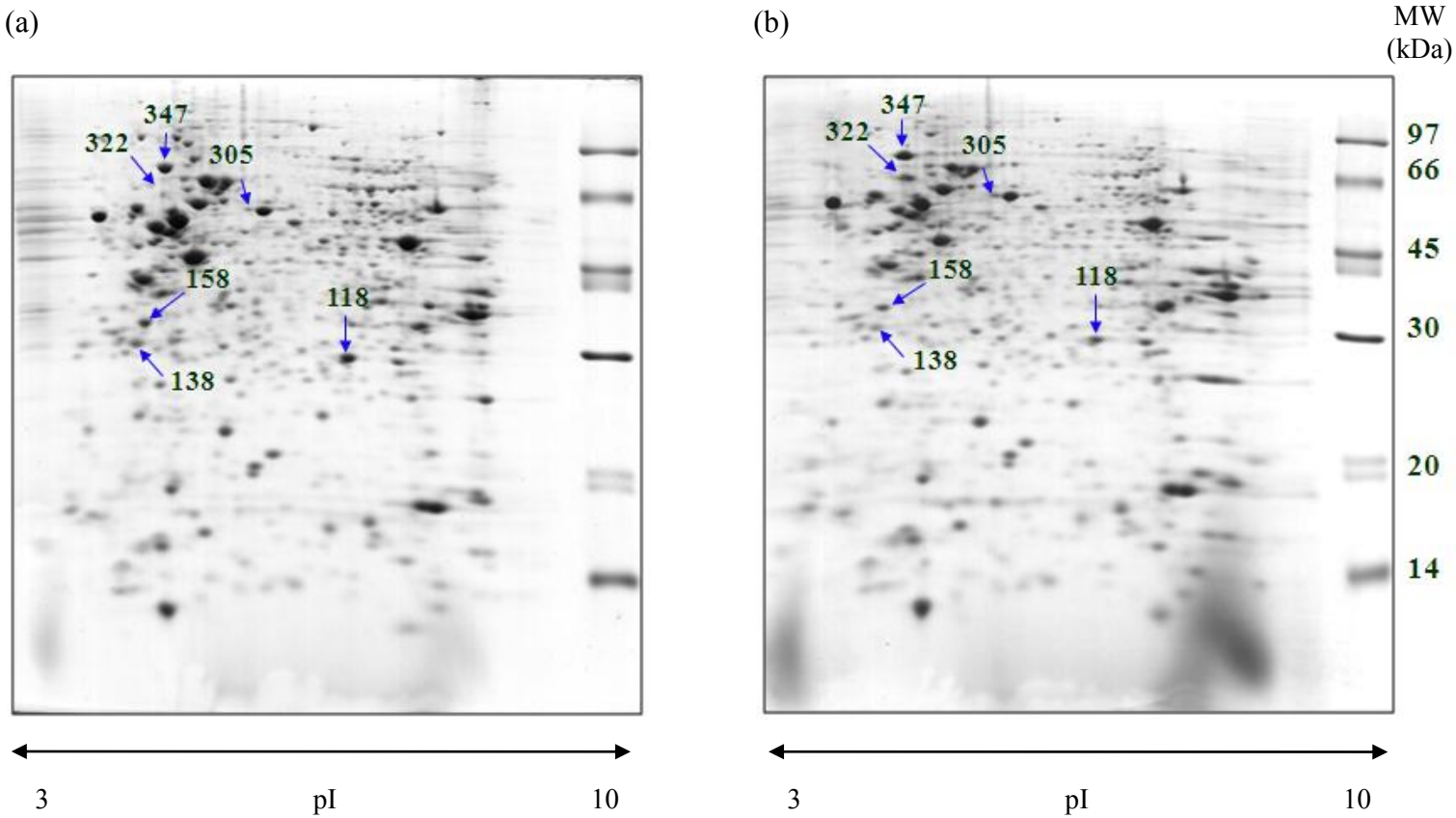


Figure 4.13 Two dimensional gel electrophoresis of the PC-3 cells using 14% SDS-PAGE, 7 cm, non-linear, pH range 3-10. Gels were stained by Coomassie blue R-250 and protein spots were analyzed by Image Master 2-DE platinum 7.0 software. Protein spots which had a statistically significant differences ($P<0.05$) were excised for LC/MS/MS analysis. Arrows show the differentially expressed proteins (a) DMSO control and (b) treated with the ethanolic crude extract of *S. erecta* for 48 h.

Table 4.5 Sequencing of differential expressed protein spots derived from PC-3 cells treated with *S. erecta* extract for 48 h. Proteins were identified by LC/MS/MS and using MASCOT search tool. Protein name was derived from the matching of amino acid sequences analyzed with mass spectroscopy with the database MASCOT MS/MS Ions Search. Mascot is a software search engine that use mass spectrometry data to identify proteins from primary sequence databases. Accession no. is the abbreviation name of the protein. Mascot score is the observed match between the experimental data and the database sequence in a random event. Peptide match is a random peptide matches produced by the database search. pI is the isoelectric point. % coverage is the matching percentage of the identified protein with the total protein sequences in the database. Sequences mean the sequences in the database that match with the identified protein.

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
118	Peroxiredoxin-6	PRDX6_HUMAN	62	9	25.019	6.00	33	K.GVFTK.E K.LAPEFAK.R K.LAPEFAKR.N K.LPFPIIDDR.N R.VVFVFGPDKK.L K.LSILYPATTGR.N K.LKLSILYPATTGR.N R.ELAILLGMLDPAEK.D K.DGDSVMVLPTIPEEEAK.K	Antioxidant	Down-regulated

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
138	14-3-3 protein zeta/delta	1433Z_ HUMAN	157	5	27.728	4.73	20	K.VFYLK.M R.NLLSVAYK.N R.YLAEVAAGDDKK.G K.FLIPNASQAESK.V K.SVTEQGAELSNEER.N	Apoptosis	Down-regulated
158	Tropomyosin alpha-3 chain	TPM3_ HUMAN	27	5	32.799	4.68	11	R.KYEEVAR.K K.LVIIEGDLER.T R.IQLVEEELDR.A R.KLVIIEGDLER.T R.IQLVEEELDRAQER.L	Cytoskeleton/ mobility	Down-regulated
305	Protein disulfide-isomerase A3	PDIA3_ HUMAN	49	2	56.894	6.23	4	R.ELSDFISYLQR.E R.FLQDYFDGNLKR.Y	Metabolism	Up-regulated
322	Sequestosome -1	SQSTM_ HUMAN	24	2	47.657	5.10	6	K.AYLLGKEDAAR.E R.VAALFPALRPGGFQAHYR.D	Apoptosis	Up-regulated

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
347	78 kDa glucose-regulated protein	GRP78_ HUMAN	179	18	72.288	5.07	25	K.IQQLVK.E K.EFFNGK.E R.LTPEEIER.M K.DAGTIAGLNVMR.I R.VEIIANDQGNR.I K.DAGTIAGLNVMR.I K.DAGTIAGLNVMR.I R.NELESYAYSLK.N K.ELEEIVQPIISK.L K.SDIDEIVLVGGSTR.I K.SDIDEIVLVGGSTR.I R.AKFEELNMDLFR.S K.TFAPEEISAMVLTK.M K.TFAPEEISAMVLTK.M R.ITPSYVAFTPEGER.L K.NQLTSNPENTVFDAK.R K.SQIFSTASDNQPTVTIK.V K.DNHLLGTFDLTGIPPAPR.G	Binding protein/ folding	Up-regulated

