ผลของทรานเรสเวออะทอลและผลิตภัณฑ์จากองุ่นแดงต่อการดูดซึมและอัลทราสตรักเจอร์ของตับของหนูเมาส์ ความเป็นพิษ การยับยั้งวัฏจักรเซลล์และการชักนำการตายแบบอะพอพโตซิสในเซลล์มะเร็งของคน

นางสาวนภาพร แก้วดวงดี

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาชีววิทยาสิ่งแวดล้อม
มหาวิทยาลัยเทคโนโลยีสุรนารี
ปีการศึกษา 2549

EFFECTS OF TRANS-RESVERATROL AND RED GRAPE PRODUCTS ON ABSORPTION AND LIVER ULTRASTRUCTURES OF MICE, CYTOTOXICITY, CELL CYCLE ARREST, AND INDUCTION OF APOPTOSIS IN HUMAN CANCER CELL LINES

Napaporn Kaewdoungdee

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Environmental Biology

Suranaree University of Technology

Academic Year 2006

EFFECTS OF TRANS-RESVERATROL AND RED GRAPE PRODUCTS ON ABSORPTION AND LIVER ULTRASTRUCTURES OF MICE, CYTOTOXICITY, CELL CYCLE ARREST, AND INDUCTION OF APOPTOSIS IN HUMAN CANCER CELL LINES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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นภาพร แก้วดวงดี : ผลของทรานเรสเวออะทอลและผลิตภัณฑ์จากองุ่นแดงต่อการดูดซึมและอัลทราสตรักเจอร์ของตับของหนูเมาส์ ความเป็นพิษ การยับยั้งวัฏจักรเซลล์และการชักนำการตายแบบอะพอพโตซิสในเซลล์มะเร็งของคน (EFFECTS OF TRANS-RESVERATROL AND RED GRAPE PRODUCTS ON ABSORPTION AND LIVER ULTRASTRUCTURES OF MICE, CYTOTOXICITY, CELL CYCLE ARREST, AND INDUCTION OF APOPTOSIS IN HUMAN CANCER CELL LINES) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.เบญจมาศ จิตรสมบูรณ์, 181 หน้า

องุ่นและสารสกัดจากองุ่นแดงเป็นสารต้านอนุมูลอิสระและสารต้านมะเร็ง การศึกษาครั้งนี้มีวัตถุประสงค์ เพื่อตรวจหาปริมาณของสารประกอบฟีนอลิกของผลิตภัณฑ์องุ่นแดงสายพันธุ์ซินฟานเดลจากฟาร์มของมหาวิทยาลัยเทคโนโลยีสุรนารี ซึ่งได้แก่ ไวน์ น้ำองุ่นและสารสกัดเอทา-นอลจากกากองุ่นและศึกษาผลของผลิตภัณฑ์องุ่น และสารทรานเรสเวออะทอลต่อการดูดซึมของสารประกอบฟีนอลิกและอัลทราสตรักเจอร์ของเนื้อเยื่อตับในหนูเมาส์ ความเป็นพิษ การยับยั้ง วัฏจักรเซลล์ และการชักนำให้เกิดการตายแบบอะพอพโตซิสของเซลล์มะเร็งของคนจากการตรวจหาปริมาณของสารประกอบฟีนอลิกในผลิตภัณฑ์องุ่น พบว่า สารประกอบฟีนอลิกที่พบในสารสกัดเอทานอลจากกากองุ่น (4,407.33±13.65 มิลลิกรัมต่อลิตร) มีปริมาณสูงกว่าไวน์แดง (3,613.00±15.13 มิลลิกรัมต่อลิตร) และน้ำองุ่น (1,102.67±21.96 มิลลิกรัมต่อลิตร) อย่างมีนัยสำคัญทางสถิติ หลังจากการป้อนด้วยน้ำองุ่นและสารสกัดเอทานอลจากกากองุ่นหนึ่งครั้ง พบการดูดซึมสูงสุดในพลาสมาของหนูเมาส์ ICR ที่ช่วงเวลา 12 ชั่วโมงเท่ากับ 0.22±0.01 กรัมต่อลิตร และช่วงเวลา 6 ชั่วโมงเท่ากับ 0.22±0.01 กรัมต่อลิตร ตามลำดับ แต่การวิเคราะห์หาปริมาณการดูดซึมของทรานเรสเวออะทอลในพลาสมาของหนูเมาส์ ICR โดยวิธี capillary electrophoresis ไม่พบระดับของทรานเรสเวออทอลในพลาสมาของหนูเมาส์ทั้งในกลุ่มที่ได้รับทรานเรสเวออะทอลและทรานเรสเวออะทอลร่วมกับไวน์

การให้ผลิตภัณฑ์องุ่นและทรานเรสเวออะทอลกับหนูเมาส์ ICR ทุกวัน ครบ 6 เดือน มีผลต่อพยาธิวิทยาระดับอัลทราสตรักเจอร์ของเซลล์ตับต่ำ เมื่อเทียบกับกลุ่มควบคุมโดยพิจารณาจากการสะสมของไขมันและไกลโคเจน และความผิดปกติของออร์แกเนลล์ในเซลล์ตับ ความเป็นพิษของทรานเรสเวออะทอลและสารสกัดเอทานอลจากกากองุ่นต่อเซลล์มะเร็งตับอ่อน Panc 2.03 และเซลล์มะเร็งตับ SNU 1079 ขึ้นกับความเข้มข้นของสาร เมื่อทดสอบโดยวิธี MTS ทรานเรส-เวออะทอลสามารถยับยั้งวัฏจักรเซลล์ของ Panc 2.03 และ SNU 1079 ในระยะ S และ G_1 ตามลำดับ ส่วนสารสกัดเอทานอลจากกากองุ่นยับยั้งวัฏจักรเซลล์ของ Panc 2.03 ในระยะ S และ

ยับยั้งเซลล์ SNU 1079 ในระยะ S และ G_2 ฤทธิ์ความเป็นพิษของทรานเรสเวออะทอล และสารสกัดเอทานอลจากกากองุ่นต่อเซลล์มะเร็งเกิดจากการชักนำให้เกิดการตายแบบอะพอพโตซิสภายในเซลล์ที่สามารถตรวจสอบโดยวิธีการย้อมด้วย DAPI และ annexin-V FITC การแตกหักของดีเอ็นเอ และการลดการแสดงออกของโปรตีน pro-caspase 3 และ โปรตีน Bcl-2 ข้อมูลจากการวิจัยครั้งนี้เสนอแนะว่ากลไกที่ชักนำให้เกิดการตายในเซลล์มะเร็งทั้งสองชนิดอาจจะเกิดผ่านทางการควบคุมของโปรตีนที่เกี่ยวข้องกับการชักนำให้เกิดการตายแบบอะพอพโตซิส และ/หรือผ่านทางการยับยั้งของวัฏจักรเซลล์

สาขาวิชาชีววิทยา ลายมือชื่อนักศึกษา _____

ปีการศึกษา 2549 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

NAPAPORN KAEWDOUNGDEE : EFFECTS OF TRANS-RESVERATROL AND RED GRAPE PRODUCTS ON ABSORPTION AND LIVER ULTRASTRUCTURES OF MICE, CYTOTOXICITY, CELL CYCLE ARREST, AND INDUCTION OF APOPTOSIS IN HUMAN CANCER CELL LINES. THESIS ADVISOR : ASST. PROF. BENJAMART CHITSOMBOON, Ph.D., 181 PP.

TRANS-RSVERATROL/ABSORPTION/ULTRASTRUCTURE/CYTOTOXICITY /CELL CYCLE/ARREST/APOPTOSIS/CANCER

Trans-resveratrol and red grape products have been known to be antioxidants and anticarcinogens. The present study investigated the total phenolic compound (TPC) contents of Zinfandel grape products, wine, juice and pomace, from the Suranaree University of Technology farm. The effects of grape products and trans-resveratrol on absorption of TPC *in vivo*, ultrastructure of mouse liver tissue, cytotoxicity, cell cycle arrest and apoptotic induction on human cancer cell lines were investigated. The TPC content of ethanolic grape pomace extract (4,407.33±13.65 mg/L) was significantly higher than those of red wine (3,613.00±15.13 mg/L) and grape juice (1,102.67±21.96 mg/ml). After single oral administration, the highest absorptions of TPC content in plasma of ICR mice were 0.22±0.01 g/L at 12 h, and 0.22±0.01 g/L at 6 h post administration of juice and ethanolic grape pomace extract, respectively. In contrast, the recoveries of trans-resveratrol absorptions as analyzed by capillary electrophoresis were not detected in the plasmas of both trans-resveratrol and trans-resveratrol-spiked wine treated groups. Exposure of ICR mice to grape

products and trans-resveratrol daily for six months reduced ultrastructural pathologic of hepatocytes, included minimal glycogen, fat accumulation, and organelle abnormality, compared to their corresponding vehicle controls. Trans-resveratrol and ethanolic grape pomace extract exhibited cytotoxic effects on pancreatic Panc 2.03 and cholangiocarcinoma SNU 1079 cells in a dose dependent manner assessed by MTS assay. Trans-resveratrol treatment of Panc 2.03 and SNU 1079 cells resulted in S and G_1 phase arrests in the cell cycle, respectively. Ethanolic grape pomace extract treatment of Panc 2.03 cells resulted in S phase arrest, while the same treatment of SNU 1079 cells resulted in both S and G_2 phase arrests of the cell cycle. The cytotoxic activity was mediated via apoptosis as demonstrated by DAPI and annexin V-FITC staining, DNA fragmentation, and decreased pro-caspase 3 and Bcl-2 protein expressions. These data suggest a possible mechanism of cytotoxicity in both cancer cell lines, at least in part, through the regulation of apoptosis-related proteins and/or cell cycle dysregulation.

School of Biology

Academic Year 2006

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Napaporn Kaewdongdee

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
BD	Becton Dickinson
BSA	Bovine serum albumin
bp	Base pair
b.wt.	Body weight
CE	Capillary electrophoresis
CHD	Coronary heart disease
cm	Centimeter
cm^2	Centimeter square
CO_2	Carbon dioxide
DNA	Deoxyribonucleic acid
DAPI	4' 6'-Diamidine-2'-phenylindole Dihydrochloride
DMRT	Duncan multiple range test
DMSO	Dimethyl sulfoxide
DTT	Dithiotrietol
DW	Distilled water
ECL	Enhanced chemiluminescence system
EDTA	Ethylenediaminetetraacetic acid
EDTA-K3	Vacutainer® EDTA-K3

LIST OF ABBREVIATIONS (Continued)

ELISA	Enzyme Linked Immunoabsorbant Assay
ER	Endoplasmic reticuum
et al.	*Et. Alii* (Latin), and others
etc.	And others
FBS	Fetal Bovine Serum
Fig.	Figure
FITC	Fluorescein isothiocyanate
g	Gram
GAE	gallic acid equivalent
h	Hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HPLC	High-performance liquid chromatography
ICR	Institute cancer research
i.d.	Internal diameter
k	Kilo (10^3)
kDa	Kilodalton
kg	Kilogram
kV	Kilovolt
L	Liter
LOD	Limit of detection

LIST OF ABBREVIATIONS (Continued)

LOQ	Limit of quantitation
m	Milli (10^{-3})
M	Molar
2 ME	2-Mercaptoethanol
min	Minute
mg	Milligram
mg/L	Milligram per litter
mg/ml	Milligram per milliliter
ml	Milliliter
mM	Milli Molar
mm	Millimeter
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt
MWs	Molecular weight
n	Nano (10^{-9})
$NaHCO_3$	Sodium Bicarbonate
NaOH	Sodium Hydroxide
ND	Not detected
nm	Nanometer
NP 40	Non-idet P 40

LIST OF ABBREVIATIONS (Continued)

OD	Optical Density
OsO_4	Osmium tetroxide
PAGE	Polyacrylamide gel electrophoresis
Panc 2.03	Pancreatic cancer cell line
PBS	Phosphate buffered saline
PI	Propidium Iodide
ppm	Part per million
p.s.i.	Pound per square inch = 6894.76 pascal
RESV	Resveratrol
rpm	Revolution per minute
RPMI 1640	Roswell Park Memorial Institute
RSW	Resveratrol spiked wine
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNU 1079	Seoul National University 1079
TEM	Transmission electron microscope
TEMED	N, N, N', N'-tetramethyl ethylene diamine
TPC	Total phenolic compound
TTS	Total solid
UV	Ultraviolet

LIST OF ABBREVIATIONS (Continued)

v/v	Volume: Volume
w/v	Weight per volume
%	Percent
ºC	Degree Celsius
µ	Micro (10^{-6})
µg	Microgram
µg/ml	Microgram per milliliter
µl	Microliter
µm	Micrometer
x g	x gravitational acceleration
x	Times

CHAPTER I

INTRODUCTION

1.1 Introduction

The use of synthetic antioxidants in food industry is severely restricted to both application and level. Hence there is a wide interest to natural antioxidants extracted from plants. Several polyphenol compounds extracted from plants possess antioxidant activity, and the research on polyphenols occurring in plants has attracted considerable interest due to the numerous and health-beneficial effects, such as antimutagenic, anticarcinogenic, antiatherogenic, etc.

Scientific discovery of many plants based foods with putative anticancer effects, such as garlic, ginger, onions, tomatoes, broccoli, cabbage, soy bean, peanut, blueberries, cranberries, and grapes have been identified to be used for human consumption. In these vegetables and fruits, there are currently more than 400 compounds being under investigations for their health promoting properties. At present, numerous medicinal plants have been identified and developed as chemotherapeutic agents and as modulators of the immune system. Several drugs derived from medicinal plants have been used widely in Asia and Europe as traditional medicine for self-medication. Following the discovery of grapes being a phytoalexin in 1976 by Langcake and Pryce, in 1992 Siemann and Creasy discovered wine. Several classes of chemical agents with antimutagenic and anticarcinogenic activities have also been identified in many foods and beverages of natural origin.

Administration of phytochemicals has been used to prevent intiation; promotion and progression in carcinogenesis (Jang et al, 1997) and may be utilized as a direct way to reduce cancer mortality and morbidity.

Recently, a wide variety of polyphenolic compounds and non-flavonoids have been found mainly in vegetables and fruits especially in grapes and their derivatives, (Frankel et al., 1993). One of the mian phytochemicals which is found in red grapes (*Vitis vinifera*) is trans-resveratrol (Fig 1). Trans-resveratrol is one of the most important sources of chemicals present in a variety of plants species. Trans-resveratrol has been found in much higher concentration in red grape varieties than white grape varieties. It is present naturally in 72 plants, and fruits, especially in grape skin. It was first isolated in 1940 as a constituent of the roots of white hellebore (*Veratrum grandiflorum* O. Loes), and later in the dried roots of *Polygonum cuspidatum*, called ***Ko-jo-kon*** in Japanese. ***Ko-jo-kon*** is used in traditional Chinese and Japanese medicine to treat suppurative dermatitis, gonorrhea favus, athlete´s foot (*tinea pedis*), and hyperlipemia (Aggarwal et al., 2004). Trans-resveratrol is a naturally stilbene-type phytoalexin synthesized in response to stress, injury, ultraviolet (UV) irradiation, pathogens, and fungal infections. The use of grape extracts for human health can be dated over 2000 years ago, to “darakchasava”, a well-known Indian herbal preparation of which the main ingredient is *Vitis vinifera* L. Also dried grapes called manakka as a cardiotonic is well documented in its properties of health promotion in reducing the risk of coronary heart disease (CHD). Specifically, its positive effect on CHD may be a result of protection against oxidation of LDL. The interest in the health promoting properties of trans-resveratrol was sparked when it was originally discovered in red wine, and thus identified as a possible key player in so called

“French Paradox”: the finding that the lower rate of CHD mortality in various parts of France compared to other industrialised countries with a similar risk factor profile, especially the high consumption of dietary fats, has been attributed to frequent consumption of red wine. The beneficial effects of trans-resveratrol consumption include suppression of lipid peroxidation and eicosanoid synthesis, inhibition of platelet aggregation, anti-inflammatory, and vasorelaxant activities. Numerous reports indicate that trans-resveratrol has antiviral effects against HIV-1 and the herpes simplex virus by synergistically enhancing the anti-HIV-1 activity of AZT, ddC, and ddI. Besides exhibiting antibacterial effects, trans-resveratrol also has anticancer activities by affecting cell signaling pathway, modulating transcription factors, gene induction, regulation of enzyme activities and protein interactions in several *in vivo* and *in vitro* study. A significant amount of trans-resveratrol is present in wine, concentration in red wine ranges from 0.2 mg/l to 7.7 mg/l and fresh grape skin contains 50-100 mg trans-resveratrol per gram. The health significance of trans-resveratrol has promoted numerous methods for its measurement in foods. The gas chromatography (GC) approach can resolve both isomers of trans-resveratrol with detection limits in the low μM range. A high-performance liquid chromatography (HPLC) method has also been developed to determine the level of trans-resveratrol in plasma. The principle of separation in capillary electrophoresis (CE) is based on the differential migration of analyses. CE has unique advantages that make it an excellent candidate for analysis of these compounds, including a very small sample size requirement, high efficiency of separation and speed.

Cholangiocarcinoma is the highest incident primary liver cancer in the Northeast of Thailand (Vatanasapt et al., 1993) and is still a major health problem of people in

this area. Pancreatic cancer is known as a cancer with poor prognosis. This malignant tumor is highly fatal and poor prognosis because there is no method for early detection and lack of effective treatments. Failure to Surgical resection of pancreatic cancer is avialable only in 15-20 % of all patients, while medical approaches, such as chemotherapy or radiation, have no cure. induce apoptosis is a major factor limiting the efficacy of common treatment for cancer: surgical treatment, chemotherapy and radiotherapy (Dive, 1997). The resistance of pancreatic cancer and cholangiocarcinoma to chemotherapeutic agents is one of the serious problems in clinical situations.

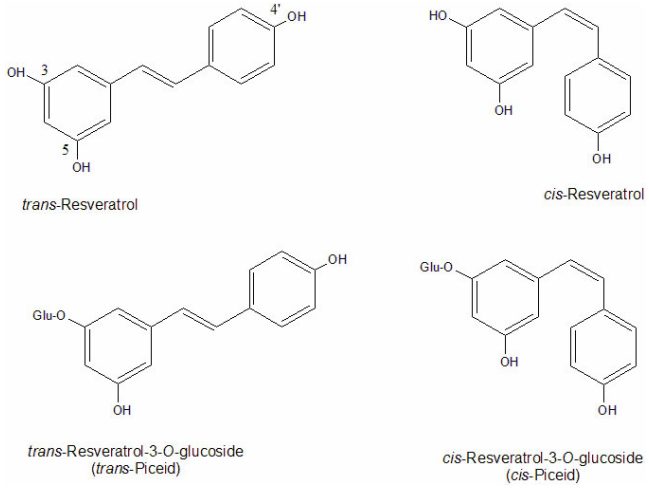


Figure 1 Structure of trans-resveratrol and its glucosides
Source: *http://lpi.oregonstate.edu/infocenter/phytochemicals/trans-resveratrol/cistrans.html*

Apoptosis, or programmed cell death, has been characterized as a fundamental cellular activity occurring under a wide range of physiological and pathological conditions. It plays an essential role as a protective mechanism against neoplastic development in the organism by eliminating genetically damaged cells or excess cells that have been improperly induced to divide by a mitotic stimulus. Therefore,

suppression of apoptosis may be a feature of tumor promotion by chemical carcinogens. Indeed, many chemopreventive agents may act through the induction of apoptosis as a mechanism of anticarcinogenic action. Apoptosis is usually mediated through the activation of caspase. Mechanistically, there are two different types of apoptosis; one that is caspase-8 dependent and receptor mediated (type I), and the other that is caspase-9 dependent and mediated through the mitochondria (type II). Trans-resveratrol induced apoptosis *via* a novel mitochondria pathway controlled by Bcl-2 (Tinhofer et al., 2001). Caspase, a family of cysteinyl aspartate-specific proteases, plays a central role in apoptosis, is synthesized as zymogens with a prodomain of variable length followed by a large subunit and a small subunit. The caspases are activated through proteolysis at specific asparagine residues that are located within the prodomain, the p20 and p10 subunits. Their important function in this process makes caspase a potential target for drug development. Cell cycle dysregulation is also a hallmark of tumor cells. The ability of normal cells to undergo cell-cycle arrest after damage to DNA is crucial for the maintenance of the genomic integrity. The biochemical pathways that stop the cell cycle in response to cellular stressors are called checkpoints. The regulation of cell signaling has also important clinical implications because the abrogation of checkpoint function can alter the sensitivity of tumor cells to chemotherapeutics. Trans-resveratrol is a new cell cycle blocker as well as a cancer chemopreventive agent since it causes S phase arrest and subsequently reversible S phase arrest (Schneider et al., 2000; Delmas et al., 2000; Kim et al., 2004), cell cycle arrest in various human cancer cell lines. The mechanisms how cancer cells evade apoptotic signal are beginning to come to light, but its upstream regulators are not as yet fully understood. Though there is enormous

amount of data supporting trans-resveratrol's and certain grape products such as wine and juice possess anticancer effects *in vitro* and *in vivo*, there is not much data on the chemopreventive and therapeutic effects of grape promace especially those that prepared from Zinfandel red grapes grown at Suranaree University of Technology. In addition, to the best of the author's knowledge, no studies of cytotoxic acitivities of trans-resveratrol and grape products against human cholangiocarcinoma SNU 1079 and pancreactic Panc 2.03 cells are conducted. Since both cholangiocarcinoma and pancreatic cancers are very poor prognosis, resistant to the available chemotherapeutic agents and hence represent the serious problems in clinical treatment, the present study aimed to explore the therapeutic potential of trans-resveratrol and certain grape products against two human cholangiocarcinoma and pancreatic cancer cell lines, SNU 1079 and pancreactic Panc 2.03 cells, respectively. In initial phase of the study, the total phenolic contents of the products of red grapes grown at Suranaree University of Technology (SUT) farm and trans-resveratrol absorption in mice were determined. Then the chemopreventive effect of the products of red grapes on ultrastructural changes of liver tissue in mice was investigated. The last part of the study was to assess the cytotoxic and antiproliferative effects of trans-resveratrol and grape products on SNU 1079 and Panc 2.03 cells. The alteration of singaling protein factors in apoptotic pathway as the molecular mechanism of cytotoxicity on the cancer cell lines was also explored.

1.2 Objectives of the study

The main objectives of this investigation are as follows:

1. To investigate the total phenolic content of red grapes products (red win, grape juice and grape pomace) and absorption of commercial trans-resveratrol and grape products in ICR mice.