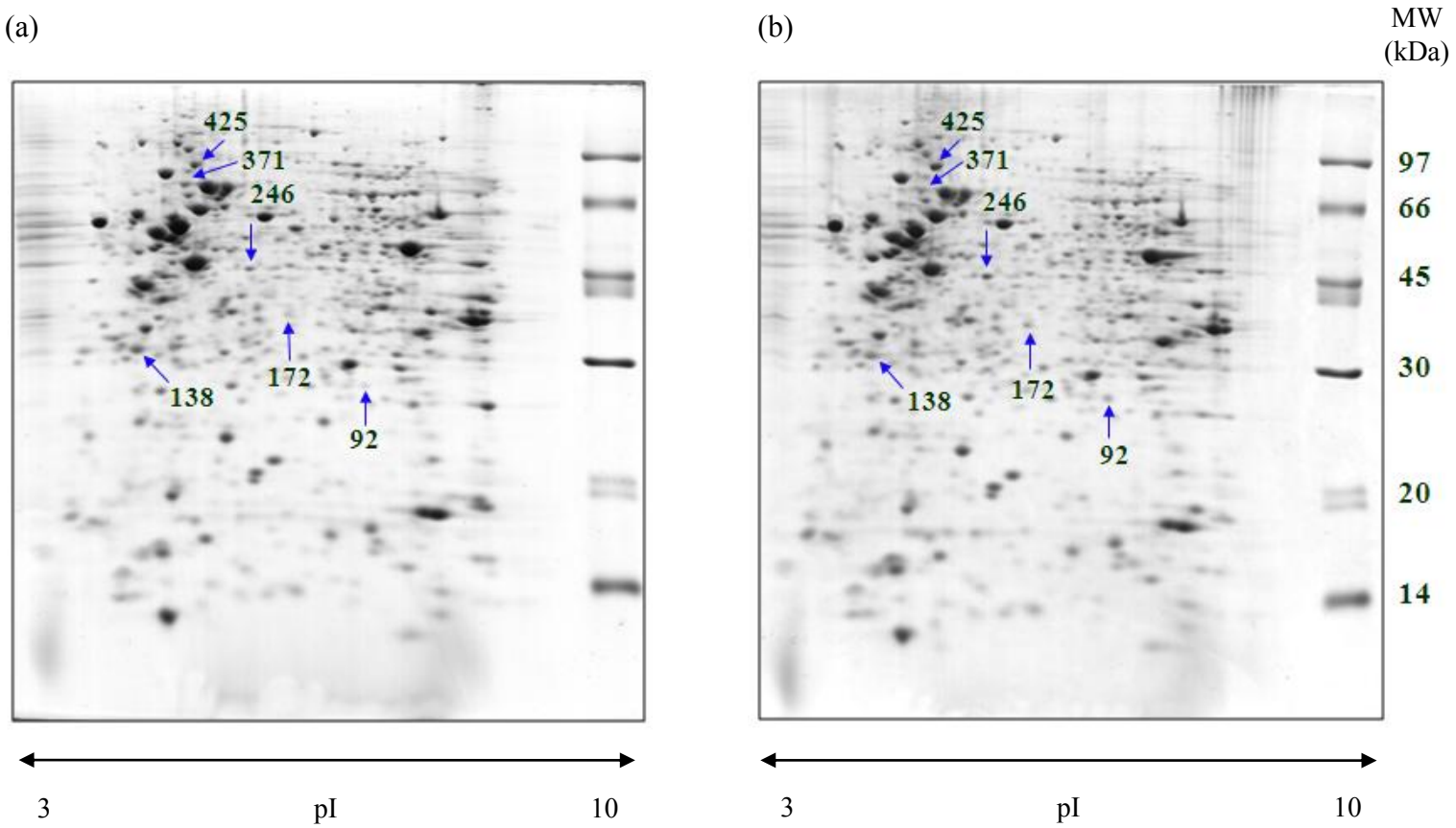


Figure 4.14 Two dimensional gel electrophoresis of the PC-3 cells using 14% SDS-PAGE, 7 cm, non-linear, pH range 3-10. Gels were stained by Coomassie blue R-250 and protein spots were analyzed by Image Master 2-DE platinum 7.0 software. Protein spots which had a statistically significant differences ($P<0.05$) were excised for LC/MS/MS analysis. Arrows show the differentially expressed proteins (a) DMSO control and (b) treated with the ethanolic crude extract of *S. venosa* for 48 h.

Table 4.6 Sequencing of differential expressed protein spots derived from PC-3 cells treated with *S. venosa* extract for 48 h. Proteins were identified by LC/MS/MS and using MASCOT search tool. Protein name was derived from the matching of amino acid sequences analyzed with mass spectroscopy with the database MASCOT MS/MS Ions Search. Mascot is a software search engine that use mass spectrometry data to identify proteins from primary sequence databases. Accession no. is the abbreviation name of the protein. Mascot score is the observed match between the experimental data and the database sequence in a random event. Peptide match is a random peptide matches produced by the database search. pI is the isoelectric point. % coverage is the matching percentage of the identified protein with the total protein sequences in the database. Sequences mean the sequences in the database that match with the identified protein.

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
92	Heterogeneous nuclear ribonucleoprotein H	HNRH1_ HUMAN	88	2	49.198	5.89	5	R.YIEIFK.S R.STGEAFVQFASQEIAEK.A	Protein synthesis/ degradation	Up-regulated
138	14-3-3 protein zeta/delta	1433Z_ HUMAN	157	5	27.728	4.73	20	K.VFYLK.M R.NLLSVAYK.N R.YLAEVAAGDDKK.G K.FLIPNASQAESK.V K.SVTEQGAELSNEER.N	Apoptosis/ Signal transduction	Down-regulated

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
172	Pyruvate kinase isozymes M1/M2	KPYM_ HUMAN	26	2	57.900	7.96	4	R.LDIDSPPITAR.N R.GDLGIEIPAEK.V	Metabolism	Up-regulated
246	Moesin	MOES_ HUMAN	34	1	56.894	6.08	1	K.ALTSELANAR.D	Protein binding	Up-regulated
371	Lamin-B1 isoform 1	LMNB1_ HUMAN	61	6	66.368	5.11	11	R.AKLQIELGK.C K.DAALATALGDKK.S K.ALYETELADAR.R K.LALDMEISAYR.K R.IESLSSQLSNLQK.E R.CQSLTEDLEFRK.S	Cytoskeleton/ mobility	Down-regulated
425	Ran GTPase-activating protein 1	RAGP1_ HUMAN	21	1	63.502	4.63	2	K.AFNSSSFNSNTFLTR.L	Protein binding/ signal transduction	Up-regulated