2. To observe the effect of prolonged administration of commercial trans-resveratrol and red grape products on ultrastructural change of liver tissue in ICR mice.

3. To find cytotoxicity of commercial trans-resveratrol and red grapes products against selected human cancer cells *in vitro*.

4. To determine certain apoptotic protein factors and their enzymatic activities in selected human cancer cell lines in response to commercial trans-resveratrol and red grapes products.

5. To investigate the molecular mechanism of the chemopreventive effects of commercial trans-resveratrol and red grapes products on cell cycle arrest in selected human cancer cell lines.

1.3 Research hypothesis

1. Commercial trans-resveratrol and red grape products could be absorbed through the mice plasma and protect ultrastructural changes of mice liver from damages caused by normal dialy stressors.

2. Commercial trans-resveratrol and red grapes products could affect signaling proteins in apoptotic pathways and their enzymatic activities in certain human cancer cell lines.

3. Commercial trans-resveratrol and red grapes products could enhance the molecular mechanism of the chemopreventive effects on cell cycle arrest.

1.4 Scope and limitations of the study

Commercially available trans-resveratrol and the extracts from Zinfandel red grapes grown in Suranaree University of Technology farm was used throughout this study. Red grapes products (red wine, grape juice, and grape pomace) were made from Zinfandel grapes and were assessed *in vitro* and *in vivo*. Grapes pomace was extracted by 80% ethanol and sterile water. The available trans-resveratrol and contents of total phenolic compounds in red grape products were determined prior administered to male ICR mice via oral gavage and determined their absorption by evaluating their recovery in blood plasma using capillary electrophoresis (CE). The effects of commercial trans-resveratrol and red grapes products on ultrastructural changes of liver tissue were studied by transmission electron microscope. The cytotoxicity of trans-resveratrol and red grapes products was investigated only *in vitro* on two human cancer cell lines: SNU 1079 and Panc 2.03. To determine whether cytotoxicity on cancer cell was mediated through apoptotic mechanism, the differential effects of trans-resveratrol and grapes products on apoptotic protein induction and their enzymatic activities, DNA contents, and cell cycle arrest in the cancer cell lines were investigated in this study.

1.5 Expected results

The anticipated outcomes from this study should:

1. Provide the basic toxicological data of medicinal plant, red grapes products, which are putatively beneficial for safety application.

2. Provide information on chemopreventive effects of trans-resveratrol and red grapes products which can be used in the future as pharmacological data for the clinical treatment.

3. Understanding the primary mechanism of their anti-cancer activity.

4. Enhance the development of medicine practice from grape products in cancer therapy.

5. Encourage the knowledge of people to realize the using of medicinal plants and alcohol consumption.

CHAPTER II

LITERATURE REVIEWS

1. Cholangiocarcinoma

1.1 Definition

Cholangiocarcinoma (CCA) is a one major kind of primary liver cancer defined as an adenocarcinoma arising form bile duct epithelium, at any portion of the biliary tree, except carcinoma of the gall bladder proper and the ampulla of Vater (Uttaravichien et al., 1999). CCA can be divided into two types including the intrahepatic or peripheral CCA and the extra hepatic CCA (Nakanuma et al., 1997).

1.2 Etiology

Several etiological factors are known to be predisposing factors for developing of CCA. These factors include parasitic infestation, inflammatory bowel disease with/without primary sclerosing cholangitis (PSC), hepatolithiaasis, cirrhosis, deposition of thorotrast, toxin, and drugs (Craig et al., 1988, Uttaravichien et al., 1994). Most of the evidence in geographics demonstrates a higher frequency of CCA within an ethnic group of Laos descendents and Esan people in the Northeast of Thailand who enjoy eating raw fish which harbor infective stage of liver flukes of Opisthorchis viverrini (OV) (Vatanasapt et al., 1995). The incidence of this cancer in Khon Kean, Thailand is very high with the standardized incident rates of 90 for male

the highest in the world (Vatanasapt et al., 1995; Parkin et al., 1997). In Thailand, epidemiological data and studies *in vivo* have supported that CCA is associated with infection of the liver fluke, *Opisthorchis viverrini* and several carcinogenic N-nitroso compounds and their precursor which exist in the daily diet and endogenous nitrosamine formation by liver fluke infection (Vatanasapt et al., 1995; Parkin et al., 1997). CCA displays a poor prognosis without established measures for treatment.

1.3 Carcinogenesis

In Thailand, OV is the major risk factor for the development of CCA (Parkin et al., 1993; Srivatanakul et al., 1991). Carcinogenesis is probably related to duration and severity of infection, host immune responses, and others such as dietary carcinogens, for example nitrosamines. Many investigators suspected that the habit of eating salted fish and plara, a fermented fish sauce product in Northeast Thailand are also contributing to cholangiocarcinogenesis. Several carcinogenic N - Nitroso compounds and their precursors exist at low levels in this daily diet (Migasena et al., 1974; 1980; Srisraluang and Boriboon, 1988). In addition endogenous nitrosamine formation by liver fluke infection has been reported. Secondary to parasite infestation, the inflammatory cells can induce nitric oxide synthase and increased nitrosation of proline and thioprolines (Srivatakul et al., 1991; Haswell-Elkins et al., 1994; Satarug et al., 1998). Both exogenous and endogenous nitrosamine formation may lead to DNA alkylation and deamination. Recemtly, Pinlaor et al. (2003) reported oxidative DNA damage occurred in biliary epithelium in OV infected hamster. It seemed that the parasites induce DNA damage and mutation as a consequence of the formation of carcinogens/free radicals and of cellular proliferation of the intrahepatic bile duct epithelium and malignant transformation.

1.4 Histopathology and histopathologic classification

CCA are classified into two major histological types: the common type of CCA (adenocarcinoma) and the special (unusual) type (Nakanuma et al., 1997). The majority of CCAs are adenocarcinoma with variable different desmoplasia (common type CCA). These include well-differentiated, moderately-differentiated and poorly - differentiated adenocarcinoma. The special types, including subtype adenosquamous and squamous cell carcinomas, mucinous carcinoma, sarcomatous type, signet ring cell carcinoma, cholangiocellular carcinoma and billiary papillomas (Nakanuma et al., 1997). The latter types have been reported to be related to poor prognosis of CCA patients.

1.5 Clinical features and diagnostic approaches

The clinical features of CCA vary with the type and the site of the tumor origin (Okuda et al., 1977; Nakeeb et al., 1996). The majority of CCA present with painless obstructive symptoms, which included pale stools, dark urine and jaundice. Right upper quadrant abdominal pain, fever and rigors are indicative of superimposed cholangitis, and require urgent treatment. Infection is often precipitated by instrumentation of biliary tree. These clinical symptoms and signs tend to occur only once the tumor is quite advanced.

The initial diagnosis of the obstructing lesion is made using imaging techniques, including ultrasound scan (US), computed tomography (CT) scan, Endoscopic retrograde cholangio pancreatography (ERCP) and magnetic resonance imaging (MRI). A recent review reports that intraductal ultrasound (IDUS) is able to

correctly diagnose 76.8% of carcinomas. Both histology and cytology plays predominantly confirmatory roles in the initial diagnosis of bile duct tumors, following from imaging of the lesion. It is still sometimes not possible to obtain material suitable for tissue diagnosis prior to surgery (Okuda et al., 1977).

Certain serum tumor markers, although not specific to CCA, may be of value, especially in patients with underlying primary sclerosing cholangitis. The most widely studies tumor markers are carcinoembryonic antigen (CEA) and carbohydrated antigen (CA) 19-9 have been reported in CCA (Nakeeb et al., 1996; Patel et al., 2001). However, CEA levels alone are neither sensitive nor specific for CCA. CA19-9 has a sensitivity of 67% - 89% and specificity of 86% - 98% with levels over 100 U/ml CCA (Nakeeb et al., 1996).

1.6 Therapy

Treatment of cholangiocarcinoma state I and II is a curative resection. Whereas the more advanced stage (III and IV), the treatment of choice is surgical bypass or non-surgical management such as percutaneous transhepatic biliary drainage (PTBD), endoscopic retrograde billiary drainage (ERBD), external radiation, brachytherapy, photodynamic therapy and chemotherapy (Terblance, 1994). Surgery remains that only intervention offering the possibility of cure. The main treatment goal should be complete excision with negative margins. However, combined treatment of preoperative chemotherapy and radiation give a high promising result. Palliative chemotherapy for CCA comprises a systemic regimen of 5-fluorouracil, doxorubicin and mitomycin C, although few good response have been reported (Alteaa et al., 1991).

2. Pancreatic cancer

Pancreatic cancer is a disease in which malignant cells form in the tissues of the pancreas. It is an important cause of cancer mortality in developed countries. The majority of pancreatic cancers start in the exocrine cells. The prognosis for pancreatic cancer is generally regarded as poor. Most pancreatic tumors are adenocarcinomas.

2.1 Causes and Risk Factors

There are various risk factors of increasing pancreatic cancer including smoking tobacco, high fat diet, chronic pancreatitis, inherited a hereditary form of pancreatitis or pancreatic cancer, and working with metals or chemicals. At present, the cause of pancreatic cancer is still unknown. Early diagnosis of pancreatic cancer is difficult because the symptoms are so non-specific and varied. Common pancreatic cancer symptoms include abdominal pain, loss of appetite, significant weight loss. Smoking seems to be the main preventable risk factor. The incidence of pancreatic cancer in Thailand is low, but there is a 3 to 5 fold variation in incidence by geographical region, with the highest rates noted in Chiang Mai (Vatanasapt et al., 1995). This may relate to the greater prevalence of smoking in the north. It is one of the most aggressive forms of cancer, leading to death in an overwhelming majority of patients within a few years despite surgery and/or chemotherapeutic treatment.

2.2 Therapy

Treatment for pancreatic cancer includes surgery, chemotherapy, radiation therapy or a combination, depending on the stage of the disease. Chemotherapy uses drugs to treat cancer, while radiation therapy uses X-rays or other kinds of radiation to

kill cancer cells. Surgery can be used to remove a tumor or to treat symptoms of pancreatic cancer. Several lines of evidence suggest the use of combinations of chemotherapy containing mitomycin C and other crosslinking agents to be beneficial for pancreatic cancer patients.

3. Anticancer and cytotoxic agent from plants

Plants have been a major source of medicine substances for thousands of years, and continue to play a major role in the primary health care. The World Health Organization (WHO) estimated that about 80% of the people in developing countries still rely on traditional medicine for their primary health care, and about 85% of traditional medicine involves plant extracts (Farnsworth, 1993; Crain, 1999).

In recent year, there has been growing interest in using natural products especially those derived from plants, for medicinal purposes including anticancer. There have been several efforts to discover new anticancer agents from plants and many of them have been established. For example, the Catharanthus alkaloids, vincritine and vinblastine, are two of the most important cancer chemotherapeutic agents in current use, followed by the podophyllotoxin derivatives, etoposide and teniposide from Podophyllum peltatum (Berberidaceae). The indole related alkaloid campothecin analogue irinotecan, and the diterpene alkaloid paclitaxel from *Taxus brevifolia* and some other *Taxus species* (Taxaceae). A number of other plant derived antiumor agents have been subjected to recent clinical evaluation, including taxol, harringtonine, homoharringtonine, 10-hydroxycampothecin, toniposide and elliptcine derivatives (Cordell et al., 1991).

3.1 Zinfandel red grape

Grapes are edible fruits within the Vitaceae family. The major species currently consumed is *Vitis vinifera* (European grape), accounting for 95% of grape production (Mazza, 1995). Grape is referred to *Vitis which is* a deciduous plant. There are more than 60 species of *Vitis* known. Currently, grapes are commercially cultivated around the world *such as Vitis vinifera.* Grape consists of a group of tissues or pericarp surrounding the seeds. The major red varieties are Cabernet Sauvignon, Merlot, Pinot Noir, Zinfandel and Shiraz. Grape was a phytoalexin in 1976 by Langcake and Pryce and later as wine by Siemann and Creasy, 1992. Several classes of chemical agents active as antimutagens and anticarcinogens have also been identified in many foods and beverages of natural origin. Administration of phytochemicals has been to prevent intiation, promotion and progression in carcinogenesis (Jang et al, 1997) and may be utilized as a direct way to reduce cancer mortality and morbidity.

3.2 Absorption

To explore and determine the mechanisms of action of antioxidant microconstituents in food and their role in disease prevention, an understanding of the factors that constrain their release from the foods in which they are contained and their extent of absorption are essential. Understanding this concept of bioavailability is important to all those involved in food production, nutritional assessment and determining diet–health relationships. Bioaccessibility is defined as the amount of an ingested compound that becomes available for absorption in the gastrointestinal tract. It is therefore an integration all the luminal events. The amount that becomes

bioaccessible may be equal to or less than the amount of the antioxidant that is liberated from the food matrix since: (i) it may not all transfer/convert to absorbable species, (ii) some may transfer to unabsorbable species, and (iii) some may interact with other components of the luminal contents to become unabsorbable.

Based upon plasma or serum assay of free resveratrol, absorption of this compound in form of resveratrol glucuronide has been demonstrated after oral administration in rats (Solea et al., 2001) and human. Quercetin in human has been found in form of glucuronide and sulfate conjugation in serum and urine (Solea et al., 2001). Bertelli et al. (1996) reported that the absorption of resveratrol concentration of rats plasma was found 175 μg/L at 15 minutes after oral dose of 0.5 mg by drawing blood at 30 and 45 minutes after dose.

3.3 Phenolic compounds

Grapes contain a variety of phenolic compounds. The specific amounts and types of phenolics present depend on a number of factors, including variety, maturity, seasonal conditions, storage, and processing (Mazza, 1995). Phenolic compounds play a major role in wine quality. Phenolic compounds undergo a number of transformations that depend on the temperature, sulfur dioxide concentration, degree of oxidation, time and the anthocyanin to tannin ratio. There are four major types of phenolic compounds found naturally in grapes and throughout the plant kingdom: phenolic acids, hydroxycinnamic acids, flavonoids and tannins. These compounds are non-nutritive secondary plant metabolites concentrated in the skins and the seeds. Most of the phenolic compounds in grapes are flavonoids such as anthocyanins are responsible for the color of red grapes. In addition, grapes contain phytoalexins, which are produced in response to tissue damage. One important phytoalexin in grapes is resveratrol.

3.4 Resveratrol

Resveratrol (3,5,4'-trihydroxystilbene) is a naturally occurring phytoalexin produced by a wide variety of plants such as grapes, peanuts, and mulberries in response to stress, injury, ultraviolet (UV) irradiation , and fungal infection. Although phytoalexins have long been inferred to be important in the defense of plants against fungal infection, few reports show its role in resistance to infection. Several plants, including grapevine, synthesize the stilbene-type phytoalexin resveratrol when attacked by pathogens. Resveratrol was first identified as a constituent of the roots of white hellebore (*Veratrum grandiflorum* O. Loes), and later in the dried roots of Polygonum cuspidatum, called Ko-jo-kon in Japanese, which is used in traditional Chinese and Japanese medicine. In 1976 langcake and Pyrce reported that resveratrol was detected in the leaf epidermis and the skin of grape berries. Fresh grape skins contain 50-100 mg resveratrol per gram, and the concentration in wine ranges from 0.2 mg/L to 7.7 mg/L.

3.4.1 Source and chemistry of Resveratrol

Resveratrol is found widely in nature. It is an off white powder. Reveratrol is insoluble in water but dissolves in ethanol and dimethylsulphoxide. The stilbene-based structure of resveratrol consists of two phenolic rings linked by a styrene double bond to generate 3,4'5,-trihydroxystilbene. Resveratrol is found in both trans and cis conformations, although it is the trans formation that is more active. It is typically found at concentrations of 1 mg/ L in red wine, and less than 0.1 mg/ L in white wine. Since it is a phytoalexin produced in response to injury, concentrations may vary widely (Siemann and Creasy, 1992).

3.4.2 Effects of resveratrol

Resveratrol has been shown to suppress proliferation of a variety of tumor cells, including lymphoid and myeloid cancers; breast, colon, pancreas, stomach, prostate, head and neck, ovary, liver, lung and cervical cancers; melanoma; and muscles (Delmas et al, 2000, Joe et al, 2002). Besides inhibiting proliferation, resveratrol also induces apoptosis and DNA fragmentations in leukemic B-cells (Billard et al., 2002), lymphoma cells (Weider et al., 2001), HL-60 (Clement et al., 1998), MCF-7 and MDA-MB-231 (Pozo-Guisado et al., 2002), PANC-1 (Ding and Andrian, 2002), human breast cancer cells line, and had no cytotoxicity against normal peripheral blood mononuclear cells (PBMC). Resveratrol is a potent anti-inflammatory (Huang et al., 1999; Wang et al., 2001), and antimutagenic/ anticarcinogenic agent (Ahmad et al., 2000). In addition, Resveratrol has been shown to have a chemopreventive role in a wide variety of tumors including skin (Jang, et al., 1997), liver (Yu, Sun, WU and Pan, 2003), colon (Tessitore et al., 2000), breast (Jang et al., 1997), lung (Hecht et al., 1999), and esophageal cancers (Li et al., 2002).

Since the response rate with all therapy is not satisfactory as mentioned above, a new and effective treatment for CCA and pancreatic cancer should be considered. One attractive strategy for CCA and pancreatic cancer therapy is the use of medicinal plants as therapeutic agent, especially in the patient with advanced stages which nearly all of them developed metastasis, the anticancer are also important because this stage is difficult to cure by surgical resection and/or localized irradiation. Therefore, the medicinal plants, grape products or trans-resveratrol were used as the anticancer drug in this study because there is no method for early detection and lack of effective treatment and recurrence of the cancer is frequently reported (Nakanuma et al., 1997).

4. Measurement of cell viability and cytotoxicity

Many experiments carried out *in vitro* are for the sole purpose of determining the potential cytotoxicity of compound understudy, either because they are being used as a pharmaceutical or cosmetic and must be shown to be non toxic or because they are designed as anticancer agents, when cytotoxicity may be crucial to their reaction.

Tetrazolium-bawed cytotoxicity assay.

Microculture tetrazolium assays are being widely used to investigate the cell viability and cell proliferation. They are colourimetric assays which are based upon the bioreduction of a tetrazolium salt to an intensely coloured formazan product. MTT-based cytotoxicity assay (MTT Colorimetric tetrazolium assay), this assay is established by Mosman (1983) and subsequently, other tetrazolium-based assay (XTT, MTS) were developed. MTT is a yellow water-soluble tetrazolium dye that is reduced by the mitochondrial enzyme succinate-dehydrogenase into a purple formarzan product that is insoluble in aqueous solutions. The conversion takes place only in living cells and amount of formazan produced is proportional to the number of cells present (Danizot and Lang, 1986). The amount of formazan product can be determined spectrophotometrically once solubilized in a suitable solvent. The advantages of this assay are easy, rapid, cheap, and the use of multiple scanning spectrophotometers (ELISA readers) permits the processing of large number of sample (Gerlier and Thomasset, 1986). The disadvantages of this assay is difficult to solubilize the formazan product, often unreliable due to protein precipitation, need to avoid the background absorbance by the phenol red indicator in the medium (Danizot and Lang, 1986).

5. Apoptosis (programmed cell death)

Apoptosis is a complex, tightly regulated, and active cellular process whereby individual cells are triggered to undergo self-destruction in a manner that will neither injure neighboring cell nor elicit and inflammatory reaction (Kerr et al., 1994). Apoptosis often occurs during development and aging of animals. It is induced by cytotoxic lymphocytes (CTL), anticancer drugs, UV irradiation, a group of cytokines called death factors, and deprivation of survival factors (Nagata, 1997; Nagata and Golstein, 1995).

5.1 Morphology of apoptosis

The following morphologic features characterize cells undergoing apoptosis (Cotran et al., 1999).

5.1.1 Cell shrinkage

The cell is smaller in size, the cytoplasm is dense, and the organelles, although relatively normal, are more tightly packed.

5.1.2 Chromatin condensation

This is the most characteristic feature of apoptosis. The chromatin aggregates peripherally, under the nuclear membrane, into well-deliminated dense masses of various shapes and sizes. The nucleus itself may break up, producing two or more fragments.

5.1.3 Formation of cytoplasmic blebs and apoptotic bodies

The apoptotic cell first shows extensive surface blebbing, then undergoes fragmentation into a number of membrane-bound apoptotic bodies composed of cytoplasm and tightly packed organelles, with or without a nuclear fragment.

5.1.4 Phagocytosis of apoptotic cells or bodies by adjacent healthy cells, either parenchymal cells or macrophages

The apoptotic bodies are rapidly degraded within lysosomes, and the adjacent cells migrate or proliferate to replace the space occupied by the now deleted apoptotic cell.

Throughout apoptosis process, the cell membrane and the membrane encasing the apoptotic fragments retain their integrity (Kerr et al., 1972, 1994). Also the lysosomes remain intact and, hence, lysosomal enzymes are not released to the surrounfing tissues. Consequently, there is no associated inflammation in apoptosis (Kerr et al., 1972, 1994). Apoptotic cells are recognized and phagocytized by either macrophages or adjacent epithelial cells (Savill et al., 1989; Kerr et al., 1994). Due to this efficient mechanism for the removal of apoptotic cell *in vivo*, no inflammatory response is elicited. *In vitro*, the apoptotic cell as well as the remaining cell fragment ultimately swell and finally lyse. This terminal phase of *in vitro* cell death has been termed “secondary necrosis” (Fig 2) (Savill et al., 1989).

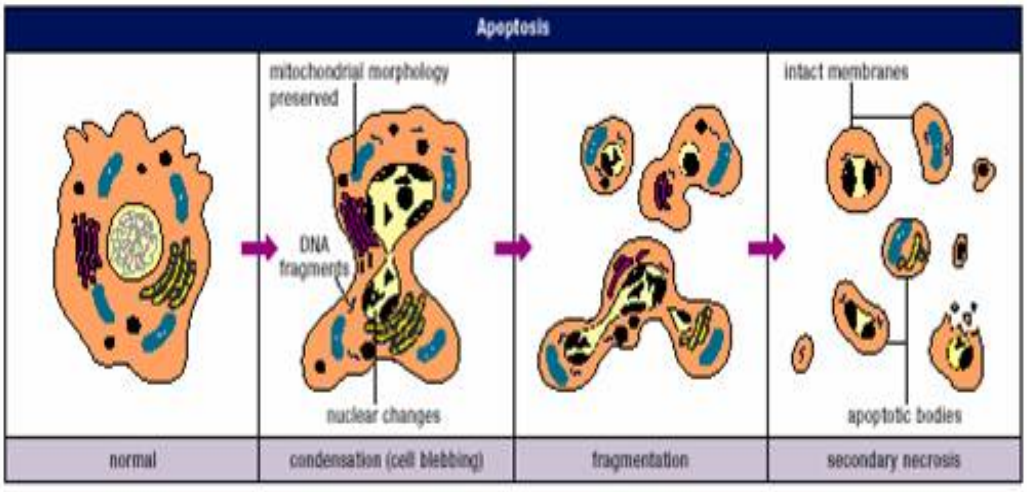


Figure 2 Illustration of the morphology feature of apoptosis (Roche Diagnostics Corporation, 2003).

6. Signal transduction for apoptosis

Inducers of apoptosis are categorized into three groups including death factors, genotoxic anticancer drugs, and factor deprivation. (Nagata, 2002).

Fas ligand, a representative of death factors, binds to Fas receptor, and causes its trimerization. The trimerized death domain in the Fas cytoplasmic region recruits pro-caspase 8 through a FADD/MORT1 adaptor, and forms a DISC. The pro-caspase 8 is autoactivated at DISC, and becomes a mature active enzyme. Two routes have been identified to activate pro-caspase 3 by cadpase 8. In one route, caspase 8 directly processes procaspase 3 in the down stream, and caspase 3 cleaves various cellular proteins including ICAD. CAD is released from ICAD, and degrades chromosomal DNA. In another route, caspase 8 cleaves Bid, a pro-apoptotic member of Bcl-2, which translocanslocates to mitochondria to release cytochrome C into the cytosol. Bcl-2 or Bcl-xL, anti-apoptotic members of Bcl-2 family, inhibits the release of cytochrome C, the mechanism of which is not well understood. The cytochrome C then activates caspase 9 together with Apaf-1, and caspase 9 in turn activates caspase 3.

The genotoxic anticancer drugs such as etoposide and X-radiation generate damage in chromosomal DNA. The signal seems to be transferred to mitochondria in a p53-dependent manner by as yet unidentify mechanism. This releases cytochrome C from mitochondrea, and activates caspase 9 as described above.

The apoptosis induced by factor-deprivation is best studied with IL-3-dependent myeloid cell lines. In the presence of IL-3 receptor causes phosphorylation of Bad, a pro-apoptotic member of the Bcl-2 family. The phosphorylated Bad is trapped by an

adaptor called 14-3-3 In the absence of IL-3, nonphosphorylated Bad is released from 14-3-3, and translocates to mitochondria to release cytoghrome C to activate caspase 9 (Fig 3) (Aggarwal et al., 2004).

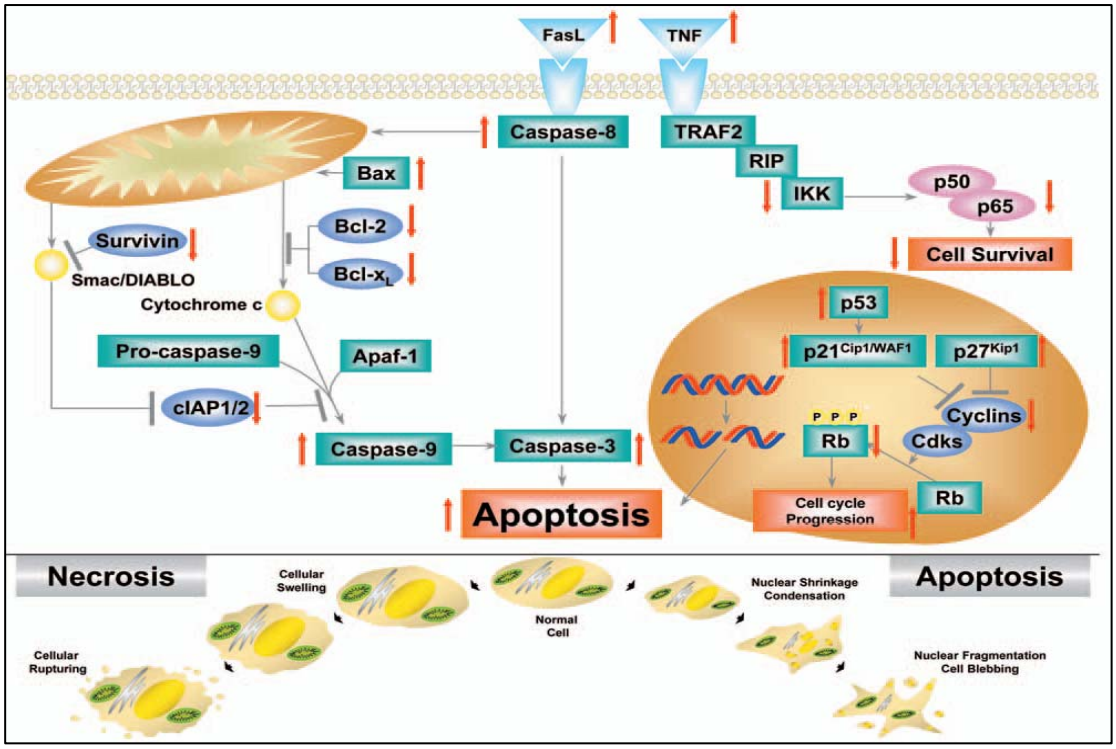


Figure 3 Schematic representation of signal transduction for apoptosis (Aggarwal et al., 2004).

7. Detection or quantitation of apoptosis

Apoptosis was initially characterized by morphological changes of dying cells. Phosphatidyl serine exposure in the outer leaflet of the cell membrane is the first evidence of morphological changes which are later seen as shrinkage, condensation of the nucleus and membrane blebbing. Cells undergoing apoptosis fragmented into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or other neighboring cells without generating an inflammatory response.

This is in contrast to necrosis, which results from gross insult to the cell, characterized by cell swelling, release of lysosomal enzymes, cellular disintegration and inflammation (Cotran et al., 1999). Apoptosis has now established its importance in numerous areas of biology particularly its major constitution in health and disease. This fact underlies the critical need for learning the most efficient and effective methods to study it. Although the morphology of death during development had been recognized as being different from necrosis for many years, characterization of this difference remained elusive. However, studies which detailed the cytological description of changes in cells undergoing "apoptosis" and it critical role in maintenance of tissue turnover, size, and shape in both normal and pathological processes (Kerr et al., 1972; Wyllie, 1980, 1985) have lead the way in establishing a need to further investigate this essential mode of cell death. It is now clear that apoptosis is a distinct mode of programmed cell death defined by a set of characteristic morphological and biochemical features. It is known to serve many critical functions, such as cell deletion during embryonic development, balancing cell numbers in continuously renewing tissues, hormone – dependent involution in the adult, immune system development, selective immune cell deletion, and many other physiologic processes. Furthermore, numerous pathologically induced conditions such as Alzheimers, autoimmune disease, cancer, and AIDS, often show varying levels of apoptosis, with greatest significance lying in whether dysregulation of apoptosis is a primary event in the pathology and subsequent clinical sequelae.

In this review, we will discuss the morphology of apoptosis and discriminate it from necrosis, discuss relevant biochemical events integral to the apoptotic process, and, finally, describe how to identify characteristic morphological and biochemical features in cells undergoing apoptosis using selective and specific techniques.

Base on these changes, various assays are designed to detect or quantitate apoptotic cells. Typical assay include annexin-V binding, caspase enzyme activity, TUNNEL (Terminal deoxynucleotidyl transferase-mediated dUTP nick-end-labeling), DAPI staining and DNA fragmentation gel electrophoresis.

7.1 Morphology: Apoptosis Versus Necrosis

In nearly all cell types apoptosis is a genetically controlled process in which scattered single cells in a tissue actively particpate in their own demise. The striking continuity of the morphological changes of apoptosis across cell types and species make its identification applicable to a variety of *in vitro* studies. Once the cell has committed to die, seemingly regardless of the mode of induction, the earliest observable ultrastructural event is condensation of chromatin to form uniformly dark, crescentic, and marginated masses adjacent to the nuclear envelope, and the nucleolar chromatin disperses forming osmiophilic aggregates in the center to the nucleus; also, the fibrillar core usually opposes the inner surface of the condensed nuclear chromatin during this time (Wyllie, 1985). Abrupt cell shrinkage, the earliest light microscopic feature, occurs almost simultaneous to nuclear changes and is evidenced as cells in culture lose contact with adjacent cells, during which time any specialized surface structures such as microvilli disappear and convolutions begin to form. The cell becomes smaller and more denses at this time. The light microscopic appearance of a shrunken cell with condensed chromatin masses appears intensely eosinophilic with a pyknotic nucleus. Pyknosis however, is a relatively early event in apoptosis as compared to necrosis, and the shape and outcome of structural changes in chromatin are distinctly different in these two types of cell death. Convolutions of nuclear and

plasma membranes occur progressively with chromatin condensation, giving the cell a bubbling or tremendously “blebbed” surface. This is best seen by scanning electron microscopy and is more common in cells with abundant cytoplasm. Observation of the intense surface blebs is restricted almost entirely to cells in culture and is rarely seen *in vivo* (Kerr et al., 1987) apparently because the duration of blebbing is too short to found in tissue sections.

Shrunken and convoluted cells now begin to pinch together at points where membrane valleys are close, sealing the broken portion behind itself. The nucleus often breaks apart to from discrete nuclear fragments enclosed within a double layered nuclear envelope, usually containing condensed chromatin which has segregated during blebbing. Since cytoplasmic blebbing is occurring simultaneously, these nuclear fragments are found within larger, oval, or round shaped cytoplasmic fragments called apoptotic bodies. It is important to note that throughout the apoptotic process, very few morphological alterations are observable in cytoplamic organelles (Kerr et al., 1972), and this is initially the case as they segregate within the apoptotic bodies. Although compaction of these organelles often accompanies cell shrinkage, and mild endoplamic reticulum dilatation and polysome disaggregation may occur in some cells, these are not necessarily hallmark features of all apoptotic cell death. As mentioned previously, apoptotic bodies are phagocytosed rapidly *in vivo,* before membrane alterations can occur. *In vitro,* however, apoptotic bodies which remain suspended in culture media undergo degenerative changes, such as organelle disruption and membrane rupture, usually within an hour this is referred to as secondary necrosis. Apoptotic bodies are initially intact structures with the ability to exclude vital dyes, but lose this ability after secondary necrosis has occurred

(Sheridan et al 1981; Wyllie, 1985). Once phagocytosed, they are degraded within lysosome and reduced eventually to unrecognizable, usually electron dense amorphous material. Interestingly, macrophages do not respond by releasing inflammatory mediators when they engulf and degrade apoptotic bodies (Meagher et al., 1992), and this likely contributes to the lack of inflammation seen in tissues containing foci of apoptosis. The fate of apoptotic cells at later stages can also be characterized by the presence or absence of lysosomal activity is (Clarke, 1990). If cell degradation is due to the action of its own lysosomes, then it is known as heterophagocytosis. When studying apoptosis *in vitro*, it is important to keep in mind that in many if not most cell types, onset of observable cellular changes to the formation of a cluster of apoptotic bodies usually occurs within minutes. However, some studies show that apoptotic structures can persist for up to an hour (Wyllie, 1992). Thus, histological quantification can be dis-interpreted if the timing parameters during experimentation are not clear.

Necrosis occurs only in response to a pathologic from of cellular injury and generally affects large groups of cells, characteristically causing an inflammatory response on the tissue level. ATP depletion is a critical precursor to the morphological changes occurring in the necrotic cell. A second and irreversible feature of the cell dying by necrosis is loss of plasma membrane integrity and function, following which organelle degeneration ensues. Unlike the shrinkage seen in apoptosis, the cell dying by necrosis rapidly swells (Table 1). Swelling is due in part, along with cytoskeletal damage, to the disappearance of ion pumping activity, which leads to loss of selective membrane permeability, changes which occur prior to morphologically identifiable necrosis (Wyllie, 1981). The nuclear chromatin also condenses, but as smaller-sized

flocculent densities in ill – defined, irregular pattern (Wyllie, 1981). Similar densities are seen in the matrix of the mitochondrial and organelle dilatation. Whereas the apoptotic nucleus fragments and segregates with apoptotic bodies, chromatin in the final stages of necrosis scatters into many loosely associated particles. Therefore, chromatin densities which may appear pyknotic at first, eventually disappear late in necrosis, as the nuclear membrane is no longer recognized

Table 1 Comparison the features of apoptosis and necrosis (Allen, William, Hunter, and Agrawal, 1997).

	Apoptosis	**Necrosis**
Occurrence	Scattered, single cells	Massive, tissue injury
Cytoplasm	Shrinkage: condensed and dehydrated; normal organelles; later fragments	Swelling: ER and mitochondria swell
Nucleus	Chromatin condenses into crescentic masses adjacent to nuclear envelope; later fragments	III defined, randomly dispersed, smaller chromatin masses; later lysed
Plasma and nuclear membrane	Intense convolutions (blebbing); apoptotic bodies containing: normal cytoplasmic organelles; nuclear chromatin	Membrane injury/lysis; leakage of intracellular contents

	Apoptosis	Necrosis
DNA breakdown	Internucleosomal; gene activation; endonuclease	Random degradation including histones; diffuse
Tissue response	No inflammation; phagocytosis of apoptotic bodies	Inflammation

7.2 Biochemical Features

Regardless of whether or not a cell requires RNA and protein synthesis for apoptosis to occur, it is without a doubt metabolically active during this time. This implies that once the program for apoptosis has been initiated, biochemical events occur, in a rather sequential order, to accomplish the cell's suicidal destiny. Because of the continuity in the morphological features of apoptosis, such as chromatin condensation, cell shrinkage, membrane blebbing and apoptotic body formation without the initial loss of membrane integrity, to these changes should occur in all cells, In the majority of cases this has been found to be true, with minor variations or exceptions occurring in a few cell types (Allen et al., 1997).

7.3 Analysis of the Apoptotic Cell

There are a number of methods that allow the researcher to accurately differentiate between apoptosis and necrosis. Assessments of membrane integrity with vital dyes, such as trypan blue dye exclusion and acridine orange/ethidium bromide fluorescent staining, are non-specific unless morphological characteristics can be

directly analyzed with the dyes. Hoechst fluorescent staining of nuclei can reveal such features of either intact or fragmented nuclei, as can the ultrasensitive DNA-binding dye YOYO-1. Other nonspecific methods for cell death detection are assays for either decreased ATP content or lactate dehydrogenase released into culture media. If accompanied by microscopy, then these are good measures of cell death; however if both necrosis and apoptosis are occurring in a system, then the above assays cannot discriminate between the two (Allen et al., 1997). It is especially important, therefore, to take advantage of those features in an apoptotic cell which are either visible or measurable. Examples of techniques used for detection of apoptosis, both in general and under specific circumstances, are CTL target cell killing, calcium flux measurements in cell death, or flow cytometric determinations of apoptosis (McGahon et al., 1995; Martin et al., 1996).

The methods described below take advantage of the most important morphological and biochemical changes occurring in apoptotic cells. The morphological criteria of apoptosis is emphasized under the technique of microscopy. Biochemical changes to be analyzed include phosphatidylserine exposure, and methods to identify three specific characteristics of DNA following its fragmentation. Apoptosis is a form of cell death initially based only on the morphology of cellular changes associated with it. Therefore, assessment of these features by microscopy should always accompany and biochemical method, regardless of its specificity. The general importance of each procedure for analysis of the apoptotic cells are discussed as following :

7.3.1 Microscopy

The morphological appearance of cells undergoing apoptosis is both distinct and specific. All experimentation, therefore, which qualifies and quantifies this mode of cell death should include microscopy. In this respect, time-lapse videomicroscopy has proved to be an extremely useful data-gathering tool; however, it can be rather expensive and difficult to maintain experimental conditions when they are required for extended periods. Although not detailed here, transmission electron microscopy (TEM) is the only definitive way to detail ultrastructural changes occurring in the apoptotic cell. Scanning electron microscopy (SEM) best illustrates intense surface blebbing and the newly formed clusters of apoptotic bodies. Since these methods are time consuming and labor-intensive, light microscopy along with other biochemical assays should be performed first, preferably followed by TEM (Allen et al., 1997).

Since a few cell types may not display all the hallmark features of apoptosis, emphasis should be placed on identifying a minimum number of characteristic changed that are unequivocal for this form of cell death. To discriminate further, the mode of cell death occurring should be specifically differentiated from necrosis. Striking apoptotic cellular changes observed by microscopy include cell shrinkage of up to 30%, crescent shaped masses of condensed chromatin adjacent to the nuclear envelope, membrane blebbing, and apoptotic bodies which are formed from cytoplasmic and nuclear fragmentation. The cytoplasmic and nuclear condensations (shrinkage) are more accurately assessed by video microscopy, although they are evident by cytospin procedures. Differential staining of the nucleus and cytoplasm should allow accurate assessment of these features to qualify, the occurrence of apoptosis (Ohyama et al., 1981).

Light microscopy is more definitive than all biochemical methods to differentiate the mode of cell death occurring in a system. It identifies features that generally occur after major biochemical events have taken place, such as phosphatidyl serine translocation and DNA fragmentation. Since the characteristic morphologies vary as to how long they will remain among different cell types, the time at which analysis of apoptosis takes place must be optimized (Allen et al., 1997).

7.4 DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride) staining assay

4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) is UV-excited, blue emitting fluorochrome (excitation 358 nm, emission 461 nm). DAPI is known to form fluorescent complexes with natural double stranded DNA, showing a fluorescence specificity for AT, AU and IC clusters. When DAPI bind to DNA, its fluorescence strongly enhanced which interpreted in term of a higly energetic and intercalative type of interaction. But there is also evidence that DAPI bind to the minor groove, stabilized by hydrogen bond between DAPI and acceptor group of AT, AU and IC base pairs. After staining of nuclei with DAPI, morphological of the cells nuclei such as chromatin condensation, nuclear shrinkage and formation of apoptotic bodies can be examined (Hotz et al., 1992; Gorman et al., 1994; Pawley, 1995).

7.5 DNA Fragmentation

For cells that fragment their DNA into oligonucleosome, DNA fragmentation analysis by agarose gel electrophoresis is considered a biochemical hallmark for the apoptotic mode of cell death. This assay is highly specific but not very sensitive, often requiring DNA from a large number of cells to analyze the fragmentation pattern.

Since in some cell types, apoptosis can occur in the absence of internucleosomal cleavage and in the presence of fragmentation of DNA about 300 and/or 50 Kbp, this method in not suitable for every cell type. If a DNA ladder is present, then death by apoptosis is almost a surety; however, if absent, then death by apoptosis cannot be ruled out since larger fragments have not been tested for. To do this, one can use pulse-field gel electrophoresis techniques, such as field-inversion gel electrophoresis, which can accurately evaluate 300- or 50-kbp fragments (Walker et al., 1991; Martin et at., 1996; Brown et al., 1993). Therefore, at least one other biochemical assay, such as TUNEL or annexin-V labeling, should be conducted with the morphological assessment by microscopy.

If a characteristic ladder pattern cannot be seen, cleavage of DNA into large fragments might be occurring and caused the exclusive of internucleosomal cleavage. Then, adjustments may need to be made to the DNA extraction procedure. Alternatively, there is always a possibility that exogenous nucleases are degrading the DNA if all equipment is not entirely sterile. In addition, DNA can be lost in the interphase during extraction. Therefore, one needs to be extremely careful when removing the upper aqueous phase (DNA) during extraction. Another way is to use more cells during the extraction procedure which should increase the extracted DNA content (Allen et al., 1997).

7.6 Annexin V and Propidium Iodide

Annexin V, an anticoagulant with other biological effects, is a member of a family of proteins which exhibits Ca^{2+}dependent binding to negatively charged phospholipids, and it binds with highest affinity to phosphatidylserine (PS) (Andree

et al., 1990). Since PS translocation occurs early in apoptosis when cell membrane integrity is still intact, flow cytometric analysis using fluorescene in isothiocyanate-(FITC) labeled annexin V is useful as a quantitative measure of early apoptosis (Homburg et al., 1995; koopmen et al., 1994).

Flow cytometric determination of programmed cell death has also been used with the DNA intercalating dye propidium iodide (PI). When cells are permeabilized, treated with PI, and analyzed by the cell-cycle method for DNA content, a sub-diploid (sub-G_0 /G_1) peak can be seen since the DNA in apoptotic cells stains particularly less intensely. Annexin V binding has been shown to reveal a much higher level of cells committed to apoptosis than PI staining, and this emphasizes the fact that PS externalization is both more sensitive than this method and that it occurs prior to any nuclear changes (Martin et al., 1995). Unlike the cell throughout most of apoptosis which retains plasma membrane integrity, the necrotic cell loses plasma membrane integrity. A benefit of annexin V and PI assay if that cells do not need to be fixed or permeabilized, thus PI staining enters necrotic cells but is excluded from apoptotic cells. Therefore, it can discriminate between necrosis and phases of apoptosis prior to secondary necrosis, one can test propidium iodide dye exclusion simultaneously with FTTC-labeled annexin V binding (Vermes et al., 1995). When annexin V binding is simultaneously occurring with PI uptake, necrosis may be the prevalent form of cell death unless cells in suspension are being collected and flow cytometry gates are set up too inclusively (too broad). Morphological analysis should resolve this issue. This assay can also be useful for measuring the kinetics of apoptosis (Martin et al., 1995).

7.7 Apoptosis as therapeutic target of diseases

The current emphasis of apoptosis in disease has lead many researchers to look for the mechanisms mediating its effect. Cellular signaling pathways, in response to internal or external apoptotic inducers, are being sought with the intentions of understanding which proteins or enzymes play central roles in modulating the apoptotic program. For example, The role of stress-induced mitogen-activated protein kinases (MAP kinases), such as JNK and p38, have recently been found to play central roles in nerve cell apoptosis upon nerve growth factor withdrawal (Xia et al., 1995). Our understanding of the genetics of apoptosis has grown immensely over the past few years. At present, the available methods allow the researcher to specifically qualify and quantify apoptosis in a variety of settings. Enzyme inhibitors or dominant-interfering mutants are used to elucidate apoptotic mechanisms making more accurate assessments of the mode and extent of cell death (Xia et al., 1995). The ultimate identification of genetic regulators of programmed cell death, combined with the knowledge of where apoptosis is implicated in the pathogenesis of certain diseases, should provide exciting and novel therapeutic targets aimed at ameliorating specific diseases or limiting their progression.