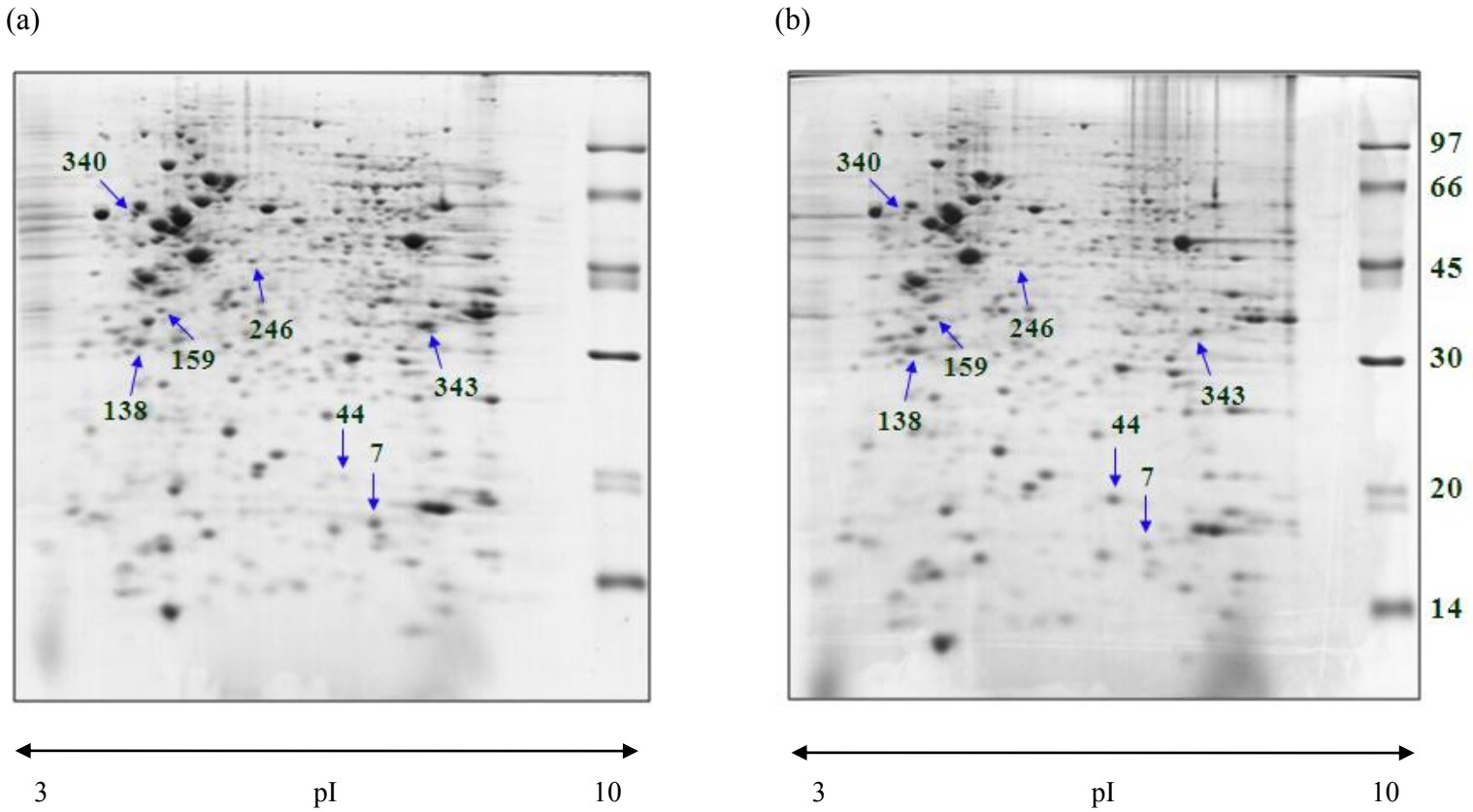


Figure 4.15 Two dimensional gel electrophoresis of the PC-3 cells using 14% SDS-PAGE, 7 cm, non-linear, pH range 3-10. Gels were stained by Coomassie blue R-250 and protein spots were analyzed by Image Master 2-DE platinum 7.0 software. Protein spots which had a statistically significant differences ($P<0.05$) were excised for LC/MS/MS analysis. Arrows show the differentially expressed proteins (a) DMSO control and (b) treated with the ethanolic crude extract of *S. asper* for 48 h.

Table 4.7 Sequencing of differential expressed protein spots derived from PC-3 cells treated with *S. asper* extract for 48 h. Proteins were identified by LC/MS/MS and using MASCOT search tool. Protein name was derived from the matching of amino acid sequences analyzed with mass spectroscopy with the database MASCOT MS/MS Ions Search. Mascot is a software search engine that use mass spectrometry data to identify proteins from primary sequence databases. Accession no. is the abbreviation name of the protein. Mascot score is the observed match between the experimental data and the database sequence in a random event. Peptide match is a random peptide matches produced by the database search. pI is the isoelectric point. % coverage is the matching percentage of the identified protein with the total protein sequences in the database. Sequences mean the sequences in the database that match with the identified protein.

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
7	DNL-type zinc finger protein	DNLZ_ HUMAN	-	1	19.192	9.88	6	K.VCGTRSSKRISK.L	DNA binding	Down-regulated
44	Cofilin-1	COF1_ HUMAN	46	2	18.491	8.22	6	R.YALYDATYETK.E R.YALYDATYETK.E	Cytoskeleton/ mobility	Up-regulated
138	14-3-3 protein zeta/delta	1433Z_ HUMAN	157	5	27.728	4.73	20	K.VFYLK.M R.NLLSVAYK.N R.YLAEVAAGDDKK.G K.FLIPNASQAESK.V K.SVTEQGAELSNEER.N	Apoptosis	Down-regulated

Spot no.	Protein names	Accession no.	Mascot score	Peptide matches	MW (kDa)	pI	% coverage	Sequences	Function(s)	Differential protein expression
159	Annexin A5	ANXA5_ HUMAN	22	2	35.754	4.89	6	K.VLTEIIASR.T K.NFATSLYSFIK.G	Signal transduction/ transcription	Up-regulated
246	Moesin	MOES_ HUMAN	34	1	56.894	6.08	1	K.ALTSELANAR.D	Protein binding	Up-regulated
340	Endoplasmin	ENPL_ HUMAN	24	3	92.411	4.76	3	K.NKEIFLR.E K.FAFQAEVNR.M R.ELISNASDALDK.I	Protein folding	Down-regulated
343	Voltage-dependent anion-selective channel protein 2	VDAC2_ HUMAN	114	7	31.547	7.49	28	R.DIFNK.G K.GFGFGLVK.L K.VGLALELEA.- R.NNFAVGYR.T K.YQLDPTASISAK.V K.VNNSSLIGVGYTQTL RPGVK.L R.TGDFQLHTNVNDGT EFGGSIYQK.V	Voltage-gated anion channel activity/ Ion transport	Down-regulated

Table 4.8 The number and percentage of the up- and down-regulated proteins derived from PC-3 cells treated with plant ethanolic crude extract. The limit of detection of up- or down-regulated proteins is based on the criterion of ≥ 1.5 fold protein spot intensity difference.

DMSO			*S. erecta*			*S. venosa*			*S. asper*		
Total protein	Up-regulated protein	Down-regulated protein	Total protein	Up-regulated protein	Down-regulated protein	Total protein	Up-regulated protein	Down-regulated protein	Total protein	Up-regulated protein	Down-regulated protein
613	-	-	599	3 (0.5%)	3 (0.5%)	615	4 (0.65%)	2 (0.33%)	602	3 (0.49%)	4 (0.66%)

4.6.5 Global network analysis of significantly difference protein spots in PC-3 cell line

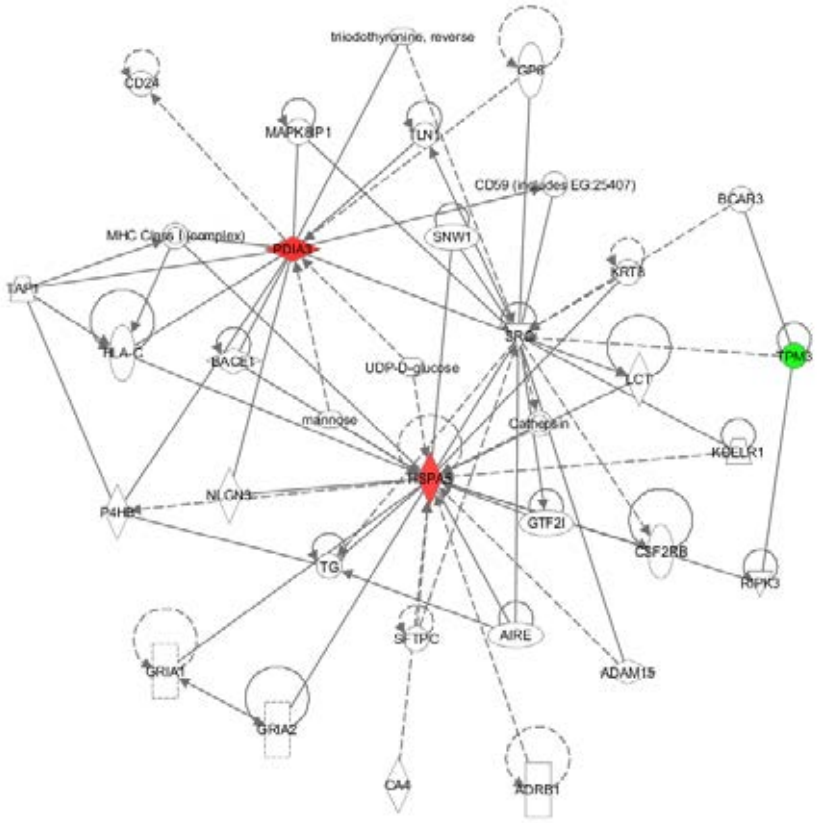
To understand the pathway of protein interaction, this study used Ingenuity Protein Analysis (IPA) software to create a mapping pathway of the identified proteins. Accession numbers and fold-changes of differential protein expression were uploaded to IPA. IPA reported the network explorer which has 35 proteins interaction. As shown in Figure 4.16, protein disulfide isomerase 3 (PDIA3) directly act on SRC (proto-oncogene encoding a tyrosine kinase), while tropomyosin 3 (TPM3) indirectly act on SRC. Moreover, PDIA3 indirectly act on Heat shock 70 kDa protein 5 (HSPA5).