8. Cell cycle

The cell cycle of mammalian cells has several checkpoints to ensure fidelity in the replication of the DNA and progression through the cell cycle. The aberration in the control of the cell cycle is central to the process of carcinogenesis (Fig 4 and 5). The cell cycle is a controlled sequence of events from one division of eukaryotic cell to the next. In eukaryotic cells, the cell cycle can be broken down to four phases, the first growth

phase (G_1), the S-phase where the DNA is replicated, a second growth phase (G_2), and finally the M-phase, where the cell actually divides into two daughter cells. Non-dividing cells are said to be in a resting phase, termed G_0 (Evan and Vousden, 2001).

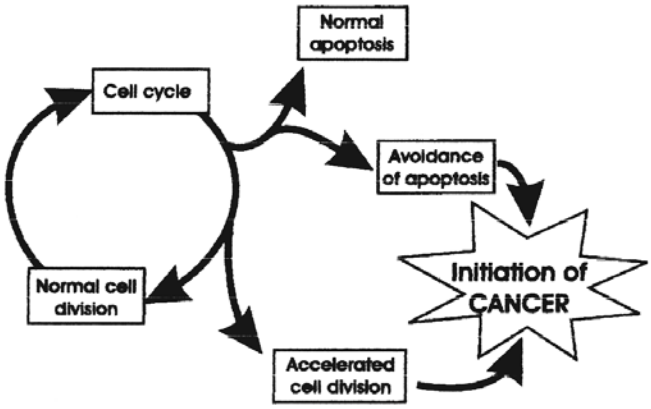


Figure 4 Simplified diagram denoting the interrelationship between abnormalities in the cell cycle and initiation of cancer. The earliest beginning of cancer most likely involes perturbation in the normal cell cycle (Evan and Vousden, 2001).

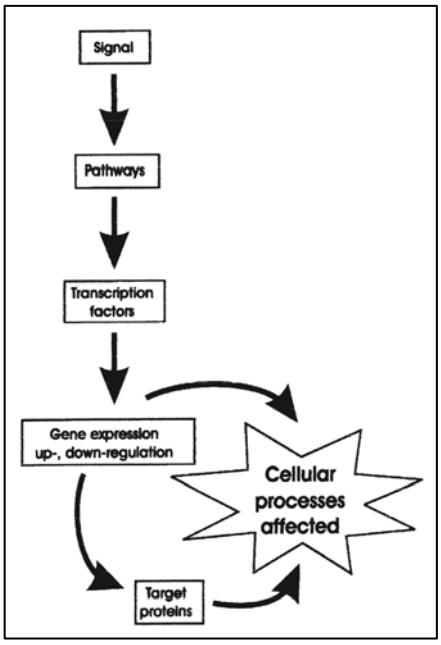


Figure 5 Flow diagram of signaling pathway of up or down regulate expression of specific genes (Evan and Vousden, 2001).

8.1 G_1 Phase During the first phase (G_1) cells grow in size and are receptive to extracellular signals, such as soluble growth factors and intracellular contact, which may trigger a commitment to entering the next phase of the cell cycle. During G_1 protein kinases, enzymes which phosphorylate other proteins, become active and thereby send a signal that make the cell division process begins. The cyclin dependent kinases (CDKs) are a group of such protein kinases whose activity is central to this process. The CDK are so named because their activity requires binding to a protein partner, a cyclin, whose levels of expression and activity varies depending on the phase of the cell cycle (Matthew, 1998).

8.2 S-Phase Soon after the restriction point, a cell begins to replicate its genetic material through the activity of a family of enzymes including the DNA polymerases, which are capable of synthesizing an exact replica of the DNA genome. In a mere 6-hours, the cell polymerizes all six billion nucleotides, the building blocks of DNA, into 48 separate strands, or chromosomes, while maintaining the same arrangement of nucleotides as the parental DNA and thus preserving the genetic information. This *"S-phase"* of the cell cycle is thus biochemically distinct from the other phases of the cell cycle and can be distinguished from other cells by their ability to stably incorporate fluorescently tagged nucleotides, the building blocks of DNA, into their nucleus. To activate the replication machinery cells depend on the protein kinase activity cyclin A coupled to CDK2 (Matthew, 1998).

. **8.3 G2 Phase** At the completion of S-phase, DNA replication ceases and cells enter the G2 phase of the cell cycle. During this phase CDK1 replaces CDK2 as the

predominant cyclin and it couples with either Cyclin A or one of the B-type cyclins to catalyze the phosphorylation of proteins specific to the G2 phase of the cell cycle.

8.4 Mitosis Mitosis is the phase of the cell cycle in which cells physically divide into two separate daughter cells. In order to do so they first dissolve the nuclear membrane which will later reform once cell division is complete. The DNA-containing chromosomes then condense into structures so compact that they can be seen with a simple light microscope. The chromosomes then precisely segregate to two sides of the cell, such that each half of the cell gets exactly one copy of each chromosome. At the completion of mitosis, cells undergo cytokinesis or separation into two halves. This occurs as a band forms around the circumference outer plasma membrane which gradual constricts like a belt until the cell pinches in two. The B-type cyclins remain active throughout M-phase, but their activity immediately ceases once cell division is complete and the two daughter cells once again enter G_1 ((Matthew, 1998).

Multicellular organisms, such as humans, contain a vast number of cells of many different types and functions. Some of these cells, e.g. those lining the stomach and intestine, are continuously growing and dividing throughout our lifetime. Another group of cells which is continuously growing and dividing are the cells of the bone marrow. Through the process of hematopoiesis, these cells grow and then differentiate into the separate types of cells which circulate in our blood stream: The red blood cells, which carry oxygen, the white blood cells which serve a defense against disease, and the platelets which initiate blood clotting. Other cells, like those in the liver divide only rarely but can increase their rate of proliferation in order to regenerate a partially

damaged organ. Still others, such as the neurons of the brain cannot grow and divide at all, except in the developing embryo (Matthew, 1998).

Cell-cycle dysregulation is a hallmark of tumor cell (Stewart et al., 2003). The ability of normal cells to undergo cell – cycle arrest after damage to DNA is crucial for the maintenance of genomic integrity. The biochemical pathways that stop the cell cycle in response to cellular stressors are called checkpoints. Defective checkpoint function results in genetic modifications that contribute to tumorgenesis. The regulation of checkpoint signaling also has important clinical implications because the abrogation of checkpoint function can alter the sensitivity of tumor cells to chemotherapeutics (Matthew, 1998).

The three major checkpoints are at the G_1 to S, S to G_2 and the G_2 to M transitions. Malfunctioning cell cycle checkpoints may lead to an accumulation of mutations and genomic instability, which in turn may lead to development of cancer. For examples, the majority of effect of a single resveratrol concentration at a single treatment time reported an accumulation in the S phase of the cell cycle in a dose dependent manner and at higher concentrations in the G_1 or G_2/M phase. The S phase accumulation may be correlated to some interference with the DNA polymerases and/or the ribonucleotide reductase. Futhermore, the effect of resveratrol on cell cycle arrest appears to depend on incubation time as well as on cell type as summarized in Table 2.

Table 2 Resveratrol stimulates cell cycle arrest in many different cell lines (Allen et al., 1997).

Model	Phase accumulation	Dose range	Treatment Time	Reference
MCF7				
	S	50μM	36 h	Pozo-Guisado et.al. (2002)
	G_0/G_1	100 and 150 μM	36 h	
SW480				
	S	30,50,100 μM	24 and 48 h	Delmas et al. (2002)
	S	30 μM	72 h	
	G_2	50,100 μM	72 h	
Caco2				
	S	25 μM	16/24 h	Schneider et.al. (2002)
	G_2	25 μM	40 h	
	none	25 μM	48 h	
	S	25 μM	64 h	

CHAPTER III

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Plant

Zinfandel red grape (*Vitis vinifera*) (Fig 6) was grown on Suranaree University of Technology (SUT) Farm, Nakhon Ratchasima province. It was harvested from SUT farm in March 2003. The plant was confirmed by Prof. Dr. Nantakorn Boonkerd, at Suranaree University of Technology.

Figure 6 Zinfandel red grapes (*Vitis vinifera*).

3.1.2 Animals

Male Institute cancer research (ICR) mice 6-8 week olds, were obtained from National Laboratory Animal Center, Salaya, Nakhon Pathom, Thailand and from Institutional Animal Care Building at Suranaree University of Technology, Nakhon Ratchasima. Mice were quarantined for at least one week prior to the experiment.

Mice were housed in a temperature-controlled environment at 24-25°C and 40-70% relative humidity, 12-hours light-dark cycle. Food and tap water were given *ad libitum* during all experiments. Procedures involving animals and their care were conducted in conformity with each institutional guideline and were in compliance with the animal protocol approved by each institutional animal care and usage committee.

3.1.3 Human cancer cell lines

Two selected cancer cell lines: SNU 1079 human cholangiocarcinoma and Panc 2.03 human pancreatic adenocarcinoma were used in cytotoxicity and apoptotic studies.

SNU 1079 (Seoul National University 1079) was developed from intrahepatic bile ducts of a Koren cancer patients since 1982.

Panc 2.03 was kindly provided by Dr. E.M. Jaffee from The Sol Goldman Pancreatic Cancer Research Center, Johns Hopkins University (Baltimore, MD)

The two cell lines were cultured in RPMI 1640. media (Gibco, Grand Island, NY) supplemented with 10% heated-inactivated fetal bovine serum (FBS), 100 Unit/ml penicillin, 100 µg/ml streptomycin. All cell lines were maintained at 37°C with 5% CO_2 in humidified air and subculture weekly. In the apoptosis experiment low serum media (RPMI 1640 containing 1% FBS with similar supplements was used).

3.1.4 Chemicals and instruments

All chemicals and reagents were at least analytical grade. The chemicals, instruments, antibodies, media and supplements employed in the present studies were listed in Tables 3, 4, 5 and 6, respectively.

Table 3 List of chemicals used in the studies.

Name	Source
Absolute Ethyl alcohol	Merck, Darmstadt, Germany
Acrylamide	Sigma, Chemical Company, St. Louis, MO
Agarose	GIBCO BRL,Paisley, Scotland
Acetic acid	Merck, Darmstadt, Germany
Acetonitrile	Sigma, Chemical Company, St. Louis, MO
Annexin V-FITC apoptosis detection kit II	BD Biosciences, BD Pharmingen™, USA
Ammonium persulfate	Merck, Darmstadt, Germany
Boric acid (H_3BO)	Merck, Darmstadt, Germany
Bromphenol blue	Sigma, Chemical Company, St. Louis, MO
Cell cycle reagent	GUAVA Technologies, Inc., USA
Chloroform	Merck, Darmstadt, Germany
Coomassie brilliant blue	Sigma, Chemical Company, St. Louis, MO

Table 3 (continued)

Name	Source
4,6-Diamidino-2'-phenylindole dihydrochloride	Roche, Germany.
disodium hydrogen phosphate	Sigma, Chemical Company, St. Louis, MO
Dimethyl sulfoxide	Sigma, Chemical Company, St. Louis, MO
DTT	Sigma, Chemical Company, St. Louis, MO
ECL reagent	Amersham Biosciences, UK
Ethidium bromide	Sigma, Chemical Company, St. Louis, MO
Ethylene diamine tetrachloroacetic acid	Merck, Darmstadt, Germany
Folin Ciocalteu's reagent	Sigma, Chemical Company, St. Louis, MO
Gallic acid	Sigma, Chemical Company, St. Louis, MO
Glacial acetic acid	GIBCO BRL,Paisley, Scotland
Glycine	GIBCO BRL,Paisley, Scotland
Glycerol	GIBCO BRL,Paisley, Scotland
HEPES(*N-2-hyddroxyethylpiperazine-N-2-ethanesulfonic acid*)	Sigma, Chemical Company, St. Louis, MO

Table 3 (continued)

Name	Source
Hybond ECL Nitrocellulose membrane	Amersham Pharmacia, UK
Hydrochloric acid	Merck, Darmstadt, Germany
Hyperfilm ECL	Amersham Biosciences, UK
Isopentylalcohol, 3-Methyl-1-butanol	Sigma, Chemical Company, St. Louis, MO
Lead acetate	GIBCO BRL,Paisley, Scotland
Methanol (HPLC grade)	Merck, Darmstadt, Germany
2-Mecaptoethanol	Sigma, Chemical Company, St. Louis, MO
MTS –based Cell Titer 96 cell proliferation assay	New-jersey Center for Biomaterial, Piscataway, NJ, USA
Non-idet P 40	Bio active Co.ltd., Bangkok
Phosphate buffered saline	GIBCO BRL,Paisley, Scotland
0.45 μm polyethersulfone membrane	Supor@Acrodisc®, Millipore, Watford, Hertfordshire
Propidium Iodide	Sigma, Chemical Company, St. Louis, MO
Proteinase K	GIBCO BRL,Paisley, Scotland
RNase A	GIBCO BRL,Paisley, Scotland
Sodium acetate	Aldrich , Gillingham, Dorset

Table 3 (continued)

Name	Source
Sodium Azide	Sigma, Chemical Company, St. Louis, MO
Sodium Bicarbonate	Wako company, Japan
Sodium carbonate	Sigma, Chemical Company, St. Louis, MO
Sodium chloride	Sigma, Chemical Company, St. Louis, MO
Sodium dodecyl sulfate	Sigma, Chemical Company, St. Louis, MO
Trisma base	Sigma, Chemical Company, St. Louis, MO
Sodium hydroxide	Famitalia Carlo Erba
Sodium tetraborate	Agilent Technologies Deutschland GmbH, Waldbronn, Germany
Triton X-100	Sigma, Chemical Company, St. Louis, MO
Trypan blue dye	GIBCO BRL,Paisley, Scotland
Tween-20	Sigma, Chemical Company, St. Louis, MO
Typsin	GIBCO BRL,Paisley, Scotland

Table 3 (continued)

Name	Source
Uranyl acetate	Sigma, Chemical Company, St. Louis, MO

Table 4 List of equipments used in the studies.

Name	Source
Becton Dickinson FACalibur	Becton Dickinson FACalibur, USA
Blender	Molinex Co., Germany
Capillary electrophoresis Agilent Technologies model G1600AX	Agilent Technologies Deutschland GmbH, Waldbronn, Germany
Centrifuge machine	Beckman, Palo Alto,CA
CO_2 incubator	BellcoGlass. Inc., USA
ELISA plate reader	BIORAD laboratories, Hecules, CA, USA
FACScalibur cell analyzer	Becton Dickinson FACSCalibur™ Flowcytometry system, NJ, USA.
Fluorescent microscope	Nikon Kogaku K.K Co., Japan
Haemocytometer and cover slip	Fisher Scientific, UK
Inverted microscope	Olympus optical Co., Japan
Laminar flow hood	Holten, Scientific Promotion Co., USA.

Table 4 (continued)

Name	Source
Mini Trans-Blot® Electrophoretic Transfer Cell	BIORAD laboratories, Hecules, CA, USA
Rotary evaporator with vacuum &water bath	Bosch, Co.,USA.
Soxhlet extraction apparatus	Pyrex Co., USA.
Transmission electron microscope	Philips CM10, Japan
Ultramicrotome	Rrichert ultracut S, Australia
Wallac Model 1420 Multilabel Counter	Wallac, Michigan, USA
WEALTEC Dolphin-DOC ultraviolet analyzer	WEALTEC, Corp., Sparks, Nevada, USA..

Table 5 List of Medias and supplements used in this studies.

Name	Source
Fetal bovine serum (FBS)	GIBCO BRL, Paisley, Scotland
Fungizone	GIBCO BRL, Paisley, Scotland
Penicillin-Streptomycin solution	GIBCO BRL, Paisley, Scotland
Roswell Park Memorial Institute 1640	GIBCO BRL, Paisley, Scotland

Table 6 List of antibodies used in this studies.

Name	Source
β-actin (mabcam 8226)	Sigma, Chemical Company, St. Louis, MO
Monoclonal Bcl-2 antibody (sc-7382)	Santa Cruzt Biotechnology, Inc., CA
Monoclonal Caspase-3 antibody (sc-7272)	Santa Cruzt Biotechnology, Inc., CA
Goat-anti-mouse-HRP conjugate (sc-2005)	Santa Cruzt Biotechnology, Inc., CA

3.2 METHODS

3.2.1 Preparation of Zinfandel red grape extracts

3.2.1.1 Collection of Zinfandel red grape

Zinfandel red grapes were harvested from SUT farm in March 2003, and were then washed and air dried at room temperature. Grape was 20°Brix of sugar content and have a pH of 4.2. Selected grapes were used to prepare juice and wine for further studies.

3.2.1.2 Preparation of grape juice and red wine

The preparation of grape juice and red wine were performed at SUT farm. Selected Zinfandel red grapes were crushed with an electric microniser and grape juice was separated from pomace residues (peel, skin and seed). Fresh grape juice was immediately filtered through a cotton wool for two times, collected into bottle, and the pH, sugar, and total phenolic content were determined prior kept at -20°C until use.

Zinfandel red wine was obtained from SUT farm and was used directly from the bottle. Total phenolic content and alcohol percentage were determined in red wine prior storage at 4°C until use.

3.2.1.3 Preparation of ethanolic ethanolic grape pomace extract extract

Ethanolic ethanolic grape pomace extract extract composed of grape peel, skin and seed which was separated from grape juice after it was crushed with electronic microniser. Pomace was homogenized and ground into homogenate residues using maximum speed of an electric blender. Blended pomace was put into a sterile pyrex and macerated overnight with 80% v/v ethanol. The extract was centrifuged at 450 × g for 15 minutes. Supernatant was collected and the pellet residues were remacerated 2-3 times with the same protocol until the supernatant had a clear color. When the extraction was completed, residues were removed by filtering through a cotton wool. The filtrates were pooled, collected and transferred to a flask. The pooled ethanol extract was concentrated under vacuum at 40°C to a final volume of 250 ml using a rotary evaporator and kept in the dark under nitrogen. The frozen extract was lyophilized in a lyophilizer at -50°C for two days. The crude pomace extract was kept at -80°C freezer until further use.

3.2.2 Determination of total phenolic compound content (TPC)

Total phenolic compounds (TPC) of red wine, grape juice and pomace were determined by a modified Folin Ciocalteu's method (Swain and Hills, 1959; Matthaus, 2002). Briefly, 100 microliter (µl) of grape juice or red wine was added into 2

milliliter (ml) of sodium carbonate, and incubated for 2 minutes. Then 100 µl of Folin-Ciocalteu's phenol reagent diluted freshly with methanol at 1:1 ratio was added into the reaction mixture. After a further 30 minutes incubation the absorbance was measured at 750 nanometer (nm) by a spectrophotometer using pure gallic acid as a standard. Samples were run in triplicates and results were expressed as as milligrams (mg) of gallic acid equivalents (GAE) per liter.

3.2.3 Absorption of total phenolic compounds *in vivo*

3.2.3.1 Reagents

1) Vehicle

Dimethyl Sulfoxide (DMSO) was used as 10% solution in sterile DW for preparing the various concentrations of the ethanolic grape pomace extract extract.

2) Test articles

Grape juice was directly used from bottle. Ethanolic grape pomace extract was dissolved in 10% DMSO solution in sterile DW.

3.2.3.2 Plasma collection

Six to eight weeks of ICR mice (25-27 gram body weight) were obtained from National Laboratory Animal Center, Salaya, Nakhon Pathom. Mice were housed at the animal facility of Suranaree University of Technology at least 1-2 week prior use. Pprocedures involving animals and their care were conducted as described in 3.1.2. Animals were weighed, randomized and were assigned into one control and three treatment groups. Food and tap water were removed from cages 4

hours before administration. Twenty four mice in each dose group were oral gavaged with 0.5 ml of vehicle, grape juice or ethanolic ethanolic grape pomace extract extract. All mice were administered once and then each group was sacrificed at each specific time point. Before treatment, blood samples were taken from each dose group by cardiac puncture. After administration, further blood samples were collected in vacutte container EDTA-K3 tube as anticoagulant. The time points for blood collection were at 0, 15, 30, 60 minutes and at a period of 3, 6, 12, and 24 h post treatment. Three mice (n=3) of each dose group were sacrificed for each time point. Blood plasma was immediately taken by centrifugation at 1500 × g for 20 min at 4°C, then carefully transferred blood plasma to a clean tube, wrapped in foil, and held at 4°C until analysis in the following morning, or held at -20°C until use. The TPC contents of plasma at each time point were analyzed by Folin- Ciocalteu's reagent as described in 3.2.2.

3.2.4 Absorption of trans-resveratrol *in vivo*

3.2.4.1 Reagents

1) Vehicle

12% v/v ethanol (HPLC grade) in sterile DW was used as solution for preparing various concentrations of trans-resveratrol.

2) Test articles

Trans-resveratrol was diluted in 12% v/v ethanol.

Trans-resveratrol spiked wine (RSW) was prepared by spiking trans-resveratrol in 12% v/v of ethanol in 100 ml of red wine.

3.2.4.2 Sample preparation

For *in vivo* study absorption of trans-resveratrol, ICR mice were obtained from National Laboratory Animal Center, Salaya, Nakhon Pathom and were housed at the animal facility of Suranaree University of Technology. Mice were taken care as described in 3.1.2. Similar protocol of absorption study of TPC as described in 3.2.3.2. Briefly, seventy two animals were divided into three dose groups. Mice were oral administered with 0.5 ml of trans-resveratrol, red wine spiked with trans-resveratrol, or 12% v/v ethanol to obtain 20 mg/kg of trans-resveratrol, 20 mg/kg of trans-resveratrol spiked red wine, and the vehicle control, respectively. All animals were administrated once and blood samples were collected. Blood samples were taken by cardiac puncture during a period to 0, 15, 30, 60 minutes and at a period of 3, 6, 12 and 24 h post treatment. Then blood plasma was taken by centrifugation at 1500 × g for 20 minutes at 4°C and held at 4°C or -20°C in freezer until use. The analysis of trans-resveratrol level in mice plasma was analyzed solely by Capillary Electrophoresis (CE) (Agilent Technologies Deutschland GmbH, Waldbronn, Germany) with a diode-array detector in Fig 7.

3.2.4.3 Capillary electrophoresis procedure

1) Instrumentations

Capillary electrophoresis analysis was carried out with a diode-array detector using an Agilent Technologies model G1600AX (Agilent Technologies Deutschland GmbH, Waldbronn, Germany). The electropherogram was recorded and integrated by Hewlet packard personal computer. An uncoated fused silica capillary tube of 50 μm internal diameter (i.d.) was obtained from Agilent Technologies with

the effective used and had a total lengths of 50 and 75 cm, respectively. The electrophoretic analyses were obtained using at an applied voltage of 15 kV at a constant temperature of 25°C. Injection was performed hydrodynamically at 5 s.