4.6.6 Global network analysis of significantly difference protein spots in PC-3 cell line

To understand the pathway of protein interaction, this study used Ingenuity Protein Analysis (IPA) software to create a mapping pathway of the identified proteins. Accession numbers and fold-changes of differential protein expression were uploaded to IPA. IPA reported the network explorer which has 35 proteins interaction. As shown in Figure 4.17, lamin-B1 isoform 1 (LMNB1) indirectly act on MYC, while annexin A5 (ANXA5) directly act on MYC.

4.6.7 Global network analysis of significantly difference protein spots in PC-3 cell line

To understand the pathway of protein interaction, this study used Ingenuity Protein Analysis (IPA) software to create a mapping pathway of the identified proteins. Accession numbers and fold-changes of differential protein expression were uploaded to IPA. IPA reported the network explorer which has 37 proteins interaction. As shown in Figure 4.18, voltage-dependent anion-selective channel protein 2 (VDAC2) and annexin A5 (ANXA5) directly act on MYC.



Abbreviations and symbols

ADAM15 = ADAM metallopeptidase domain 15
ADRB1 = Adrenergic, beta-1-, receptor
AIRE = Autoimmune regulator
BACE1 = Beta-site APP-cleaving enzyme 1
BCAR3 = Breast cancer anti- estrogen resistance 3
CA4 = Carbonic anhydrase IV
Cathepsin = --
CD24 = CD24 molecule
CD59 (includes EG:25407) = CD59 molecule
CSF2RB = Colony stimulating factor 2 receptor, beta
GP6 = Glycoprotein VI
GRIA1 = Glutamate receptor, ionotropic, AMPA 1
GRIA2 = Glutamate receptor, ionotropic, AMPA 2
GTF2I = General transcription factor IIi
HLA-C = Major histocompatibility complex, class I, C
HSPA5 = Heat shock 70kDa protein 5
KDELR1 = KDEL endoplasmic reticulum protein retention receptor 1
KRT8 = Keratin 8
LCT = Lactase
mannose = --
MAPK8IP1 = Mitogen-activated protein kinase 8 interacting protein 1
MHC Class I (complex) = --
NLGN3 = Neuroligin 3
P4HB = Prolyl 4-hydroxylase, beta polypeptide
PDIA3 = Protein disulfide isomerase family A, member 3
RIPK3 = Receptor-interacting serine-threonine kinase 3
SFTPC = Surfactant protein C
SNW1 = SNW domain containing 1
SRC = v-src sarcoma
TAP1 = Transporter 1, ATP-binding cassette
TG = Thyroglobulin
TLN1 = Talin 1
TPM3 = Tropomyosin 3
triiodothyronine, reverse = --
UDP-D-glucose = --

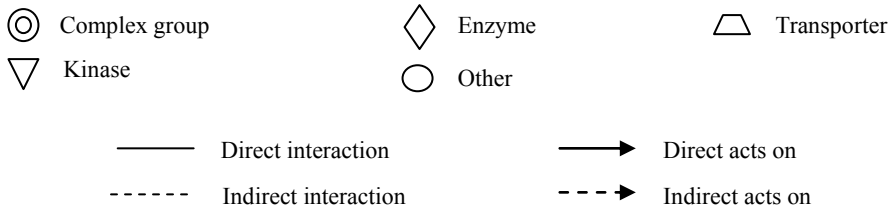
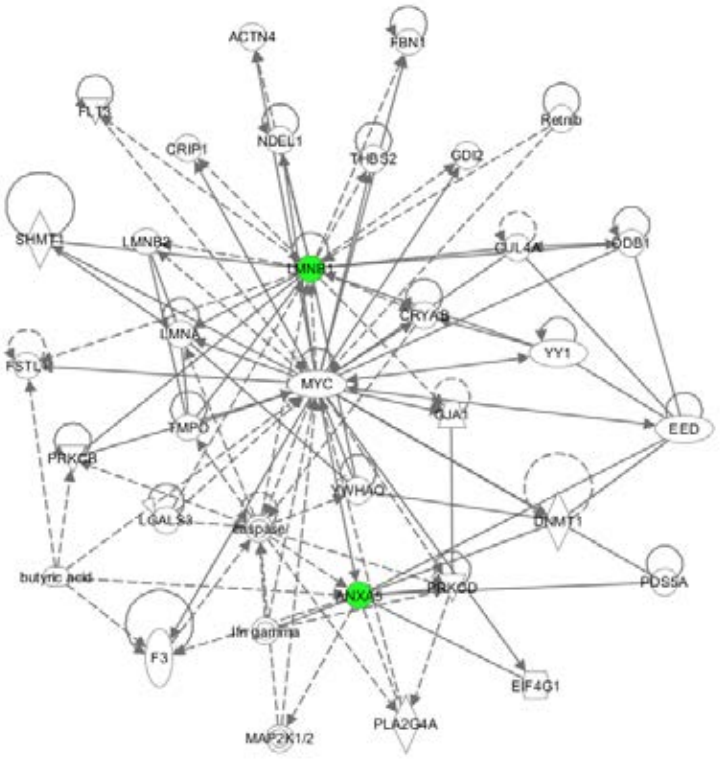


Figure 4.16 The network analysis of proteins had a differential expression due to exposure by PC-3 cells using Ingenuity Pathway Analysis (IPA). Proteins that increase in expression were shown in red color while, proteins that decrease in expression were shown in green color.



Abbreviations and symbols

ABCG8 = ATP-binding cassette, sub-family G, member 8
AEBP1 = AE binding protein 1
CRABP1 = Cellular retinoic acid binding protein 1
CRIP1 = Cysteine-rich protein 1
DCAF6 = DDB1 and CUL4 associated factor 6
DCAF8 = DDB1 and CUL4 associated factor 8
glycochenodeoxycholate = --
GPC4 = Glypican 4
HS6ST2= heparan sulfate 6-O-sulfotransferase 2
Lamin = --
Lamin b = --
LARGE = Like-glycosyltransferase
LBX2 = Ladybird homeobox 2
LEMD3 = LEM domain-containing 3
LMNB1 = Lamin B1
LMNB2 = Lamin B2
MBIP = MAP3K12 binding inhibitory protein 1
miR-23b-3p/miR-23a-3p/miR-23c (includes others) = --
PAPPA = Pregnancy-associated plasma protein A
POLM = Polymerase (DNA directed), mu
PRRX2 = Paired related homeobox 2
RCN1 (includes EG: 19672) = Reticulocalbin 1, EF-hand calcium binding domain
REEP5 = Receptor accessory protein 5
RTCD1 = RNA terminal phosphate cyclase domain 1
S100A13 = S100 calcium binding protein A13
SERINC1 = Serine incorporator
SHMT1 = Serine hydroxymethyltransferase 1
SLIT3 = Slit homolog 3
SPG20 = Spastic paraplegia 20
SUN1 = Sad1 and UNC84 domain containing 1
SUN2 = Sad1 and UNC84 domain containing 2
TLN1 = Transforming growth factor, beta-induced, 68 kDa
THBS2 = Thrombospondin 2
URB2 (includes EG: 292087) = URB2 ribosome biogenesis 2 homolog
ZFR = Zinc finger RNA binding protein