Figure 7 Capillary electrophoresis (CE) (Agilent Technologies Deutschland GmbH, Waldbronn, Germany).

2) Buffer preparation

Stock solution of 40 mM sodium tetraborate was prepared by diluting accurate amount of 100 mM sodium tetraborate in deionized water. The Electrophoretic buffer was a mixture of 40 mM sodium tetraborate, 20% acetronitrile at pH 9.32. The buffer was obtained from Agilent Technologies Deutschland GmbH (Waldbronn, Germany) and adjusted to the desired pH by using 2 N sodium hydroxide (NaOH).

3) Plasma Preparation

Plasma samples were prepared by diluting 1 to 10 with 20 mM sodium tetraborate pH 9.32 and filtered through 0.45μm polyethersulfon membrane filter (Supor@Acrodisc®). 2-100 mg/L Trans-resveratrol were spiked into plasma samples to prepare for a calibration curve.

4) General procedure of capillary electrophoresis

All new capillaries were initially conditioned by 0.5 M NaOH for 10 min, deionized water for 3 min, followed by 40 mM sodium tetraborate for 10 min. Before performing each experiment, the column was pre-rinsed with 0.1 M NaOH for 1 min, deionized water for 2 min and then equilibrated with running buffer for 3 min. Sample was hydrodynamically to 0.5 psi and injected for 5 s. Between analysis, the column was flushed with 0.1 M NaOH for 1 min, deionized water for 2 min and running buffer for 3 min. All CE analyses were performed in term of reproducibility and repeatability of concentration and peak area. The repeatability (intra-day) was achieved by replicating injection of standard derivative mixtures in a day (n=8) for trans-resveratrol spiked plasma and for plasma samples (n=3). The recovery was studied by spiking known concentration of standard mixtures into samples before derivatisation and was then analysed in three replicates. The recoveries of samples were established on separated run. All solutions and samples were filtered through 0.2-0.45 μm polyethersulfon filter membrane prior use.

3.2.4.4 Capillary electrophoresis analysis

Calibration curve of trans-resveratrol spiked plasma was

established using concentrations ranging from 2-10 mg/L plotted against peak areas. Each sample concentration was calculated by program chemstation Rev.A.09.01 (1206) (Agilent Technologies 1990-2001). Six replicate samples were used for analysis in calibration curve of each standard compound.

3.2.5 Effects of grape products and *trans-resveratrol* on ultrastructural changes of liver tissues

3.2.5.1 Reagents

1) Vehicles

DMSO was used as 10% Dimethyl Sulfoxide (DMSO) solution in sterile DW for preparing the various concentrations of ethanolic grape pomace extract extract.

12% v/v ethanol in sterile DW was used as solution for preparing the various concentrations of trans-resveratrol.

2) Test articles

Grape juice was used directly from bottle.

Ethanolic grape pomace extract was dissolved in 10% DMSO solution in sterile DW.

Trans-resveratrol was used to make 20 mg/kg oral administration within 12% v/v ethanol.

Red wine was obtained from Suranaree University of Technology farm. It is 12% ethanol and was used directly from bottle.

3.2.5.2 Sample preparation

For all *in vivo* studies, male mice were daily exposed in the morning through oral gavage using 18 guage feeding tube for six months. All animals were manually restrained and dosed with 0.5 ml per mouse of grape products; juice, pomace, red wine, or commercial trans-resveratrol, and their respective vehicle controls (12% v/v ethanol, 10% DMSO, water). The animals were administered every morning.

Before administration, animals were weighed and assigned into homogenous weight groups. Mice were given with 0.5 ml per 25 g mouse via oral gavage and oral administration was daily adjusted to the actual weight of each mouse. Every two months liver tissues were taken in order to study of ultrastructural examination under the standard techniques for Transmission electron microscope (TEM) (Bozzola and Russell, 1999). After 24 hours of last exposure, animals were sacrificed and were then immediately fixed by vascular perfusion with karnovsky's fixative. Liver was collected and dissected into 1x1 mm and fixed in fixative and stored overnight at 4°C. Then liver specimens were washed for 5 min three times with phosphate buffered saline (PBS), stained with osmium tetroxide for 2 hours, then repeated with washing buffer for 5 min three times. After washing, specimens were dehydrated by acetone at different series of ascending concentrations of 50, 70, 80, 85, 95 for 5 min and 100% v/v acetone in sterile water for 5 min twice. Then specimens were infiltrated with plastic: acetone 1:1 for 2 hours and then pure plastic was added overnight. Before embedding step, placed liver specimens on filter paper to dry, embedded in araldite 502 and then placed in oven at 70°C. After embedding media, specimens were cured for 3 days. Specimens were then trimmed and sectioned by

ultramicrotome. Sections that had been cut were picked up using a specimen grid made of copper and dried in dust-free environment. The grids were stained by dipping in uranyl acetate and lead acetate for 15 min in the dark. Dipped grids were quickly rinse with deionised water 10 times and repeated three times. Then specimen grids were placed on filter paper to dry. Ultrastructural examinations were observed and autoradiographed under TEM (Philips CM10) (Fig 8).

Figure 8 Transmission electronmicroscope (TEM) (Philips CM10).

3.2.6 *In vitro* cytotoxicity studies

MTS cell proliferation assay (Biomaterial, Piscataway, NJ, USA) was used to evaluate cytotoxicity of ethanolic grape pomace extract and trans-resveratrol to specific type of cancer cell lines.

3.2.6.1 Maintenance of cell lines

The normal human fibroblast, SNU 1079 and Panc 2.03 cell lines were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 Unit/ml penicillin and 100 µg/ml streptomycin. All cell lines were maintained at 37°C in 5% CO_2 humidified incubator and were subcultured weekly. They were used up to 25-30 passages

For preparatrion of single cell suspension, the cell monolayer was washed once with PBS pH 7.2 and trypsinized with 2 ml of 0.25% trypsin / 0.03 % EDTA (GIBCO BRL, Paisley, Scotland). After 5 min incubation at 37°C, 10 ml of RPMI 1640 complete medium was added. The cell suspension was centrifuged at 200 × g for 5 min and the cell pellet was reconstituted in 2 ml RPMI 1640 complete medium. A number of cells were determined using trypan blue dye exclusion test. The percent viable cells were calculated using the formula below. Cells in complete media were reconstituted in complete medium containing 20% DMSO at a ratio 1:1 at a concentration of $1x10^6$ cells/ml, and 1.8 ml of cell mixture was added to 2 ml. cryotube. The vials were placed at -20°C freezer for 60 minutes, and transferred to -80°C freezer until used.

$$\% \text{ viable cells} = \frac{\text{number of viable cells}}{\text{Total cell}} \times 100$$

3.2.6.2 *In vitro* Cytotoxicity test

The MTS assay was used in this study to indirectly determine cytotoxic effects of ethanolic grape pomace extract and trans-resveratrol on normal human fibroblast, SNU 1079 and Panc 2.03 cells. Cells at the exponential growth

phase were detached with 0.25% trypsin-EDTA (Sigma) to make single cell suspension. The number of cells was determined by trypan blue dye exclusion test using a haemocytometer (Freshney, 1987). Cells were diluted with RPMI 1640 complete medium to give final concentration of $1x10^4$ cells/ml and seeded in a 96-well microtiter plates in the volume of 200 µl/well. The cultures were incubated at 37°C in a 5% CO_2 humidified incubator. Twenty four hours later, media was removed, 200 µl of RPMI 1640 containing 0.5% FBS was added to each well and incubated for further 24 hours. After incubation, media was removed, 100 µl of trans-resveratrol or ethanolic grape pomace extract was added to each well in triplicates to obtain the final concentrations of 2.5, 5, 10 or 20 µg/ml of trans-resveratrol or 50, 100, 200 or 400 µg/ml of ethanolic grape pomace extract. Cells treated with final concentration of 0.1% DMSO in complete RPMI 1640 media containing 1 % FBS were used as a vehicle control. After 48 hour incubation, 20 µl of 3-(4,5-dimethyldiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS reagent) was added to each well. Cultures were incubated in the dark at 37°C in a 5% CO_2 incubator for 1 hour. The absorbance (OD) of each well was measured at 490 nm using ELISA plate reader (Wallac Model 1420 Multilabel counter, michigan, USA). The intensity of color developed in each well was corresponding to the cell number. Percentage of cell viability was calculated using a formula below. IC_{50} value was expressed as concentration of extract in microgram per milliter that caused a 50% growth inhibition comparing with controls.

$$\% \text{ cell viability} = \frac{\text{OD (test sample)-OD(medium)}}{\text{OD (DMSO control)–OD (medium)}} \times 100$$

3.2.7 Cell cycle analysis

Cell cycle analysis was determined by staining the DNA content with propidium iodide (PI) followed by flowcytometry analysis according to the manufacturing instruction (GUAVA Technologies, Inc., USA), Lee et al. (2004), and Watkins and Norbury (2004) with slight modification. Briefly, two human cancer cell lines ($1x10^6$ cells) were cultured in a 100 mm^2 culture dish in RPMI 1640 complete medium and incubated at 37°C for 24 hours. Cells were treated with trans-resveratrol, ethanolic grape pomace extract, etoposide or vehicle control and incubated at 37°C for 48 hours. The treated cells were trypsinized, washed twice with cold PBS and centrifuged at 200 × g for 5 min. Cells were resuspended to a single cell suspension in 200 µl PBS and fixed by adding dropwise into 4 ml of cold 70% ethanol, and incubated at 4°C for at least 12 hours or overnight. Fixed cells were washed once with 2 ml cold PBS and stained with 200 µl GUAVA® cell cycle reagent (PI staining) (GUAVA Technologies, Inc., U.SA) per sample at room temperature for 30 min in the dark. After incubation, 400 µl of cold PBS was added and cells were immediately analyzed within one hour by Becton Dickinson FACScalibur cell analyzer (Fig 9). Data from 10,000 events per sample were collected and analyzed using the Cell analysis program. Stages of cell cycle, G_O/G_1, S and G_2/M were analyzed from 100% gated cells. The distribution of different phases of cell cycle was measured.

Figure 9 Becton Dickinson FACScalibur cell analyzer. (BD FACSCalibur™ Flowcytometry system, NJ, USA).

3.2.8 Apoptosis assay

The concentrations of ethanolic grape pomace extract and trans-resveratrol which induced high cytotoxicity to cell line tested were used for investigating whether the cell death was mediated through apoptosis mechanism by using DAPI staining, detection of DNA fragmentation and annexin V-FITC staining for quantification of apoptotic cells.

3.2.8.1 DAPI (4', 6-Diamidine-2'-phenylindole dihydrochloride) staining

Apoptosis was determined by the detection of nuclear morphology using DAPI staining as described by Hotz et al. (1992), German et al. (1994) and Pawley (1995) with slight modification. Two human cancer cell lines at a density of $1x10^6$ cells were seeded in 25 cm^2 flasks and were incubated at 37°C in a 5% CO_2

incubator for 24 hours to give a 60-80% confluent monolayer cell. Cells were incubated with RPMI 1640 containing 1% FBS at 37°C for 24 hours. Cells were then treated with trans-resveratrol at final concentration of 10, 20, 40 and 80 μg/ml or with ethanolic grape pomace extract at final concentration of 100, 200, 400 and 800 μg/ml. Cells cultured with 0.1% DMSO in RPMI 1640 containing 1% FBS were used as a vehicle control. Cells treated with 10 μg/ml etoposide were used as a positive control. Cells were incubated at 37°C for 48 and 72 hours. Thereafter, cells were trypsinized, washed twice with PBS and centrifuged at 400 x g at 25°C for 5 min. Cells were then fixed with methanol and stained with 1 μg/ml DAPI solution (Roche, Germany) for 15 min in the dark. Cells were mounted with 10% glycerol in PBS. The nuclear morphology was observed under fluorescence microscope (excitation at 372 nm and emission 454 nm). Apoptotic nuclei can be identified by condensed chromatin and fragmented apoptotic nuclei. Nuclei were considered to be normal phenotype when glowing bright and homogenously.

3.2.8.2 DNA fragmentation assay

DNA fragmentation was analyzed by agarose gel electrophoresis as described by Lee et al. (2004), Basil et al. (1996), and Herrmann et al. (1994) and genomic DNA detection Kit (Real genomic DNA detection, CA, USA) with slight modification. Two human cancer cell lines ($1x10^{6}$ cells) were pre-incubated in RPMI 1640 complete medium in a 100 mm^2 culture dish (Corning Incorporated, Life science, MA, USA) at 37°C for 24 hours. Cells were then treated with trans-resveratrol (10, 20, 40 and 80 μg/ml or ethanolic grape pomace extract (100, 200, 400 and 800 μg/ml) for 48 hours. Cells treated with 10 μg/ml etoposide were used as positive control. The

treated cells were harvested and washed twice with PBS by centrifugation. Cell pellets were lysed with lysis buffer (50 mM Tris-HCl, pH 7.5, 20 mM EDTA, 1% NP-40) for 10 seconds and were centrifuged at 1600 × g for 5 min. Supernatant was collected and treated with RNase A at final concentration of 5 µg/µl (Herrmann et al., 1994) at 56°C for 2 hours and followed by digestion with 5 µg/µl protrinase K (Herrmann et al, 1994) at 37°C for 1 hour. DNA was precipitated with 2.5 volume of cold ethanol. The DNA pellet was dissolved with 50 µl of TE buffer (Apendix). The DNA was loaded in 1.6% agarose gel containing 0.1 mg/ml ethidium bromide in 1x TBE buffer and electrophoresed at 80 volts for 90 min with 100 bp DNA markers (Promega, Madison, WI). The gel was visualized with a WEALTEC Dolphin-DOC ultraviolet analyzer (WEALTEC, Corp., Sparks, Nevada, USA).

3.2.8.3 Annexin V-FITC assay

Apoptotic cells were determined by annexin V-FITC apoptosis detection kit (BD Biosciences, BD Pharmingen^{TM}) as described by Casciola-Rosen et al. (1996) with some modification. Briefly, cells ($5x10^5$ cells/well) were cultured in RPMI 1640 complete medium in a 6-well culture plate (Corning Incorporated, Life science, MA, USA) in duplicate. Plates were incubated at 37°C in a 5% CO_2 incubator for 24 hours. After removing medium, 2 ml of RPMI 1640 containing 1% FBS was added and incubated for 24 hours. Cells were washed once with PBS and then treated with trans-resveratrol at final concentrations of 10, 20, 40 or 80 µg/ml or with ethanolic grape pomace extract at final concentrations of 100, 200, 400, 600 or 800 µg/ml. Cells treated with 0.1% DMSO in RPMI 1640 containing 1% FBS were used as a vehicle control. Plates were incubated at 37°C for 24 hours. Thereafter, cells were

washed once with PBS and trypsinized to make a single celll suspension. Then cells were washed once with cold PBS and were centrifuged at 300 × g for 5 min at room temperature (RT). Cell pellet was resuspended with free serum medium and centrifuged at 300 x g for 5 min at RT. Cell pellet was resuspended in 100 µl of 1x binding buffer in a 5 ml tube. Then 3 µl of annexin V-FITC and 5µl of PI solution were added and incubated at RT for 15 min in the dark. Four hundred microliters of 1x binding buffer were added to each tube and the cells were mixed by gently vortexing to make a single cell suspension. Cells were then analyzed using Becton Dickinson FACScalibur cell analyzer (BD FACSCalibur™ Flowcytometry system, NJ, USA) within one hour (Fig 6). Data were collected and analyzed using the Cell analysis program.

3.2.9 Determination of apoptotic protein

3.2.9.1 Cell extraction

Cell extraction was prepared according to Santa Cruzt Biotechnology manufacturer's instruction. The SNU 1079 and Panc 2.03 cell lines ($2x10^6$ cells) were cultured with RPMI 1640 complete medium in 100 mm^2 culture dish and were incubated at 37°C for 24 hours. Cells were then treated with trans-resveratrol (10, 20 or 40 µg/ml), ethanolic grape pomace extract (100, 200 or 400 µg/ml) and 0.1 % DMSO (a vehicle control) in duplicate and were incubated at 37°C for 48 hours. After treatment, cells were washed twice with cold PBS and were extracted on ice with 250 µl cold RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP40, 1mM EDTA, 1mM DTT) containing 2mM leupeptin, 1 mM E-64 and 200 mM phenylmethylsulphonylfluoride (PMSF) pH 7.4 for 30 min. The cell

lysates were further disintegrated by passing through 21 gauge to break up the aggregated cells. After centrifugation at 10,000 x g at 4°C for 10 min, supernatant was then separated and kept in small aliquots at -80°C until analysis.

3.2.9.2 Protein determination

Protein content of cell lysate was estimated by the Bradford protein assay using Coomassie Protein Assay Kit Reagent (Pierce Biotechnology, Inc., Rockford, USA). The assay was performed according to a manufacturer's instruction with minor modification. The cell lysate was prediluted into 1:200 with RIPA buffer. Ten micro-liters of prediluted cell extract were mixed with 160 µl of coomassie protein assay kit reagent. The mixture was kept at room temperature for 10 min, then the absorbance was measured at 595 nm against blank. The concentration of protein in the sample was estimated from a standard curve that constructed by using 50, 100, 150, 200 and 250 µg/ml of bovine serum albumin (BSA) as standards.

3.2.9.3 Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The cell lysates were subjected for polyacrylamide gel Electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) as slightly modification method described by Laemmli system (Pozo-Guisado et al., 2002) (Appendix D). Four volumes of sample were homogeneously mixed with one volume of 5x SDS-PAGE sample buffer to obtain 1x final concentration of sample buffer. The treated sample was boiled at 95°C for 5 min and then 90 µg of protein was loaded into individual well of SDS-polyacrylamide mini-slab gel (8x7x0.75 cm) with 12%

acrylamide separating gel and 4% acrylamide stacking gel. The loaded gel was electrophoresed in running buffer (Appendix D) at a constant current of 20 mA until the tracking dye front reached the bottom of the gel.

3.2.9.4 Electrophoretic blotting onto nitrocellulose membrane

Proteins in electrophoresed polyacrylamide gel were transferred onto a Hybond ECL Nitrocellulose membrane (0.2 μm) (Amersham Pharmacia Biotech, UK) using a transferring buffer (Appendix D). Prior to transferring, the nitrocellulose membrane and filter paper were pre-equilibrated in the transferring buffer for 20-30 min. Then, proteins in the gel were transferred onto a nitrocellulose membrane at a constant voltage of 80 volts at 4°C for 60 min using Mini Trans-Blot® Electrophoretic Transfer Cell (Biorad laboratories, Hecules, CA, USA). The blotted nitrocellulose membranes were stored at -20°C until use.

To determine the efficiency of the electrophoretic transfer, the transferred proteins on the membrane were revealed by staining with 0.1% amido black for 5-10 min and destaining with destaining buffer (Appendix D). To reveal remained proteins on the blotted gel, the gel was stained with Coomassie® brilliant blue (Sigma) overnight and destained with destaining solution (Appendix D).

3.2.9.5 Immunodetection of antigens on the nitrocellulose membrane

The blotted nitrocellulose membrane was cut into strips. The nonspecific protein binding sites on the blotted nitrocellulose membrane were blocked with 5% non fat dry milk in 0.1% Tween-20 in PBS, pH 7.4 (Appendix D) at 4°C

overnight. Each strip of the blocked nitrocellulose membrane was then incubated with monoclonal antibodies to Bcl-2, caspase-3 and β-actin at the dilution of 1:150, 1:200 and 1:200 respectively at 4°C overnight with gently shaking. After 3 washed (5 min each) with PBS-Tween-20, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa cruz Biotechnology, Inc., CA, USA) at RT for 1 hour. After 2 washes (5 min each) with PBS-Tween-20 and wash once (10 min) with PBS, the Super-signal luminal substrate (Pierce Biotechnology, Inc., Rockford, USA) was added to membrane and exposed for 5 min. Protein was detected and visualized by using Ecl chemiluminescence system as described in the manufacturing instruction (Pierce Biotechnology, Inc., Rockford, USA). The protein bands were quantified by autoluminographs (Nicolini et al., 2003 and Niles et al., 2003). The amount of protein in each well was confirmed by stripping the membrane with stripping buffer (at 70°C for 1 hour) and reprobing with monoclonal antibody to β-actin by following the manufacturer's instruction (Santa cruz Biotechnology, Inc., CA, USA). Pre-Stained protein markers of molecular weights (MWs) 6.5-175 kDa were electrophoresed in the same gel and subsequently transferred onto the same nitrocellulose membrane. The apparent MW of each antigen on the immunoblot was determined from the pre-stained protein markers

3.3 Statistic analysis

Statistic analysis was performed on commercial computerized statistic software, Sigma Stat® 2.0 and Sigma plot® 5.0, (Jandel Scientific Software, Jandel Corporation, San Rafael, CA, USA), and data were expressesed as mean ± SD. Student's test (unpaired) was used to compare between DMSO-treated control cells

and trans-resveratrol or ethanolic grape pomace extract-treated cells. Duncan's Multiple Range Test (DMRT) was used to compare the total phenolic compound contents between grape products.

CHAPTER IV

RESULTS

4.1 The total phenolic content

Initial studies were conducted to investigate and determine the total phenolic compound contents (TPC) in Zinfandel red grape products which were grown and produced in SUT farm at Suranaree University of Technology, Nakhon-Ratchasima province. Phenolic compound determination was performed by harvesting the Zinfandel grape in March 2003 and produced the grape products; juice, ethanolic grape pomace extract and wine. Meanwhile, phenolic compounds occur in all vegetables and fruits as a diverse group of secondary metabolite which is a component of human diet although the data for dietary intakes and metabolites is limited. They had been characterized by chemical reactivity and their quantitative complicated analysis. The amount of TPC in grape products such as juice, ethanolic grape pomace extract, wine, were determined by using absorbance measurements of the samples using UV/VIS 916 spectrophotometer equipped with a Peltier thermocell (GBC, Dandenong, Australia) at 750 nm. The determination of TPC content from grape products was measured by Folin-Ciocalteau's phenol reagent as modified from the method of Matthaus (2002). The data of individual TPC contents is shown in Fig. 10. The amount of TPC in ethanolic grape pomace extract (4,407.33±13.65 mg/L) was higher than red wine (3,613.00 ±15.13 mg/L) and juice (1,102.67±21.96 mg/L). They were significant differences ($p \leq 0.01$). The amount of alcohol content in red wine was equal to 12 % and the value of total soluble solid (°Brix) was decreased to 7.11±0.02 and the pH value was 3.28±0.04.