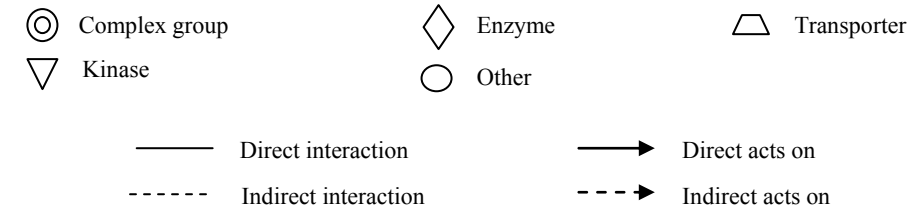
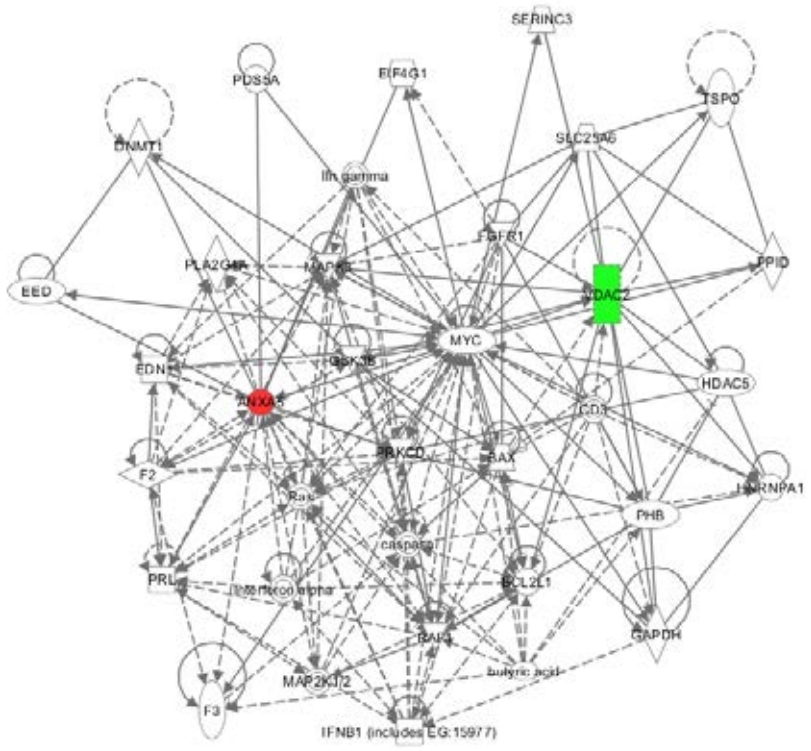


Figure 4.17 The network analysis of proteins had a differential expression due to exposure by PC-3 cells using Ingenuity Pathway Analysis (IPA). Proteins that increase in expression were shown in red color while, proteins that decrease in expression were shown in green color.



Abbreviations and symbols

ANXA5 = Annexin A5
BAX = Bcl-2–associated X protein
BCL2L1 = BCL2-like 1
butyric acid = --
caspase = --
CD3 = --
DNMT1 = DNA (cytosine-5)-methyltransferase 1
EDN1 = Endothelin 1
EED = Embryonic ectoderm development
EIF4G1 = Eukaryotic translation initiation factor 4 gamma, 1
F2 = Coagulation factor II (thrombin)
F3 = Coagulation factor III (thromboplastin, tissue factor)
FGFR1 = Fibroblast growth factor receptor 1
GAPDH = Glyceraldehyde 3-phosphate dehydrogenase
GSK3B = Glycogen synthase kinase 3 beta
HDAC5 = Histone deacetylase 5
HNRNPA1 = Heterogeneous nuclear ribonucleoprotein A1
Ifn gamma = --
IFNB1 = Interferon, beta 1, fibroblast
Interferon alpha = --
MAP2K1/2 = --
MAPK3 = Mitogen-activated protein kinase 3
PHB = Prohibitin
PLA2G4A = Phospholipase A2, group IVA (cytosolic, calcium-dependent)
PPID = Peptidylprolyl isomerase D
PRKCD = Protein kinase C, delta
PRL = Prolactin
RAF1= V-raf-1 murine leukemia viral oncogene homolog 1
Ras = --
SLC25A6 = Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 6
TSPO = Translocator protein
VDAC2 = Voltage-dependent anion channel 2
SFTPC = Surfactant protein C
SNW1 = SNW domain containing 1
SRC = v-src sarcoma
TAP1 = Transporter 1, ATP-binding cassette
TG = Thyroglobulin

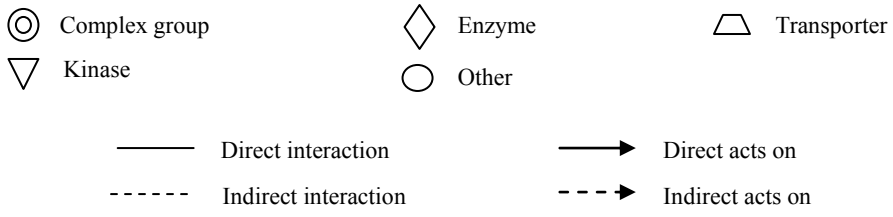


Figure 4.18 The network analysis of proteins had a differential expression due to exposure by PC-3 cells using Ingenuity Pathway Analysis (IPA). Proteins that increase in expression were shown in red color while, proteins that decrease in expression were shown in green color.

4.6.8 Immunoblotting analysis (Western blot) of the differential expressed proteins

Four antibodies including pan 14-3-3, 14-3-3σ, GAPDH and PKM2, were applied to the 1-DE gel loaded with the protein extracts derived from the DMSO and treated cells, to determine the differential expression of some selected proteins.

4.6.8.1 Immunoblotting analysis (Western blot) of pan 14-3-3

The expression of pan 14-3-3 protein was down-regulated in a similar level following the 3 plant ethanolic crude extract treatment as compared with the control (Figure 4.19).

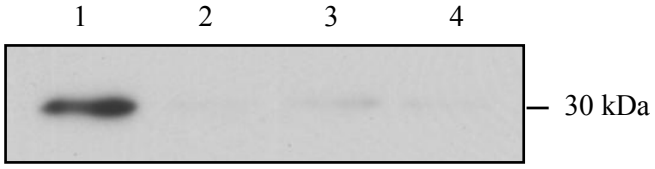


Figure 4.19 Immunoblotting analysis of the expression of pan 14-3-3 protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated, lane 3: *S. venosa* treated, lane 4: *S. erecta* treated.

4.6.8.2 Immunoblotting analysis (Western blot) of 14-3-3σ

There was no significant different in expression level of 14-3-3σ protein followed the plant ethanolic crude extract treatments (Figure 4.20).

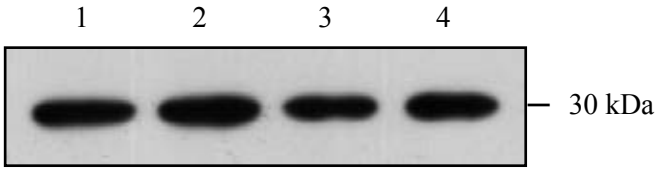


Figure 4.20 Immunoblotting analysis of the expression of 14-3-3σ protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated (intensity fold increased 13.12%), lane 3: *S. venosa* treated (intensity fold decreased 16.24%), lane 4: *S. erecta* treated (intensity fold decreased 9.25%).

4.6.8.3 Immunoblotting analysis (Western blot) of GAPDH

There was no significant different in expression level of GAPDH followed the plant ethanolic crude extract treatments (Figure 4.21).

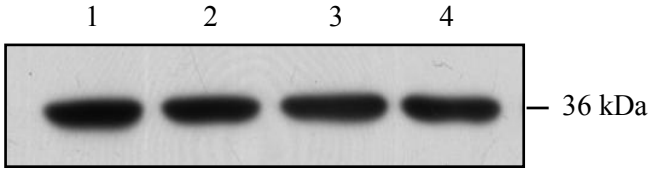


Figure 4.21 Immunoblotting analysis of the expression of GAPDH protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated (intensity fold decreased 35.73%), lane 3: *S. venosa* treated (intensity fold decreased 36.42%), lane 4: *S. erecta* treated (intensity fold decreased 38.12%).

4.6.8.4 Immunoblotting analysis (Western blot) of PKM2

There was no significant different in expression level of GAPDH followed the plant ethanolic crude extract treatments (Figure 4.22).

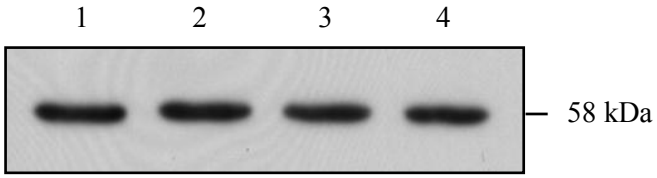


Figure 4.22 Immunoblotting analysis of the expression of PKM2 protein in PC-3 cells after treatment with plant crude extract for 48 h. lane 1: DMSO control, lane 2: *S. asper* treated (intensity fold decreased 38.80%), lane 3: *S. venosa* treated (intensity fold decreased 38.79%), lane 4: *S. erecta* treated (intensity fold decreased 22.41%).

CHAPTER V

DISCUSSION

Medicinal or herbal plants are sources of phytochemical with human health benefits. Screening of a potential herbal 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 exhibits strong IC_{50} is selected and submitted to further studies including pure compound isolations and bioassays. At present, analysis at proteomics level is a challenge assay due to its rapid and broadens results on differential protein proliferation of those proteins related to the cellular response to the phytochemical treatment.

The ethanolic crude extracts derived from 22 plants were evaluated for the antiproliferative effect against PC-3 cells. The results from the study indicated that the plant extracts expressed different degrees of antiproliferative activity against PC-3 cells according to the criteria of cytotoxicity activity 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).

In terms of quantitative analysis, the ethanolic crude extract of *S. erecta* (tuberous root), *S. venosa* (tuber) and *S. asper* (seed) exhibited the highest antiproliferation activity against PC-3 cells (IC_{50} = 4.30, 4.47 and 10.78 μg/ml, respectively).

In the previous reports, *S. asper* exhibited anticancer activity (Rastogi and Dhawan, 1990). Two cytotoxic cardiac glycosides, strebloside and mansonin, were isolated from *S. asper* stem bark with significant activity against KB (human epidermoid carcinoma) cells with IC_{50} values of 32 and 42 μg/ml, respectively. The volatile oil isolated from fresh leaves of *S. asper* showed significant antiproliferation activity (IC_{50} < 30 μg/ml) against P388 (mouse leukemia) cells (Phutdhawong *et al*., 2004).

Tubers from *S. venosa* exhibited anti-proliferation against breast cell culture. 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). The ethanolic crude extract from *S. venosa* showed moderate cytotoxic activity against

SKOV3 (human ovarian cancer cell line), with IC_{50} of 35.11 µg/ml and SKBR3 (human breast cancer cell line), with IC_{50} 39.67 µg/ml. 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).

The ethanolic extracts of *P. nigrum* (seed), *S. multiflorum* (leaf), *T. crispa* (whole stem) and *A. procera* (stem bark) exhibited moderate antiproliferative activity against PC-3 cells with IC_{50} values of 124.37, 127.36, 331.01 and 339.18 µg/ml, respectively.

The ethanolic extracts of *S. multiflorum* (stem), *P. nigrum* (fruit), *K. parviflora* (rhizome), *D. rhodocalyx* (stem bark) and *B. alnoides* (whole stem) exhibited weak antiproliferative activity against PC-3 cells (IC_{50} = 508.54, 529.50, 692.65, 911.22 and 985.33 µg/ml, respectively).

The ethanolic extracts of *A. farnesiana* (root), *L. leucocephala* (root), *B. superba* (tuberous root), *P. mirifica* (tuberous root), *M. collettii* (whole stem), *F. fragrans* (whole stem), *A. luzonensis* (whole stem), *D. conferta* (whole stem), *V. trifolia* (stem bark), *P. emblica* (fruit), *M. azedarach* (whole stem) and *C. rotundus* (rhizome) exhibited no antiproliferative activity against PC-3 cells with an IC_{50} values were greater than 1,000 µg/ml.

There were a limited number of publications of the effective herbal treatment against PC-3 cells. There was only a report of ethanolic extract of *Dryopteris crassirhizoma* induced cell cycle arrest and apoptosis in prostate cancer (PC3-MM2) cells (Chang *et al.*, 2010). The study on anti-proliferation activity of the plant ethanolic crude extract against PC-3 cells was verified by flow cytometric analysis which is based on measuring the externalization of phosphatidylserine (PS). In many cell types, induction of apoptosis is associated with plasma membrane changes where PS is translocated from the inner layer of plasma membrane to the outer leaflet (Engeland *et al.*, 1998). PC-3 cells were successively stained with both annexin V-FITC and PI after exposure to plant ethanolic crude extracts prior to the flow cytometric analysis. This assay could distinguish the plant extracts-induced apoptosis from other forms of cell death. The induction of apoptosis was clearly indicated by the proportions of apoptotic cells of the treated cells against that of the untreated cells. 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 extract.

In terms of qualitative analysis, the 3 plant extracts exhibited the survived cells at the same 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.

This 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 immunoblotting system worked well in the test systems.

Bcl-2 is a member of the Bcl-2 family of proteins that involved in the regulation of apoptosis (Reed, 1996). The overexpression 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 in comparison with the control, in which *S. venosa* treated PC-3 cells showed the least intensity. This implies that apoptosis regulator protein was significantly down-regulated by the plant ethanolic crude extract treatment and Bcl-2 has anti-apoptotic activity in PC-3 cells.

Bax is a family of Bcl-2-related proteins (Reed, 1996). Bcl-2 expression and the level of apoptosis and Bax expression appeared to be inversely proportional (Lichnovsky *et al.*, 2000). The overexpression of Bax promotes apotosis (Hasnan *et al.*, 2010). In this study there was a significant increased intensity of Bax protein in the 3 tested samples in comparison with the control, in which *S. venosa* treated PC-3 cells showed the highest intensity. This implies that Bax was significantly up-regulated by the plant ethanolic crude extract treatment.

Caspase-3 is a cytosolic protein. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis (Nicholson *et al.*, 1995). There was a significant increased intensity of cleaved caspase-3 protein in the 3 tested samples in comparison with the control, in which *S. asper* treated PC-3 cells showed the highest intensity. This implies that cleaved caspase-3 was significantly up-regulated by the plant ethanolic crude extract treatment.

Poly (ADP-ribose) polymerase (PARP) is a family of proteins involved in DNA repair in response to environmental stress (Satoh and Lindahl, 1992). PARP helps cells to maintain their viability; cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (Oliver *et al.*, 1998). There was a significant increased intensity of cleaved PARP protein in the 3 tested samples in comparison with the control, in which *S. asper* treated PC-3 cells showed the highest intensity. This implies that cleaved PARP was significantly up-regulated by the plant ethanolic crude extract treatment.

Proteomic analysis of PC-3 cells after exposure to *S. erecta* extract with the aid of Image Master 2-DE Platinum software revealed that 6 protein spots were statistically different in expression. There were increasing in expression of 3 proteins including protein disulfide-isomerase A3, sequestosome-1 and 78 kDa glucose-regulated proteins. There were decreasing in expression of 3 proteins including peroxiredoxin-6, 14-3-3 protein zeta/delta and tropomyosin alpha-3 chain.

Proteomic analysis of PC-3 cells after exposure to *S. venosa* extract with the aid of Image Master 2-DE Platinum software revealed that 6 protein spots were statistically different in expression. There were increasing in expression of 4 proteins including heterogeneous nuclear ribonucleoprotein H, pyruvate kinase isozymes M1/M2, moesin and Ran GTPase-activating protein 1. There were decreasing in expression of 2 proteins including 14-3-3 protein zeta/delta and lamin-B1 isoform 1.

Proteomic analysis of PC-3 cells after exposure to *S. asper* extract with the aid of Image Master 2-DE Platinum software revealed that 7 protein spots were statistically different in expression. There were increasing in expression of 3 proteins including cofilin-1, annexin A5 and moesin. There were decreasing in expression of 4 proteins including DNL-type zinc finger protein, 14-3-3 protein zeta/delta, endoplasmin and voltage-dependent anion-selective channel protein 2.

Among these proteins, pan 14-3-3, 14-3-3σ, GAPDH and PKM2 proteins were confirmed to exhibit differential expression by immunoblotting assay.