Ethanolic grape pomace extract has a total soluble solid and pH values of 22.48±0.58 mg/kg and 4.25±0.05, respectively. The total soluble solid and pH of juice are 15.90±0.10 mg/mL and 3.18±0.06, respectively. The result is shown in Fig 11.

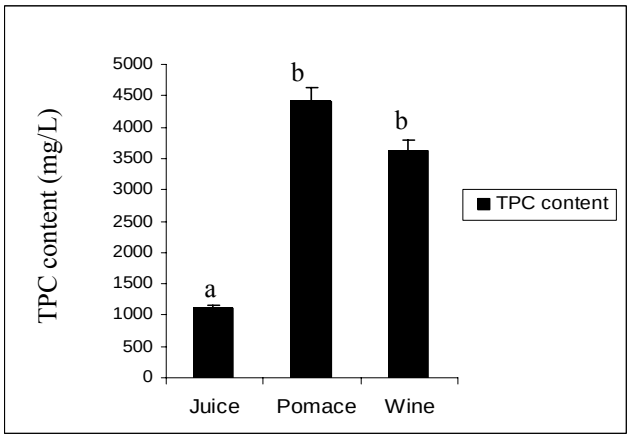


Figure 10 Comparison of TPC contents of Zinfandel grape products: juice, ethanolic grape pomace extract and wine expressed as mg/L GAE. Each sample was measured in triplicates. All values are mean±S.D. Values with differing alphabets are significantly different from each other at $p \leq 0.01$.

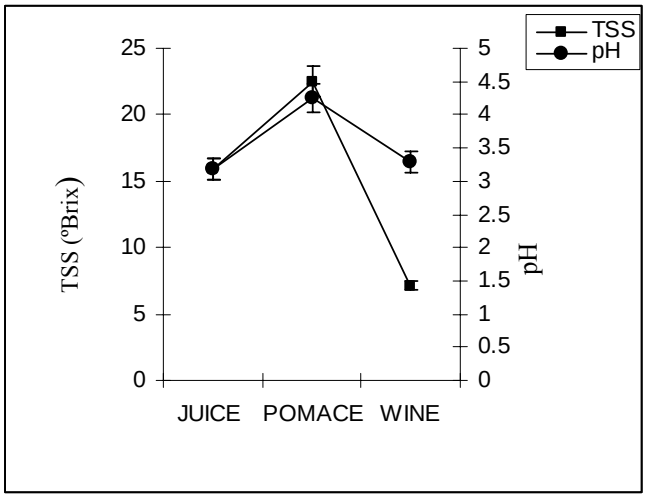


Figure 11 Comparison of total soluble solid (TSS) (°Brix) of juice, ethanolic grape pomace extract and wine in Zinfandel grape products expressed as mg/L GAE. Each sample was measured in triplicates. All values are mean±S.D. Values with differing alphabets are significantly different from each other at $p \leq 0.01$.

4.2 Optimization of extraction of ethanolic grape pomace

To evaluate the optimum condition for the extraction of TPC content from ethanolic grape pomace extract, different methods of extraction were investigated and compared for TPC contents of the obtained extracts.

After separation from juice, ethanolic grape pomace extract (containing peel, skin and seeds) was collected and pooled into a sterile pyrex. It was then dried in a hot air oven which the temperature controlled at 65°C for one week. The hot air oven method was compared with the freeze-dried under vacuum at -50°C and the soxhlet extraction procedure. The TPC content was determined by Folin-Ciocalteau's method. Both grape pomace extraction methods determined the amount of TPC concentration by dissolving in 80% ethanol and macerated overnight. They were then centrifuged and evaporated by a rotary evaporator under vacuum at 40°C. After evaporation, grape pomace was frozen and dried to make powder and kept at -80°C until use. The result of TPC contents is shown in Fig 12.

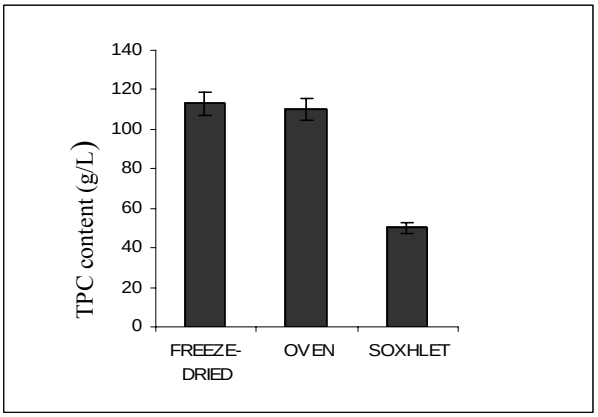


Figure 12 Comparison of TPC content of ethanolic grape pomace extract in different extraction methods expressed as g/L GAE. All values are mean±S.D. of n=3. Values with differing alphabets are significantly different from each other at $p \leq 0.01$.

4.3 Absorption of TPC content *in vivo*

The results of absorption of grape juice and ethanolic grape pomace extract as determined by measuring the absorption of TPC in mice plasma after oral garaged with grape products are shown in Fig 6. The concentration of TPC absorbed over a 24 hours period was determined. After single oral administration, the absorption of TPC contents of both juice and ethanolic grape pomace extract in ICR mice was measured by Folin-Ciocalteu's method using pure gallic acid as a standard. There were different significant absorption of TPC ($p \leq 0.05$) at different period of time from the beginning of the administration up to 24 hours post administration and the absorption of ethanolic grape pomace extract at all time points were significantly higher than the behicle control. The result showed that TPC had been promptly absorbed in plasma. It was found that the highest absorption of TPC content of juice and ethanolic grape pomace extract were 0.2152±0.01 g/L at 12 h, and 0.2157±0.01 g/L at 6 h, respectively. By comparing the TPC content between juice and ethanolic grape pomace extract at the same period of time, the result indicated that there was an interaction between two groups as summarized in Fig 13. TPC concentrations between two groups were significant different from control around 30 min ($p \leq 0.01$).

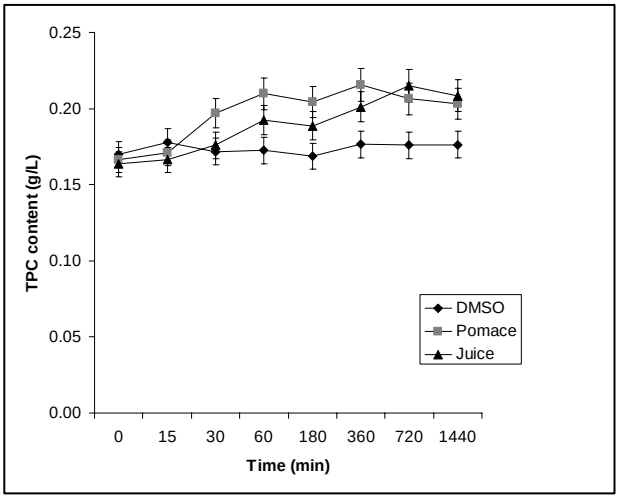


Figure 13 TPC concentrations in mice plasma treated with juice, ethanolic grape pomace extract or vehicle control at 0, 15, 30, 60 min, 3, 6, 12 and 24 h. TPC concentrations were detected by Folin-Ciocalteu's method. Data represents the mean ± S.D. (n=3). The experiment was analyzed by design-expert plot. * Denote significant differences from control values at $p \leq 0.01$.

4.4 Absorption of trans-resveratrol *in vivo*

The amount of pure phenolic compounds, trans-resveratrol, resveratrol spiked wine and selected Zinfandel red wine from SUT Farm were used as reference to determine whether they were presented in mice plasma. The amount of trans-resveratrol in mice plasma was determined by CE as summarized in 4.4.1

4.4.1 Determination of trans-resveratrol by CE

4.4.1.1 Optimisation of CE for the determination of trans-resveratrol

To evaluate the optimum condition for the determination of trans-resveratrol by CE, the following parameters were studied including buffer

(pH, type and concentration), organic modifier and applied voltage.

pH plays a very important role in CE. Because pH is not only affect the ionization of the analystes to provide charged analytes but also influences on electrophoretic mobilities of the analytes. Thus, pH is the most important parameter used to optimise selectivity, resolution and peak shape. In the present study, the effect of pH was studied in the range of 9.00 -10.0 using sodium tetraborate buffer. The results indicated that an increase of pH caused a decrease of the apparent mobility. The decreased apparent mobility results in slow migration of trans-resveratrol in capillary. However, increases of pH and current in capillary were observed. In order to achieve a good resolution for all peaks, a pH of 9.32 was chosen throughout. Besides the pH of buffer, the concentration of buffer needs to be considered, the optimum ionic strength of electrolyte must be in balance between an acceptably low current to minimise noise and good peak shape. The effect of a concentration of sodium tetraborate buffer ranging from 20 - 50 mM was investigated. In general, increasing the borate concentration results in the migration time being prolonged with low apparent mobility. To optimise sensitivity, migration time and resolution, 40 mM sodium tetraborate (pH 9.32) was selected as the running buffer.

The addition of organic modifiers to the electrolyte buffer can control electroosmotic flow, improve resolution, and enhance analyte solubility. Acetonitrile (ACN) was used in this study. The results showed that when content of ACN was increased to 20%, a good resolution of resveratrol was observed and then was used throughout. The applied voltages were investigated in the range of 20 – 25 kV. Increasing applied voltage results in the increasing of apparent mobility and electroosmosis giving sharper peak. However, it was shown that too high voltage

(higher than 25 kV) leads to the formation of current in capillary and the reduction of resolution. Low applied voltage results in a peak broadening and a prolonging migration time. Therefore, to obtain high efficiency, short analysis and extend the use of capillary, the applied voltage of 25 kV was then used. The optimum condition of CE for the determination of trans-resveratrol is summarized in Table 7. Figure 14 shows the typical of electropherogram of standard trans-resveratrol.

Table 7 The optimum conditions for CE of trans-resveratrol

Parameters	Optimum condition
Electrolyte buffer	40 mM sodium tetraborate and 20% acetonitrile
pH	9.32
Applied voltage (kV)	25
Hydrodynamic Injection (0.5 p.s.i.)	5 s
Detection wavelength (nm)	220

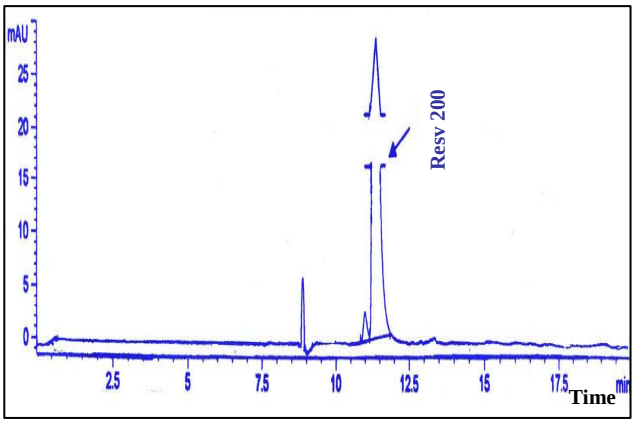


Figure 14 Electropherogram of standard trans-resveratrol 200 mg/L was performed with a diode-array detector using an Agilent Technologies model G1600AX. The capillary was uncoated fused silica of 50 μm i.d. with effective and total lengths of 50 and 75 cm. Electrophoretic analyses were performed in 40 mM sodium tetraborate, 20% acetonitrile, pH 9.32, at 25 kV, 25°C. Samples were injected by hydrodynamic (0.5 p.s.i.; 1 p.s.i.=6894.76 Pa) for 5s. Detection wavelength was 220 nm.

Under optimum conditions, the calibration curve was constructed by the three replications of a series of concentration of trans-resveratrol (diluted with 20 mM sodium tetraborate pH 9.32) in the range of 1.0 – 100.0 mg/mL. The relationship between the obtained peak area and its corresponding concentrations was plotted. The linear equation, linearity and correlation coefficient (R^2) are summarized in Table 9. The results indicated a linear range of 4.0 – 100.0 mg/mL and the correlation coefficient was 0.99931. The limit of detection (LOD) and the limit of quantitation (LOQ) were evaluated based on the concentrations that give a signal-to-noise ratio of 3 and 10, respectively. The obtained LOD and LOQ were 2 and 4 mg/mL.

The recovery was studied in order to evaluate the accuracy of theselected condition. A known amount of standard trans-resveratrol was spiked into both running electrolyte and plasma. The percentage recovered for running electrolyte and plasma were 92.49±1.46 and 110.57±2.99, respectively. The quantitative data of trans-resveratrol standard in electrolyte and plasma are summarized in Table 8 Electropherograms for the study of recovery are shown in Fig 15.

Table 8 Calibration and % recovery of trans-resveratrol determined by capillary electrophoresis.

Compounds	Regression equation[a]	Absorbance (nm)	Correlation Coefficient	Recovery (%)[b]
1. *trans*-resveratrol in 12 % ethanol (v/v)	y = 5.52351x – 4.28145	220	0.99931	92.49±1.46
2.*trans*-resveratrol in plasma	y = 5.54481x – 6.25118	220	0.99922	110.57±2.99

[a] x is the concentration in mg/L and y is the peak area

[b] Recovery due to the performance of CE instrument, n =8

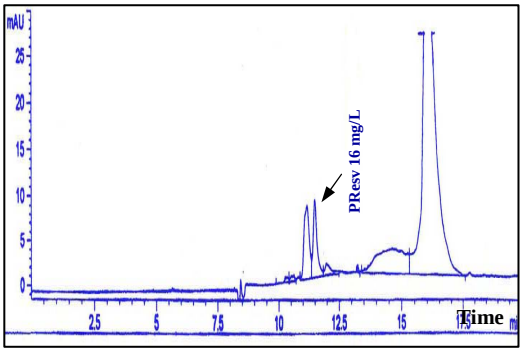


Figure 15 Electropherogram of standard trans-resveratrol (16 mg/L) spiked plasma was performed with a diode-array detector using an Agilent Technologies model G1600AX. The capillary was an uncoated fused silica of 50 μm i.d. (Agilent). Electrophoretic analyses were performed in 40 mM sodium tetraborate, 20% acetonitrile, pH 9.32, at 25 kV, 25°C. Samples were injected by hydrodynamic 0.5 p.s.i. for 5s. Detecion wavelength was 220 nm.

4.4.1.2 Analysis of trans-resveratrol in mice plasma

The amount of trans-resveratrol in mice plasma was determined by CE. All animals were treated with trans-resveratrol and resveratrol spiked wine once a day. The presence of trans-resveratrol in samples was confirmed by comparing the migration time and absorption spectra that were obtained from samples and standard trans-resveratrol. Figure 16 and 17 show electropherograms of the samples. However, no peak of trans-resveratrol was observed in all of the spiked samples, as summarised in Table 9.

Table 9 The content of trans-resveratrol in mice plasma by CE.

Samples	Amount of trans-resveratrol (mg/L)								N
	minute				hour				
	0	15	30	60	3	6	12	24	
Sample[a] plasma	ND	ND	ND	ND	ND	ND	ND	ND	3
Sample[b] plasma	ND	ND	ND	ND	ND	ND	ND	ND	3

ND = not detected

Sample [a] =plasma from mice treated with trans-resveratrol

Sample [b] = plasma from mice treated with trans-resveratrol spiked wine

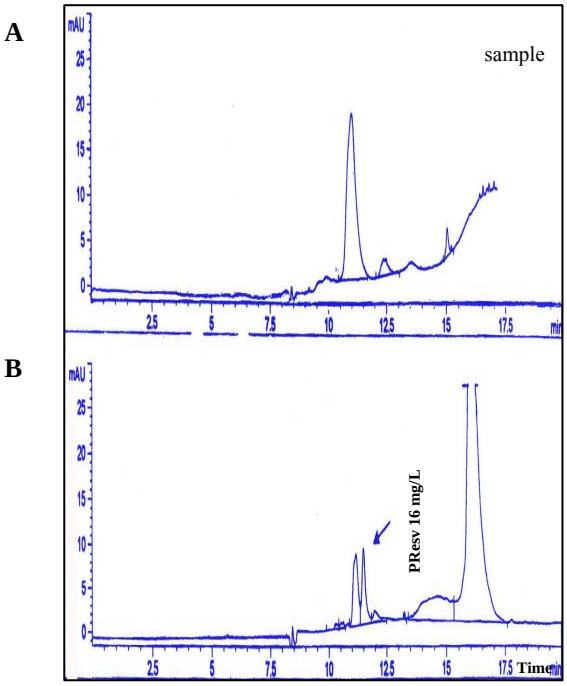


Figure 16 Electropherogram of sample plasma (diluted with buffer 1:10) from mice treated with trans-resveratrol (A), trans-resveratrol (16 mg/L) spiked plasma (B). Electrophoretic separation was performed with a diode-array detector using an Agilent Technologies. The capillary was an uncoated fused silica of 50 μm i.d. Electrophoretic analyses were performed in 40 mM sodium tetraborate, 20% acetonitrile, pH 9.32, at 25 kV, 25°C. Samples were injected by hydrodynamic 0.5 p.s.i. for 5s. The detection wavelength was 220 nm.

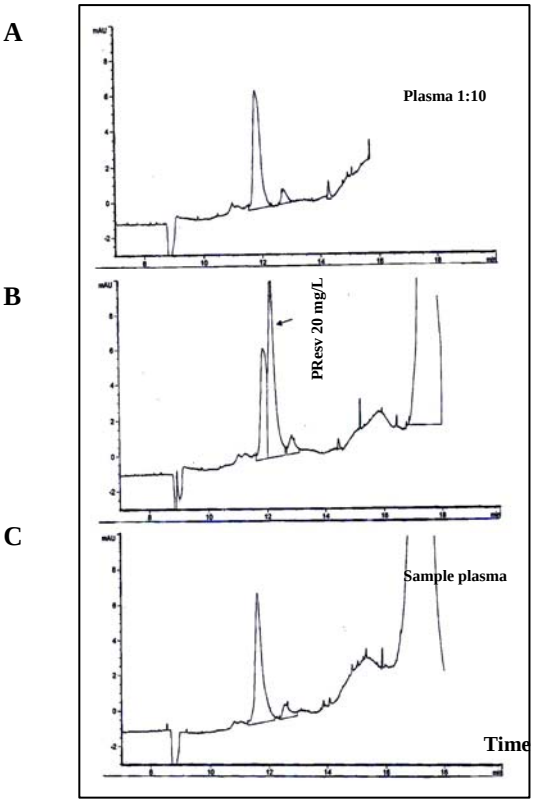


Figure 17 Electropherogram of plasma (A), trans-resveratrol (20 mg/L) spiked plasma (B) and sample of plasma (C) from mice treated with resveratrol spiked wine. Electrophoretic separation was performed with a diode-array detector using an Agilent Technologies. The capillary was an uncoated fused silica of 50 μm i.d. Electrolyte buffer was 40 mM sodium tetraborate, 20% acetonitrile, pH 9.32, at 25 kV, 25°C, 0.5 p.s.i., 5s injection. The detection wavelength was 220 nm.

4.5 Effect on Ultrastructural pathologic changes study

A study of ultrastructural changes of liver cells morphology was conducted at Suranaree university of Technology. The detailed changes in the photomicrograph of liver cells were demonstrated by electron microscopy. The liver has the structure of an endocrine gland and is the heaviest gland in the body. It receives all material absorbed from the intestinal tract with the exception of lipid, most of which was transported to the lymphatic system, the digested material was stored and excreted by the liver. In the present study, mice were treated with grape products: juice, ethanolic grape pomace extract, wine, trans-resveratrol or vehicle control. Mice were sacrificed and livers were collected for every two months. The hepatocytes were examined under TEM and liver tissues were examined under low power (20X) of microscope. The liver was seen to be composed of masses of epithelial, parenchyma cells (hepatocytes) arranged in anastomosing and branching plates which form a three dimensional lattice. Between the plates were sinusoidal blood spaces, such as lobule, hepatic lobule, which had several portal canals and in its center was a central vein. Hepatic cells were polygonal with six or more surfaces, usually 20 to 35 μm in size, and clearly a cell membrane.

Ultrastructural pathologic changes of hepatocytes treated with grape products and trans-resveratrol administration was observed under Transmission electron microscopy. This data is showed in Table 10 and Fig 18. Result from Transmission electron microscope (TEM) showed the different ultrastructural pathology of hepatocytes was examined. Findings included a degenerated margin of cells with vacuolized cytoplasm, loss of cellular structure and increased of fat cells. Endoplasmic reticulum and mitochondria showed irregular shape and swelling.

Ultrastructures in morphology of mice hepatocytes were comparable in all treatment groups; grape products, trans-resveratrol and vehicle control. In comparison with normal controls (Fig18 A and B), 12% ethanol (Fig 18 C and D) and 10% DMSO (Fig 18 E and F) administration caused ultrastructural pathologic changes in mice hepatocytes at the first time point evaluated, first 2 months and was more changes with a prolonged time of six months (Table 10). The prominent pathology were lipid droplets and glycogen accumulation, organelle abnormality, and focal cytoplasmic degeneration. One to several large fat droplets, surrounded by glycogen granules, was found in the cytoplasm. Mitochondria abnormalities were observed, including variation in size and shape. The endoplasmic reticulum was disarranged. Focal cytoplasmic degeneration was often found, and the condensation of chromatin was also occasionally observed.

ICR mice administered with juice, pomace and wine everyday for up to six months, exhibited the ultrastructural pathology of hepatocytes from the beginning treatment till the end of administration. In comparison to the hepatocytes of normal control, the ultrastructure of juice-treated hepatocytes was very similar (Fig 18 I and J). Liver from ethanolic grape pomace extract and wine-treated mice showed lipid droplets and glycogen accumulations through the cytoplasm and condensation of chromatin in hepatocytes was also occasionally observed. However, ultrastructural pathology of livers in the pomace and wine-treated groups were comparable to their corresponding vehicle controls, DMSO and ethanol, respectively. Ethanolic grape pomace extract caused much less lipid droplets accumulation and mild disorganization of the ultrastructure of organelles in hepatocytes (Fig 18 G and H). Wine caused much more lipid droplets accumulation than pomace, but caused much less than 12% ethanol-treated control (Fig 18 K and L).

Trans-resveratrol-treated livers caused much less mature lipid droplet accumulation and disorganization of organelles (Fig 18 M and N) than 12% ethanol and wine which had much more immature and mature lipid droplets accumulation and disorganization of the ultrastructure of the organelles in hepatocytes. Focal cytoplasmic degeneration was found in 12% ethanol and wine much more than trans-resveratrol treated livers. Several large lipid droplets surrounded glycogen granules could be found in cytoplasm.

Table 10 Ultrastructural pathologic characteristic of hepatocytes from mice treated with grape products and trans-resveratrol during different period of time post administration.