14-3-3 protein is a family of conserved proteins consisting of seven isotypes in human cells (β, ε, γ, ε, σ, τ, δ). These isotypes were found to be overexpressed in cancer cells (Neal *et al.*, 2009) which plays roles in the regulation of cellular processes i.e., the maintenance of cell cycle, the prevention of apoptosis (Wilker *et al.*, 2004). Interference with 14-3-3 function is one of the therapeutic approaches in cancer cells (Hermeking, 2006). In this experiment, pan 14-3-3 was down-regulated. Thus it is one of the mechanisms in apoptotic induction to PC-3 cells.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a key regulatory enzyme of glycolysis. GAPDH involved in several biological processes, including control of gene expression, DNA replication and repair, and apoptosis (Tarze *et al.*, 2007). It has evidenced that GAPDH expression is substantially increased in human cancers of various origins such as the lung (Tokunaga *et al.*, 1987), pancreas (Schek *et al.*, 1988) and breast (Révillion *et al.*, 2000). In this experiment, GAPDH was down-regulated. Thus it is one of the mechanisms in anti-proliferation induction to PC-3 cells.

Pyruvate kinase (PKM) is an isoenzyme of the glycolytic enzyme with four isoenzymes including M1, M2, L and R. Proliferating cells and tumor cells express PKM2 (Mazurek *et al.*, 2005). PKM2 protein was decreased by the 3 plant ethanolic crude extract treatment. Thus it is one of the mechanisms involved in anti-proliferation against PC-3 cells.

To understand the interaction among the differential protein profiles, proteins with significant differences in expression were uploaded to the IPA software. The program generated the biological pathways of protein inter-relation. From IPA (Figure 4.16), protein disulfide-isomerase 3 (PDIA3) was found up-regulated. This protein directly acts on SRC (proto-oncogene encoding a tyrosine kinase). SRC is a tyrosine kinase that plays a role in the regulation of cell development such as cell morphology, motility, proliferation, and survival (Roskoski, 2004). In this experiment, SRC might be disturbed by the over expressed PDIA3. Thus it might be one of the mechanisms in anti-proliferation against PC-3 cells.

Tropomyosin 3 (TPM3) was down-regulated. This protein indirectly acts on SRC. Thus SRC might be affected by the under expressed TPM3.

PDIA3 was up-regulated. This protein indirectly acts on SRC. Thus SRC might be affected by the over expressed PDIA3.

From IPA (Figure 4.17-4.18), the upstream of the identified proteins mainly interact with Myc. Lamin-B1 isoform 1 (LMNB1) was down-regulated. This protein is one of the cytoskeletons. Myc is a regulator gene that codes for a transcription factor. Myc induced pathways that contributed to the apoptotic response (Nilsson and Cleveland, 2003). In this experiment, Myc was disturbed with the down-regulated LMNB1. It might be one of the mechanisms in anti-proliferation of PC-3 cells.

Voltage-dependent anion-selective channel protein 2 (VDAC2) was down-regulated. VDAC2 functions as ion transport protein. Thus the ion transport of the cell membrane was disturbed with the down-regulated VDAC2. It might be one of the mechanisms in anti-proliferation of PC-3 cells.

Annexin A5 (ANXA5) was down-regulated. ANXA5 is a signal transduction protein. ANXA5 is linked to Myc. Myc was disturbed with the down-regulated ANXA5. Thus it might be one of the mechanisms in anti-proliferation of PC-3 cells.

In conclusion, the ethanolic crude extracts of the herbal plants used in the Thai traditional rejuvenating remedies were screened for antiproliferative activity against PC-3 cells. Among the 24 plant extracts tested, 3 plant extracts including *S. erecta*, *S. venosa* and *S. asper* exhibited a potential antiproliferation against human prostate cancer cells (PC-3). The 3 plant ethanolic crude extracts were confirmed with immunoblotting to exert anti-cancer activity through inducing cancer cell apoptosis. Proteomics study also demonstrated possible cellular proteins affected by the plant extract treatment. The plant materials in this study have long been presented as ingredients in the Thai traditional rejuvenating remedies. Hence, this is the proof that one of the rejuvenating efficacies of the plants might be related to their anti-proliferation effect against at least prostate cancer cells.

PERSPECTIVES

Anti-proliferation assay in cell cultures of the plant extracts is a conventional and rapid screening method. Thus, more plant samples in other groups of traditional remedies should be brought to this type of investigation. More different cell type is also needed to verify other bioactivities of the plant materials in this group. Flow cytometry and proteomic study show a dramatic advance tools for this type of study and could explain in detail upon the anti-proliferation activity of certain plant crude extract against the prostate cancer cells. The study of proteomic also help targeting specific proteins involve in cancer development. This could initiate a new strategy for cancer treatment via up-regulate or down-regulate of those specific proteins after a phytotherapy.

Even the selected plant crude extracts could be manufactured into traditional medicines, beverages, foods or cosmetics, however, the crude extract *per se* is not convincing for development of medicines for a specific treatment. Thus, identification as well as purification of bioactive chemicals is urgent needed in the further study. The new intellectual property should derive from the further study. Finally, new commercial products are possible to be developed from this massive study.

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APPENDICES

Appendix A: Chemicals used in cell culture

Chemical	**Company**
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma, USA
Dimethy sulfoxide (DMSO)	E.MERK, Germany
Dimethy sulfoxide (DMSO) (cell culture grade)	Sigma, USA
RPMI 1640	Gibco-Invitrogen, USA
Fetal Bovine Serum (FBS)	Gibco-Invitrogen, USA
Penicillin/Streptomycin	Gibco-Invitrogen, USA
Antibiotics/Antimycotics	Gibco-Invitrogen, USA
Sodium Bicarbonate	Sigma-Aldrich, Germany
Trypsin	Gibco-Invitrogen, USA
Trypan blue	Sigma-Aldrich, Germany
Sodium Hypoclorite	Clorox, USA
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)	Sigma, USA

Appendix B: Instruments and consumables used in cell culture

Instrument/consumable	**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

Appendix C: Chemicals used in flow cytometry

Chemical	**Company**
Annexin-V-fluorescein isothiocyanate (FITC)	Gibco-Invitrogen, USA
Propidium Iodide (PI)	

Appendix D: Instruments used in flow cytometry

Instrument/ consumable	**Company**
1.5 ml Microtube	Axygen, USA
15 ml centrifuge tube	Cloning, Mexico
Auto Pipettes	Gilson, USA
Centrifuge	Heraeus, UK
Flow cytometer FC500	Beckman Coulter, USA

Appendix E: Chemicals used in proteomics and 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
Acetonitrile	CH_3CN	41.05	E. Merck, Germany
Acrylamide	C_3H_5NO	71.08	Bio-Rad, USA
Agarose	-	-	CAMBREX, USA
Ammonium persulfate	NH_4HCO_3	228.20	Sigma, USA
Ampholyte (pH 3-10)	-	-	SERVA, Germany
Bradford protein assay	-	-	Bio-Rad, USA
Bromophenol Blue	-	670.00	Sigma, USA
Calcium chloride	$CaCl_2$	147.02	E. Merck, Germany
3-[(3-cholamidopropyl)-methylammonio] Propanesulfonate (CHAPS)	$C_{32}H_{58}N_2O_7S$	614.90	USB, USA
Coomassie brilliant blue R-250	-	826.00	SERVA, Germany
Dithiothreitol (DTT)	-	154.20	USB, USA
Drystrip cover fluid (mineral oil)			GE Healthcare, UK
ECL plus Western blotting detection system			GE Healthcare, UK
Ethylenediamine tetraacetic acid (EDTA)		372.24	E. Merck, Germany
Glycerol	-	92.10	USB, USA
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
Iodoacetamide (IAA)	-	185.00	Sigma, USA
Low molecular weight calibration kit for SDS			GE Healthcare, UK
N, N'methylenebisacrylamide		154.20	Bio-Rad, USA
N, N, N', N'-Tetra-methyl ethylenediamine (TEMED)	$C_4H_{11}NO_3$	116.20	Invitrogen, USA
Polysorbate 20 (Tween-20)	$C_{58}H_{114}O_{26}$	1,227.54	Sigma, USA
Protease inhibitor cocktail	-		Sigma-Aldrich, Germany
Potassium chloride	KCl	74.56	E. Merck, Germany
Potassium dihydrogen phosphate	KH_2PO_4	136.09	E. Merck, Germany
Sequencing Grade Modified Trypsin			Promega, USA
Sodium chloride	NaCl	58.44	RANKEM, India
Sodium dodecyl sulfate (SDS)	$C_{12}H_{25}O_4SNa$	288.38	Sigma, USA
Sodium hydrogen phosphate	Na_2HPO_4	141.959	E. Merck, Germany
Sucrose	$C_{12}H_{22}O_{11}$	342.30	Fisher Scientific, USA
Trichloroacetic acid	$C_2HCl_3O_2$	163.4	E. Merck, Germany
Thiourea	CSN_2H_4	76.12	Sigma-Aldrich, Germany
Tris (Hydroxymethyl aminomethane)	$H_2NC(CH_2OH)_3$	121.10	USB, USA
Urea	CH_4N_2O	60.06	Bio-Rad, USA