Treatment	Ultrastructural pathologic characteristic of hepatocytes					
	2 months		4 months		6 months	
	1	2	1	2	1	2
Normal control	-	-	-	-	-	-
10 % DMSO	++	++	+++	+++	+++	+++
12 % Ethanol	++	++	+++	+++	++++	++++
Grape juice	-	-	-	-	-	-
Ethanolic grape pomace extract	+	+	++	++	++	++
Wine	+	+	++	++	++	+++
Trans-resveratrol	+	+	++	++	++	++

++++= most change

+++ = mild change

++ = less change

- = normal

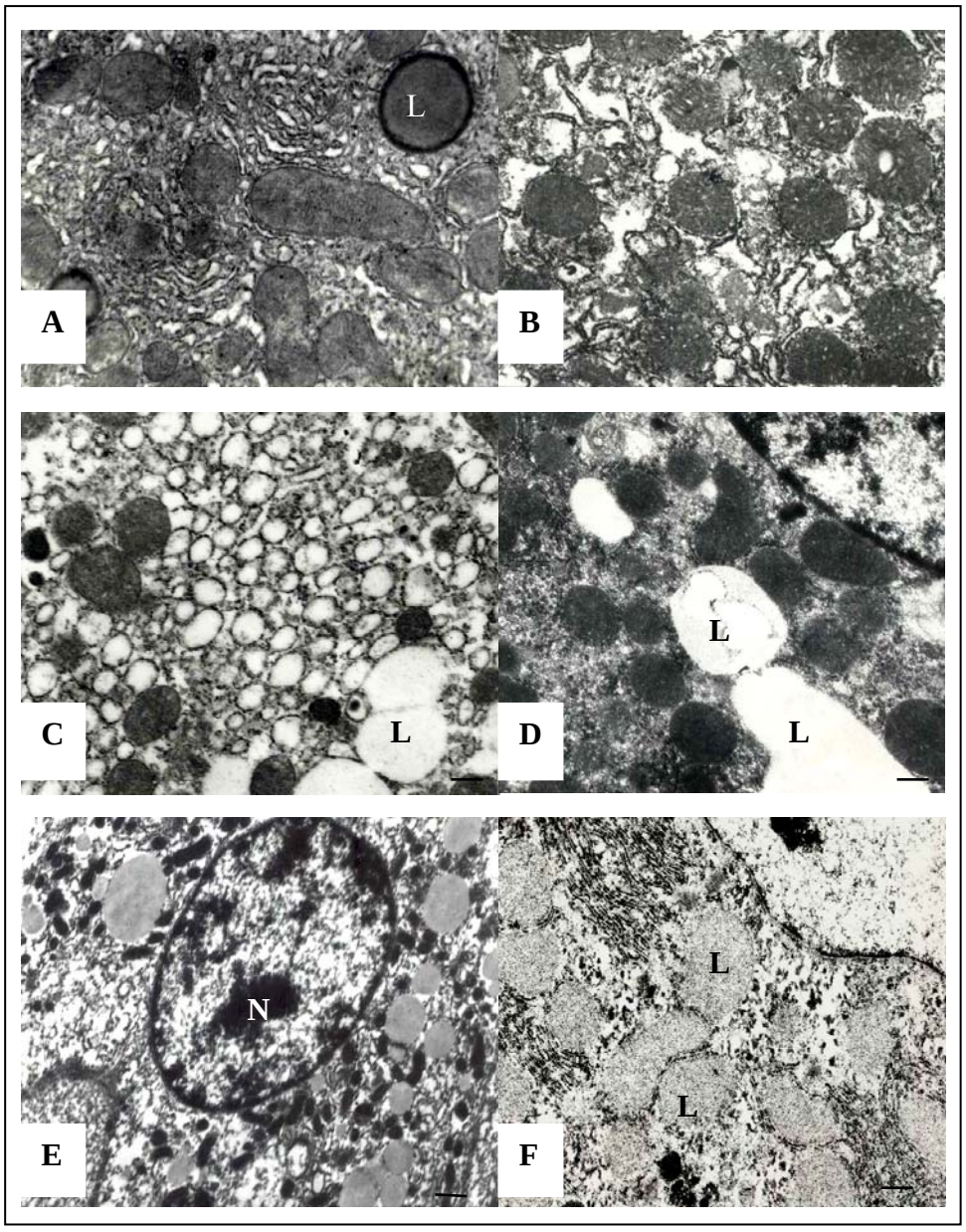
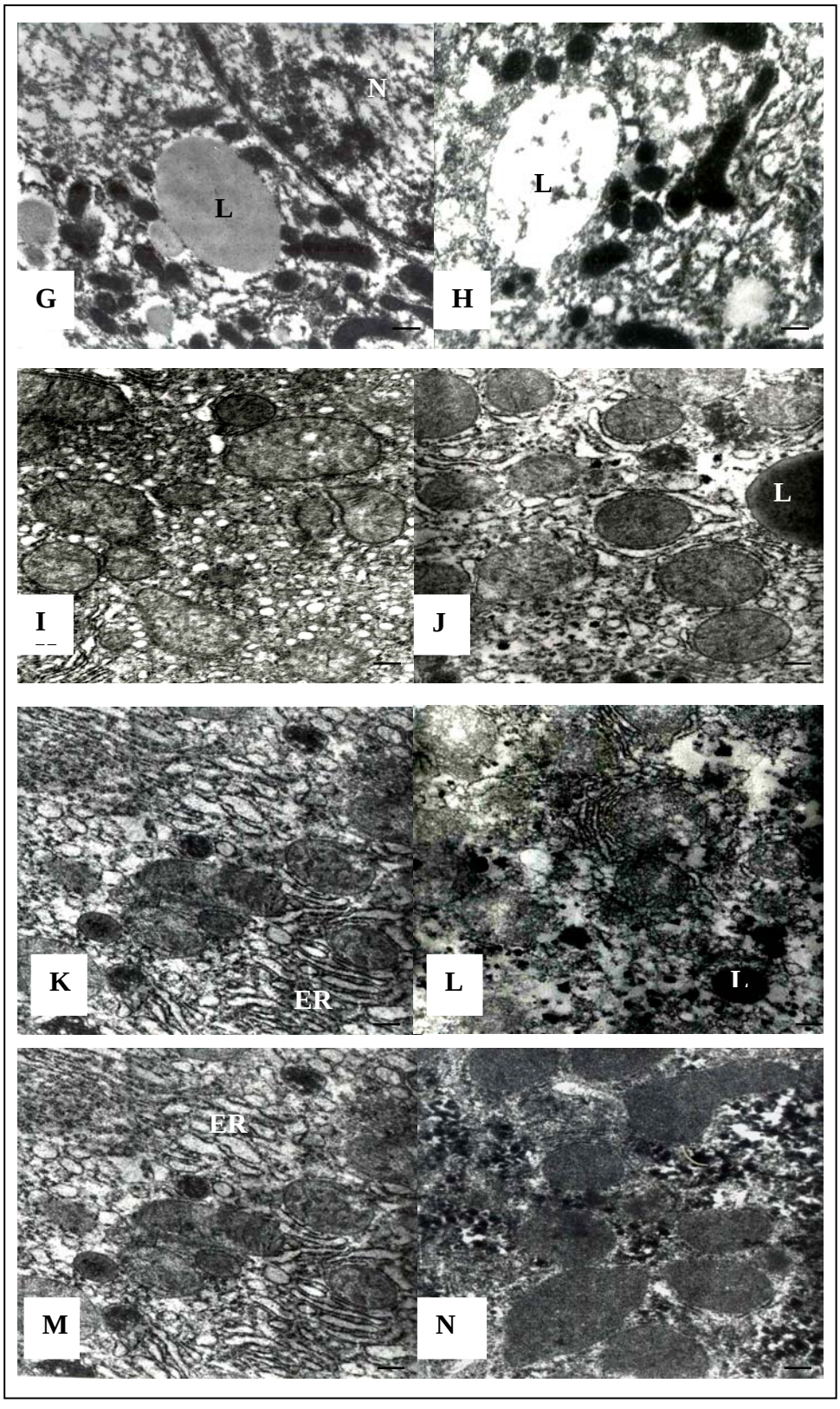


Figure 18 Electronmicrograph of mice hepatocytes treated with test compounds: vehicle control, grape products and trans-resveratrol for up to 6 months. Photographs show normal control (A, B), 12% ethanol (C, D), DMSO (E, F), ethanolic grape pomace extract (G, H), juice (I, J), wine (K, L) and trans-resveratrol (M, N) during 2 and 6 months post administration. L= lipid droplet, N=nucleus, ER=endoplasmic reticulum.

Figure 18 (continued)



4.6 *In vitro* Cytotoxicity studies

An *in vitro* cytotoxic effects of trans-resveratrol and ethanolic grape pomace extract on two human cancer cell lines, Panc 2.03 and SNU 1079, and normal human fibroblast cells were investigated. Trans-resveratrol and ethanolic grape pomace extract were selected for this *in vitro* cytotoxic study because both compounds produced the least ultrastructural changes of hepatocytes in the previus study.

4.6.1 Cytotoxic effect of trans-resveratrol on normal human fibroblast cell line

The treatment of normal human fibroblast cells with trans-resveratrol at various concentrations for 48 hours markedly decreased cell viability in a dose-dependent manner (Fig 19 and Table 11), and pronounced cytotoxic activity was exhibited with IC_{50} values of 16.29±2.69 μg/ml.

4.6.2 Cytotoxic effect of ethanolic grape pomace extract on normal human fibroblast cell line

After exposure to the ethanolic grape pomace extract, cell viability of normal human fibroblast cells was examined using the MTS assay. The treatment of various concentrations of ethanolic grape pomace extract on normal human fibroblast cells for 48 hours, markedly decreased the cell viability in a dose-dependent manner (Figure 19 and Table 11), with the IC_{50} values of 467.78±3.50 μg/ml.

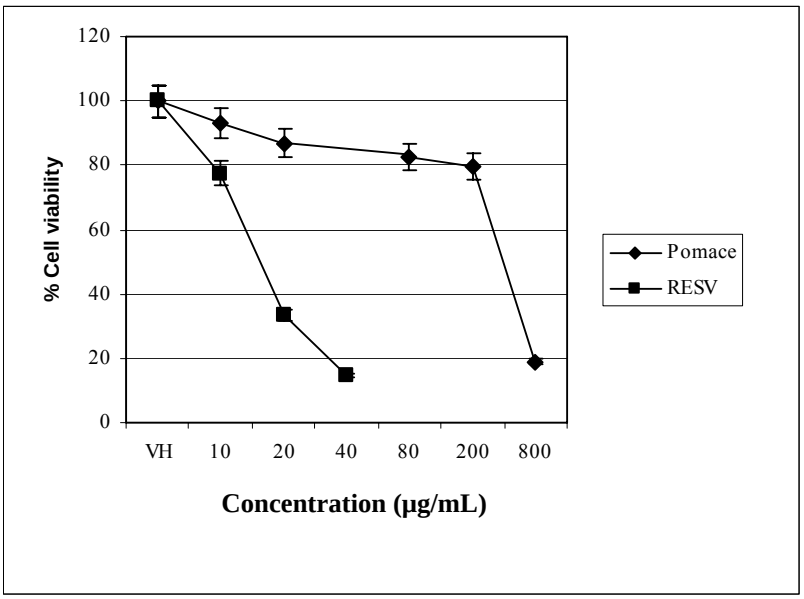


Figure 19 Cytotoxic effects of trans-resveratrol and ethanolic grape pomace extract on normal human fibroblast cells. Cells were treated with different concentrations of trans-resveratrol or ethanolic grape pomace extract for 48 hours. Cell viability was determined by MTS assay. Each value represents the means±S.E. of three independent experiments.

Table 11 Effect of trans-resveratrol and ethanolic grape pomace extract on viability of normal human fibroblast cell was performed. Cells were treated with various concentrations of trans-resveratrol or ethanolic grape pomace extract for 48h. The cell viability was measured by MTS assay. Data represents means± S.E., n=3.

Concentration (µg/mL)	% Cell viability of cancer cell lines mean± S.E.	
	Ethanolic grape pomace extract	Trans-resveratrol
10	-	77.46±7.18[b]
20	86.92±7.71[b]	33.28±9.12[a]
40	-	14.77±2.24[a]
80	82.62±6.78[b]	-
200	79.69±4.50[b]	-
800	18.92±1.09[a]	
IC_{50} values	467.78±3.50	16.29±2.69

n=number of independent experiments

Values with differing alphabetic superscripts are significantly different from each other at $p \leq 0.05$.

4.6.3 Cytotoxic effect of trans-resveratrol on cancer cell lines

Effect of trans-resveratrol on SNU 1079 and Panc 2.03 cell lines were studied by using MTS cell proliferation assay. The treatment of both cancer cells with trans-resveratrol at various concentrations for 48 hours markedly decreased cell viability in a dose-dependent manner (Fig 20 and Table 12). Pronounced cytotoxic activity was observed on SNU 1079 and Panc 2.03 cells with IC_{50} values of 18.10±0.11 and 5.09±0.04 µg/ml, respectively.

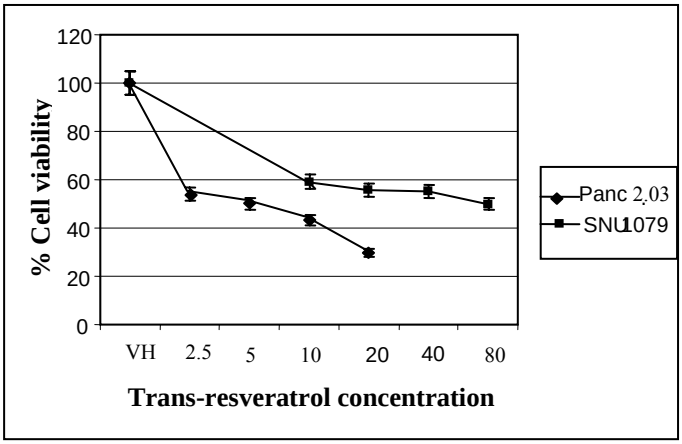


Figure 20 Cytotoxic effect of trans-resveratrol on SNU 1079 and Panc 2.03 cell lines. Cells were treated with different concentrations of trans-resveratrol for 48 hours. Each value represents the means±S.E. of three independent experiments.

Table 12 Effect of trans-resveratrol on viability of SNU 1079 and Panc 2.03 cells. Trans-resveratrol was incubated with two cancer cell lines for 48 hours. The percentage of cell viability was measured by MTS cell proliferation assay. Data represent the mean ± S.E. (n=3)

Exposure	Conc (μg/mL)	Mean± S.E. of % Cell viability	
		Panc 2.03	SNU 1079
Trans-resveratrol	2.5	53.92±0.46^{d}	-
	5.0	50.13±0.63^{c}	-
	10	43.17±0.55^{b}	58.35±0.04^{c}
	20	29.82±0.57^{a}	48.79±0.09^{b}
	40	-	47.44±0.02^{b}
	80	-	44.54±0.02^{a}
IC_{50} values		5.09±0.04	18.10±0.11

n=number of independent experiments

Values with differing alphabetic superscripts are significantly different from each other at $p \leq 0.05$

4.6.4 Cytotoxic effect of ethanolic grape pomace extract on cancer cell lines.

After exposure to the ethanolic grape pomace extract, cell viabilities of SNU 1079 and Panc 2.03 cell lines were examined using MTS cell proliferation assay. Twenty four hours treatment of ethanolic grape pomace extract markedly decreased cell viability of both cell lines in a dose-dependent manner (Fig 21 and Table 13). Pronounced cytotoxic activity was observed on SNU 1079 and Panc 2.03 cells with IC_{50} values of 716.42±0.05 and 274.46±0.82 μg/mL, respectively.

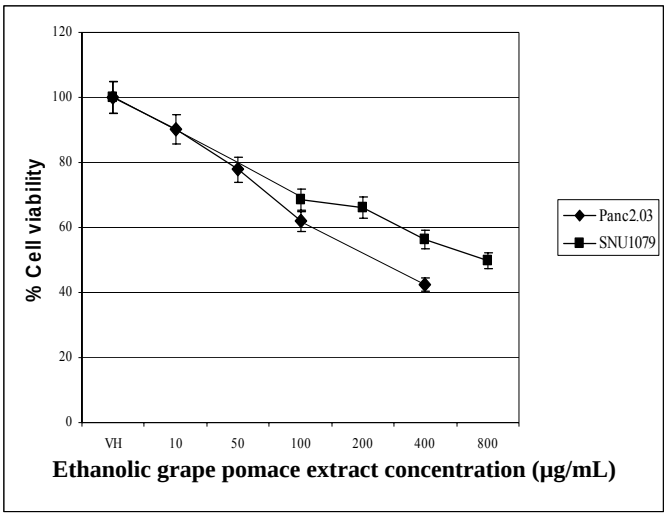


Figure 21 Cytotoxic effect of ethanolic grape pomace extract on SNU 1079 and Panc 2.03 cell lines. Each value represents the means±S.E. of three independent experiments.

Table 13 Effect of ethanolic grape pomace extract on viability of SNU 1079 and Panc 2.03 cells lines. Ethanolic grape pomace extract was incubated with two cancer cell lines for 48 hours. The parcentage of cell viability was measured by MTS cell proliferation assay. Data represent the mean ± S.E. (n=3)

Exposure	Conc (μg/mL)	Mean± S.E. of % Cell viability	
		Panc 2.03	SNU 1079
Ethanolic grape pomace extract	10	90.23±1.32^{d}	-
	50	77.88±4.27^{c}	-
	100	62.02±1.11^{b}	67.00±0.04^{d}
	200	-	64.90±0.06^{c}
	400	41.35±1.70^{a}	55.35±0.04^{b}
	800	-	48.06±0.08^{a}
IC_{50} values		274.46±0.82	716.42±0.05

n=number of independent experiments

Values with differing alphabetic superscripts are significantly different from each other at $p \leq 0.05$

4.7 Cell cycle assay

Cell-cycle dysregulation is a hallmark of tumor cells. The ability of normal cells to undergo cell-cycle arrest after DNA damage is crucial for the maintenance of genomic integrity. The biochemical pathways that stop the cell cycle in response to cellular stressors are called checkpoints. Defective checkpoint function results in genetic modifications that contribute to tumorigenesis. The regulation of checkpoint signaling also has important clinical implications because the abrogation of checkpoint function can alter the sensitivity of tumor cells to chemotherapeutic. This experiment is to assess whether trans-resveratrol and ethanolic grape pomace extract induced inhibition of proliferation is mediated via alterations of cell cycle regulation.

4.7.1 Cell cycle arrest in Panc 2.03 cells

To further examine the effect of trans-resveratrol and ethanolic grape pomace extract on the mechanism of cell proliferation inhibition, cell cycle distribution was evaluated. Flow cytometric analysis revealed that trans-resveratrol and ethanolic grape pomace extract treatment induced changes in cell cycle distribution. After treatment with various concentrations of test compounds for up to 48 hours, results showed that the dysregulation of cell cycle is one of the mechanism responsible for cell proliferation inhibition in trans-resveratrol and ethanolic grape pomace extract-treated Panc 2.03 cells (Fig 22 and Table 14). Trans-resveratrol and ethanolic grape pomace extract caused an accumulation of cells in S phase. Both test compounds caused a significant increase of cell population in the S phase of cell cycle in Panc 2.03 cells . The increases from vehicle controls were 9, 10, 11 and 28% cells at 10, 20, 40, 80 µg/mL trans-resveratrol, respectively. At high of concentration of trans-resveratrol (80 µg/mL) the increased cell population in S phase of Panc 2.03 was related to the concomitant decrease of the cells in G_0/G_1 and G_2 phase. The S phase arrest was increased about fourfold as compared to the control (Fig 22 A). Similarly, ethanolic grape pomace extract-treated cells caused an increase of cell population in S phase at 7, 8, 9 and 52% when compared to control at 50, 100, 200 and 400 µg/mL ethanolic grape pomace extract, respectively. At high concentration of ethanolic grape pomace extract (400 µg/mL), the cell cycle was affected in the G_0/G_1 phase, whereas the number of cells in G_2 phase did not significantly change when compared with the corresponding control. In comparison with the control, S phase arrest of ethanolic grape pomace extract-treated cells was about sevenfold increase in population (Fig 22 A).

4.7.2 Cell cycle arrest in SNU 1079 cells

The effects of trans-resveratrol and ethanolic grape pomace extract induced cell cycle arrest in SNU 1079 cells are shown in Fig 24 and Table 14. The result showed that trans-resveratrol caused increased accumulation of cells in G_1 phase. At high dose of trans-resveratrol (80 μg/mL), the increase of G_1 cell population was about 32% when compared to corresponding control (Table 14).

Ethanolic grape pomace extract decreased cell population in G_0/G_1 phase and increased cell population in both S and G_2 phase when compared to corresponding control. At high dose of ethanolic grape pomace extract (800 μg/mL), the increase of S and G_2 phase were about 32 and 58%, respectively (Table 14).

Table 14 Cell cycle distribution of human cancer cell lines after treatment with trans-resveratrol and ethanolic grape pomace extract for 48 hours. Data are presented as mean of percent PI stained cells of two independent experiments. Experiment was repeated with similar results.