Appendix F: Instruments used in proteomics

Instrument	**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 supply	Bio-Rad, USA
Ettan IPGphor III IEF System	GE Healthcare, USA
High performance chemiluminescen film	GE Healthcare, UK
High Speed Micro Refrigerated Centrifuge	Sigma, USA
IPG gel pH 3-10, NL, 7 cm	GE Healthcare, USA
Magnetic stirrers	IKA, Germany
Magnetic stirrers hotplate	IKA, Germany
Milli-Q plus	Millipore, USA
Multi-block heater	Lab-line, India
Nitrocellulose membrane	GE Healthcare, 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 G: Reagents used in cell culture

G.1 RMPI 1640

One pack of RPMI 1640 was dissolved in 1 L of Milli Q water, added 2.2 g of sodium bicarbonate and 5.95 g of HEPES, then adjusted pH to 7.2 and sterilized by filtration through cellulose acetate membrane (pore size 0.22 μm) and stored at 4ºC.

G.2 Phosphate buffer saline (PBS) pH 7.4

NaCl	8.00	g
KCl	0.20	g
Na_2HPO_4	1.44	g
KH_2PO_4	0.20	g
Milli Q water to total of	1.00	L

The buffer was mixed well, sterilized by autoclave at 121ºC, 15 lb/square inches for 15 mins and stored at 4ºC

G.3 Trypsin solution (1X Trypsin in PBS)

10X trypsin	5.00	ml
1X PBS	45.00	ml

G.4 Complete medium (10% FBS, 0.5% Pen G/Streptomycin, 0.5% Amphoterich B in RPMI 1640 media)

FBS	10.00	ml
Penicillin/Streptomycin	0.50	ml
Antibiotic/Antimycotic	0.50	ml
RPMI 1640 media to total volume of	100.00	ml

The solutions were mixed well and stored at 4ºC

Appendix H: Reagents used in flow cytometry

H.1 Binding buffer

HEPES	2.40	g

NaCl	8.20	g
$CaCl_2$	0.30	g
Milli Q water to total of	1.00	L

The buffer was mixed well, then adjusted pH to 7.2 and sterilized by autoclave at 121ºC, 15 lb/square inches for 15 mins and stored at 4ºC

H.2 Annexin V-FITC + PI Solution (for each sample)

binding buffer	50	µl
annexin V-FITC	5	µl
Propidium iodide (PI; 50 µl/ml)	5	µl

The buffer was mixed well (prepare before use)

Appendix I: Reagents used in proteomics

I.1 0.25 M Sucrose

Sucrose	85.58	g
Milli Q water to total of	1.00	L

The solution was mixed well, sterilized by autoclave at 121ºC, 15 lb/square inches for 15 mins and stored at 4ºC

I.2 5X sample buffer (reducing condition-SDS-PAGE)

Milli Q water	4.00	ml
0.5 M Tris-HCl, pH 6.8	1.00	ml
Glycerol	0.80	ml
10% (w/v) SDS	1.60	ml
B-mercaptoethanol	0.40	ml
0.1% (w/v) bromophenol blue	0.20	ml

I.3 2-DE lysis buffer

Urea	0.42	g
Thiourea	152.24	mg
CHAPS	40.00	mg
DTT	20.00	mg
Ampholytes (pH 3-10)	50.00	µl
Protease inhibitor	10.00	µl

Dissolved in Milli Q water and adjusted the volume to 1 ml.

I.4 Rehydration buffer

Urea	0.48	g
DTT	2.80	mg
CHAPS	20.00	mg
IPG buffer (pH 3-10)	5.00	µl
Milli Q water	320.00	µl

I.5 Equilibration buffer I (1.5 ml per 7 cm. strip)

DTT	15.00	mg
SDS	15.00	mg
Urea	0.54	g
Glycerol	0.45	ml
0.5 M Tris-HCl, pH 6.8	0.15	ml
Milli Q water	0.50	ml

I.6 Equilibration buffer II (1.5 ml per 7 cm. strip)

Iodoacetamide (IAA)	37.50	mg
SDS	15.00	mg
Urea	0.54	g
Glycerol	0.45	ml
0.5 M Tris-HCl, pH 6.8	0.15	ml
Milli Q water	0.50	ml

I.7 30% (w/v) Acrylamide/0.8% N, N′- Methylenebis-acrylamide

Acrylamide	30.00	g
N, N′- Methylenebis-acrylamide	0.80	g

These chemicals were dissolved in distilled water, adjusted the final volume to 100 ml and stored at 4ºC

I.8 10% (w/v) Sodium dodecyl sulfate

10 grams of sodium dodecyl sulfate (SDS) was dissolved and adjusted the final volume to 100 ml with distilled water

I.9 10% (w/v) Ammonium persulfate

1 gram of Ammonium persulfate (APS) was dissolved in Milli Q and adjusted the final volume to 10 ml

I.10 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

I.11 14% separating gel (11 ml/gel)

Milli Q water	3.08	ml
1.5 M Tris-HCl, pH 8.8	2.75	ml
10% SDS	0.11	ml
30% Acrylamide/0.8% N, N′- Methylenebis-acrylamide	5.00	ml
10% Ammonium persulfate	55.00	µl
TEMED	5.50	µl

I.12 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

I.13 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 J: Antibodies used in immunoblotting

Antibody	**Company**
Bcl-2	Cell Signaling Tech, USA
Bax	Cell Signaling Tech, USA
Caspase-3	Cell Signaling Tech, USA
PARP	Cell Signaling Tech, USA
Pan 14-3-3	Santa Cruz Biotechnogy, USA
PKM2	Santa Cruz Biotechnogy, USA
14-3-3σ	Abcam, UK
GAPDH	Abcam, UK
α-tubulin	Sigma, USA
Polyclonal swine anti-rabbit immunoglobulins	Dako, Denmark
Polyclonal rabbit anti-mouse immunoglobulins	Dako, Denmark

Appendix K: Reagents used in immunoblotting

K.1 2X lysis buffer

500 mM Tris-HCL, pH 7.5	800.00	µl
2 M NaCl	1.50	ml
50% glycerol	4.00	ml
100% NP-40	200.00	µl
100 mM Na_2VO_4	200.00	µl
1 M β-glycerophosphate	200.00	µl

Dissolved in Milli Q water and adjusted the volume to 10 ml

K.2 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

K.3 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

K.4 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

K.5 10% Skim milk (1° and 2° antibody)

Skim milk	1.00	g
TBS/T	10.00	ml

BIOGRAPHY

Miss Chutima Chaisanit was born on September 27, 1986 in Bangkok, Thailand. She was graduated with a Bachelor degree of Science in Marine Science, Faculty of Science, Chulalongkorn University in 2008. She has enrolled in the Graduate School, Chulalongkorn University for Master Degree of Science in Biotechnology during 2008-2011.

Research presentation

Chaisanit, C., Srisomsap, C., Panriansaen, R. and Cherdshewasart, W. 2011. Antiproliferation against PC-3 prostate cancer cells by rejuvenating Thai herbal plants. Abstract *the 16th Biological Sciences Graduate Congress*, 12th-13th December, National University of Singapore, Singapore.

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