		Distribution (% cells)					
Exposure	Conc	Panc 2.03			SNU 1079		
	(μg/mL)	G_0/G_1	S	G_2	G_0/G_1	S	G_2
Control	0	82.11	7.37	10.53	71.58	12.63	15.79
Tans-resveratrol	10	77.66	9.57	12.77	71.58	15.79	12.63
	20	77.42	10.76	11.83	71.28	15.96	12.77
	40	79.38	11.34	9.28	72.34	15.96	11.70
	80	47.54	45.90	6.56	94.29	2.86	2.86
Grape pomace	50	82.11	7.36	10.53	-	-	-
	100	80.62	8.16	11.22	79.12	10.99	9.89
	200	81.05	9.47	9.47	72.04	11.83	16.13
	400	15.79	68.42	15.79	66.30	14.13	19.56
	800	-	-	-	58.3	16.67	25.00

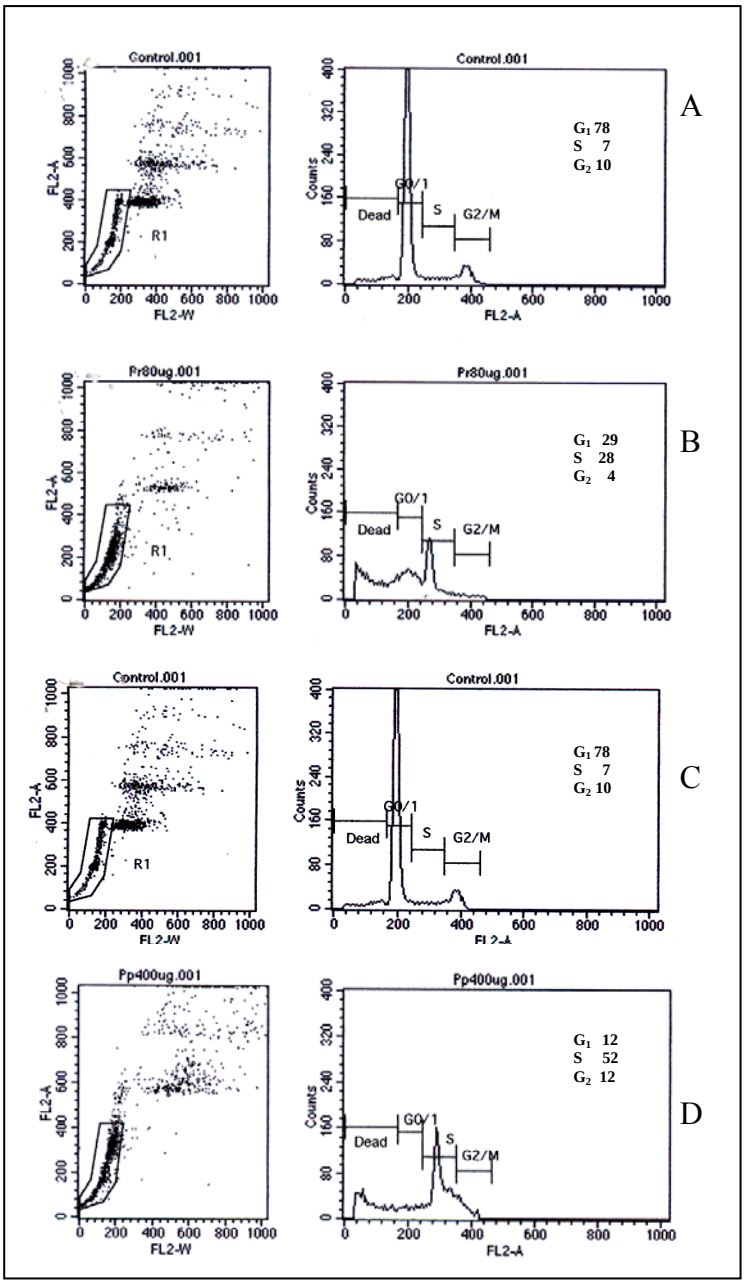


Figure 22 Flow cytometric analysis of cell cycle distribution in Panc 2.03 cells treated with various concentrations of 10, 20, 40 and 80 µg/mL trans-resveratrol and 50, 100, 200 and 400 µg/mL ethanolic grape pomace extract. After 48 hours of treatment, Cells were fixed and stained the DNA with PI and analyzed. Figure shows the results of one of these experiments, control (A), 80 µg/mL trans-resveratrol (B), vehicle control (C) and 400 µg/mL ethanolic grape pomace extract (D). The tests were performed in two independent experiments and gave similar results.

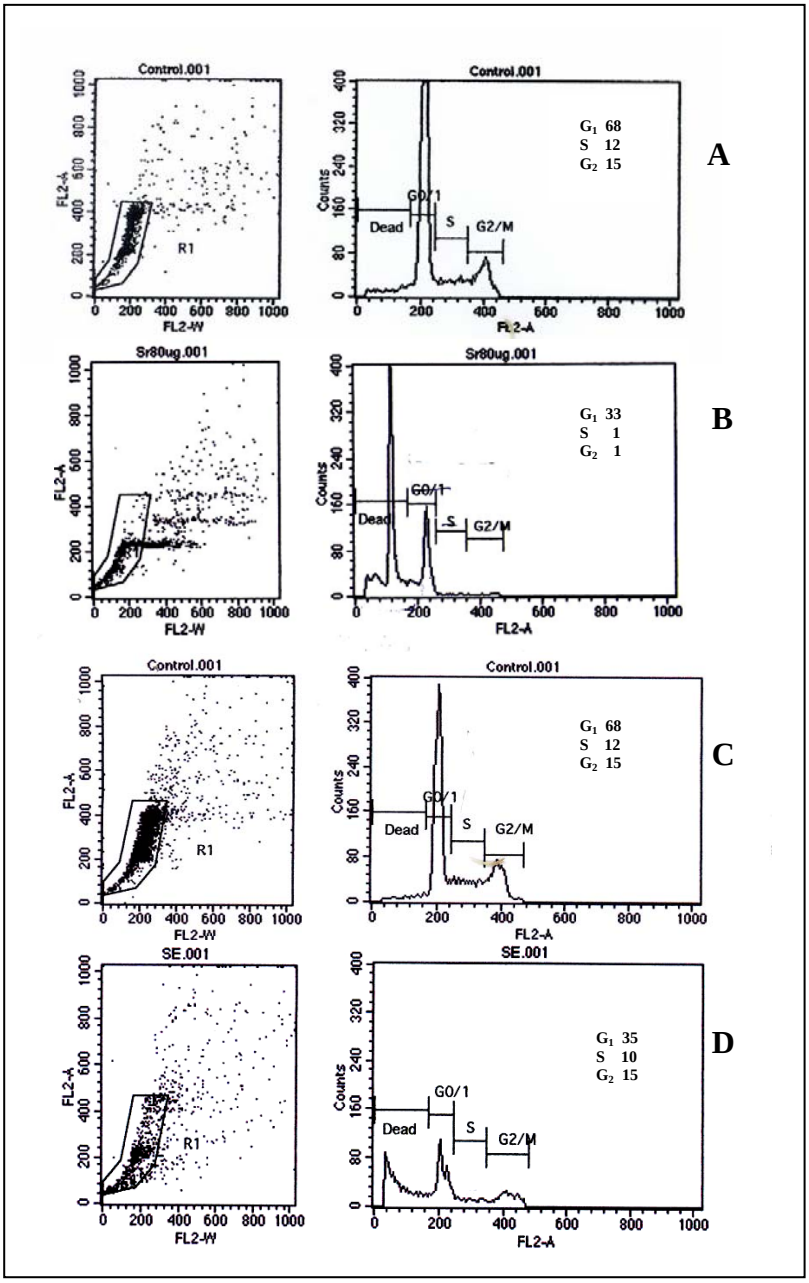


Figure 23 Flow cytometric analysis of cell cycle distribution in SNU 1079 cells treated with various concentrations of 10, 20, 40 and 80 μg/mL trans-resveratrol and 100, 200, 400 and 800 μg/mL ethanolic grape pomace extract. After 48 hours of treatment, Cells were fixed and stained the DNA with PI and analyzed. This figure shows the results of one of these experiments, control (A), 80 μg/mL trans-resveratrol (B), vehicle control (C) and 800 μg/mL ethanolic grape pomace extract (D). The tests were performed in two independent experiments and gave similar results.

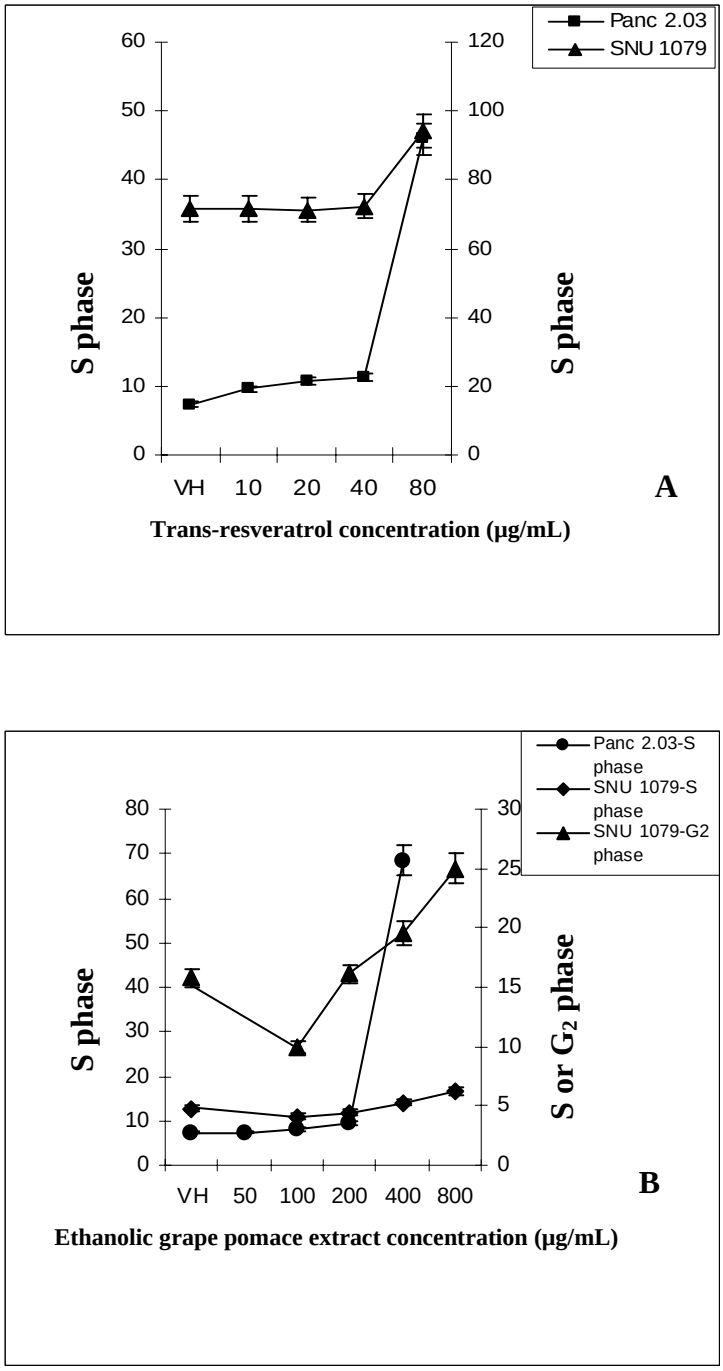


Figure 24 % S phase of Panc 2.03 or SNU 1079 cells (A) and % G_0/G_1 or S phase or G_2 phase of Panc 2.03 or SNU 1079 cells (B) after treatment with trans-resveratrol and ethanolic grape pomace extract for 48 hours. Data are presented as mean of percent PI stained cells of two independent experiments. Experiment was repeated with similar results.

4.8 Apoptotic assay

Cell death can occur by either of two distinct mechanisms, necrosis or apoptosis. In addition, certain chemical compounds are said to be cytotoxic to the cell, that is to cause its death. Cytotoxicity may also be used, as it is in this guide, to denote a laboratory method for detecting dead cells, regardless of the mechanism of their death. Two possible cytocidal mechanisms have been proposed for cell mediated cytotoxicity: (i) the apoptotic mechanism and DNA fragments; (ii) cell lysis. Dead cells are unable to metabolize various tetrazolium salts. This allows the use of the colorimetric assays, including MTT, XTT, MTS, and WST-1 to measure cell survival. Apoptosis, however, is an active mode of cell death requiring the metabolism of cell. The assay may underestimate cellular damage and detect cell death only at the later stages of apoptosis when the metabolic activity of the cell is reduced.

In order to determine whether the cytotoxic effect of ethanolic grape pomace extract and trans-resveratrol, it was due to the induction of apoptotic cell death. The ability of trans-resveratrol and ethanolic grape pomace extract to induce the apoptoic cell death was estimated by analyzing its effect on cell morphology under bright field inverted microscope and nuclear morphological evidence of apoptosis using DAPI staining. The induction of apoptosis was confirmed by DNA fragmentation assay and annexin V-FITC binding assay and with propidium iodide (PI) staining. All experiments were performed in two independent experiments and the results were similar.

4.8.1 Effect of trans-resveratrol on cell and nuclear morphology

After treatment of Panc 2.03 and SNU 1079 cells with various concentrations of trans-resveratrol (2.5, 5, 10 and 20 μg/ml) for 48 and 72 hours, respectively. The morphological alterations in Panc 2.03 and SNU 1079 cells were observed under bright filed inverted microscope. Untreated Panc 2.03 cells were polygonal in normal shape while untreated SNU 1079 cells were spindle shape (Fig 25). Exposure of both cell lines to trans-resveratrol led to retraction, rounding and some sensitive cells were detached from the surface. Membrane blebbing (Fig 25, arrow head) was observed.

Following DAPI staining, it was found that after treatment of Panc 2.03 and SNU 1079 cells with trans-resveratrol, apoptotic cells could be clearly seen under fluorescent microscope as shown in Fig 26. These apoptotic cells exhibited characteristic of nuclear morphological changes, including chromatin condensation, segmentation of nuclear chromatin of irregular size in treated cell (Fig 26). This was clear contrast to the spherical and regular form of control nuclei (Fig 26).

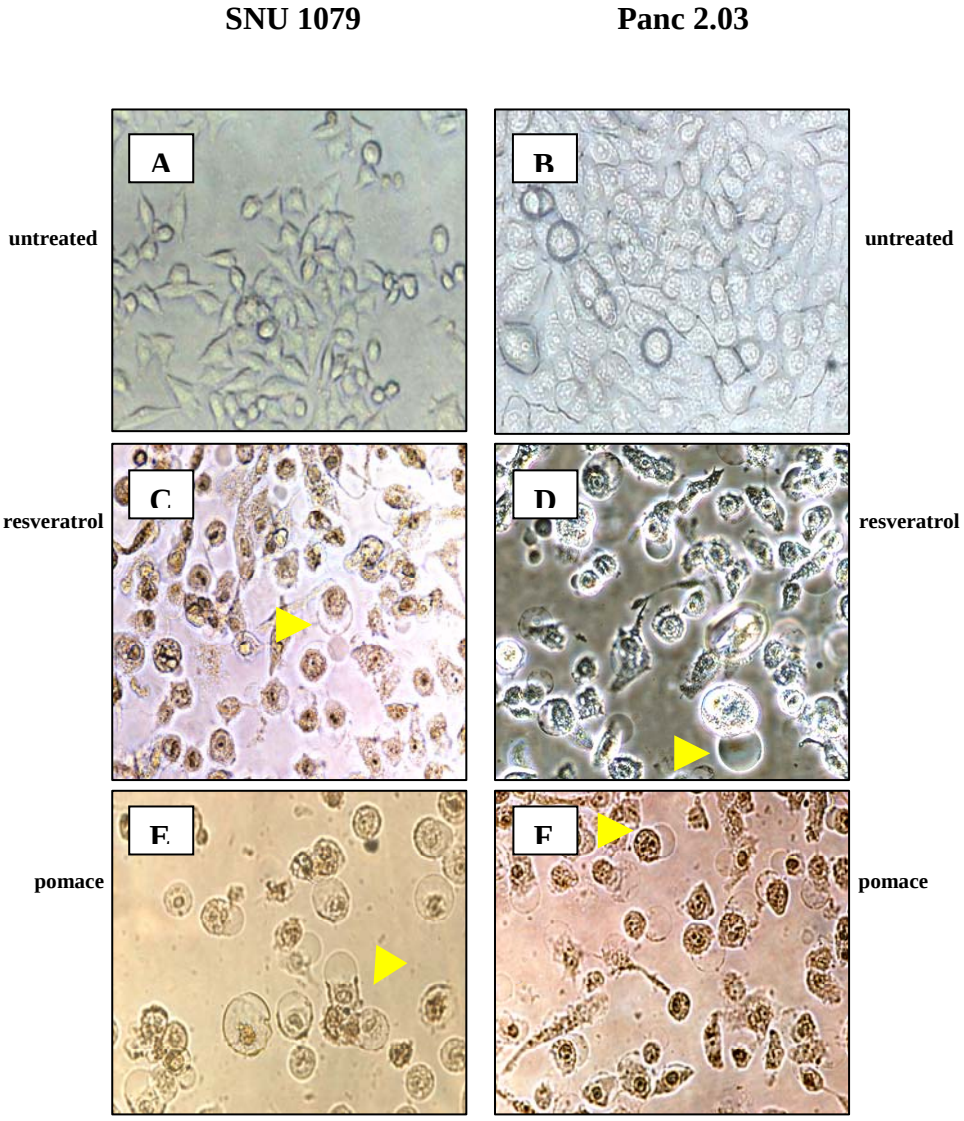


Figure 25 Morphological changes of Panc 2.03 and SNU 1079 cells after treated with trans-resveratrol for 48 h and 72 h when observed under bright field microscopy. Untreated SNU 1079 (A) cells were spindle in normal shape whereas untreated Panc 2.03 (B) cells were polygonal. Trans-resveratrol or ethanolic grape pomace extract-Panc 2.03 and SNU 1079 treated-cells (C, D, E, F) showed retraction, rounding, and detached from surface, membrane blebbing (arrow head) and apoptotic bodies were observed.

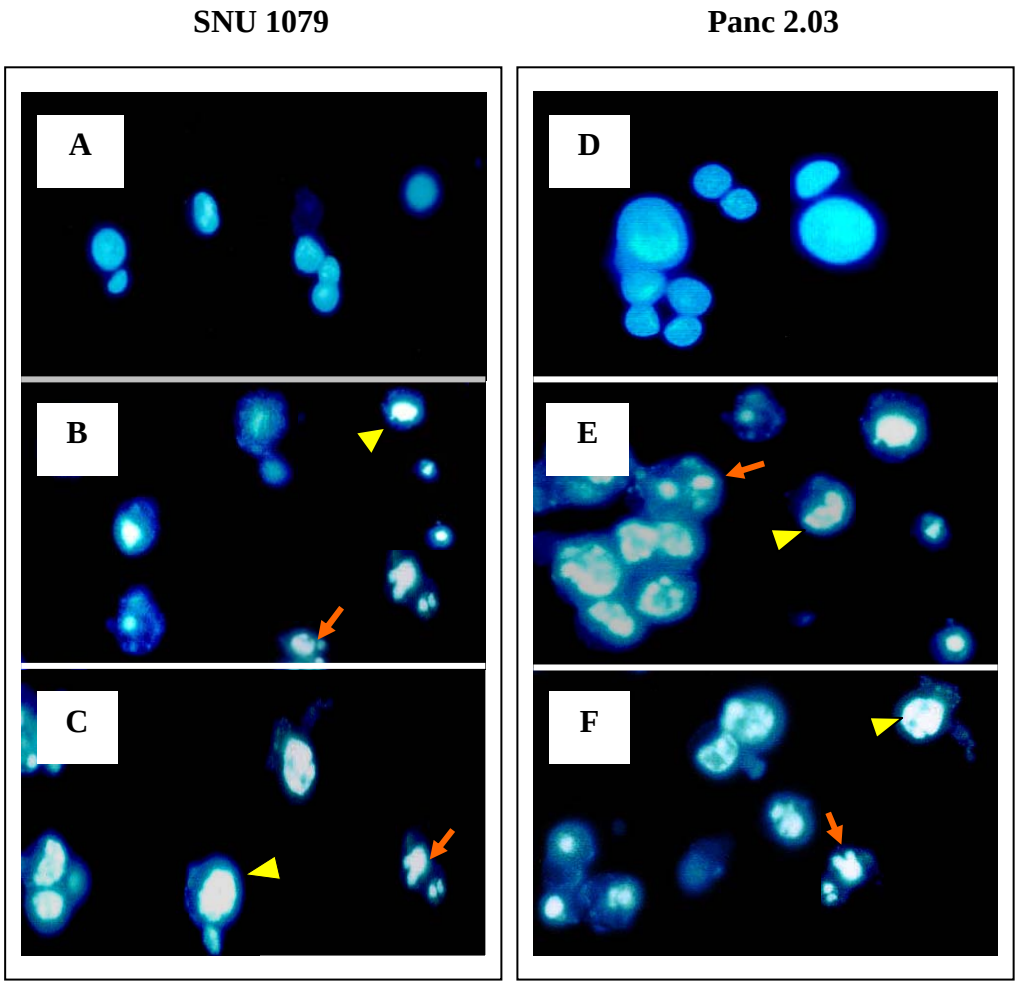


Figure 26 Effects of trans-resveratrol on nuclear morphology of Panc 2.03 and SNU 1079 cells for 48 h and 72 h respectively. Cells were fixed with methanol, and stained with DAPI. Nuclear morphology was observed under fluorescent microscope. Nuclei of control cells were round with a normal contour of nuclei in SNU 1079 cells (A), Trans-resveratrol-treated SNU 1079 cells (B), Ethanolic grape pomace extract-treated SNU 1079 cells (C) and Panc 2.03 cells (D), Trans-resveratrol-treated- Panc 2.03 cells (E) and Ethanolic grape pomace extract-treated Panc 2.03 cells (F) showed chromatin condensation (arrow head) and nuclear fragmentation (arrow).

4.8.2 Effect of ethanolic grape pomace extract on cell and nuclear morphology

After the treatment of Panc 2.03 and SNU 1079 cells with various concentrations of ethanolic grape pomace extract (10, 50, 100, 200, 400 μg/ml) for 48 and 72 hours, the morphological alteration in Panc 2.03 and SNU 1079 cells were observed under bright field inverted microscope. Untreated Panc 2.03 cells were polygonal in normal shape (Fig 27) while untreated SNU 1079 cells were spindle shape (Fig 25). Treatment of both cell lines with ethanolic grape pomace extract led to retraction, rounding and some cells were detached from the surface. Membrane blebbing (Fig 26, arrowhead) and apoptotic body (Fig 26, arrow) were observed.

Following DAPI staining, it was found that after treatment of Panc 2.03 and SNU 1079 cells with ethanolic grape pomace extract, apoptotic cells could be clearly seen under fluorescent microscope as shown in Fig 26. These apoptotic cells exhibited characteristic of nuclear morphological and size changes (Fig 26). This was clear contrast to the spherical and regular form of control nuclei.

4.8.3 Effect of trans-resveratrol and ethanolic grape pomace extract on DNA fragmentation

After treatment of Panc 2.03 cells with trans-resveratrol and ethanolic grape pomace extract for 48 hours, the DNA fragmentation was detected on agarose gel eletrophoresis. The fragmented DNA was clearly observed in treated cells in a dose dependent manner (Fig 27, lane 3-8). Whereas DNA content of control cells did not provide ladders (Fig 27, lane 2).

Similar results were observed when SNU 1079 cells were treated with trans-resveratrol and ethanolic grape pomace extract for 72 hours. The fragmented DNA was also clearly observed only in treated cells in a dose dependent manner (Fig 28, lane 3-9), but not in the control cells (Fig 28, lane 2).

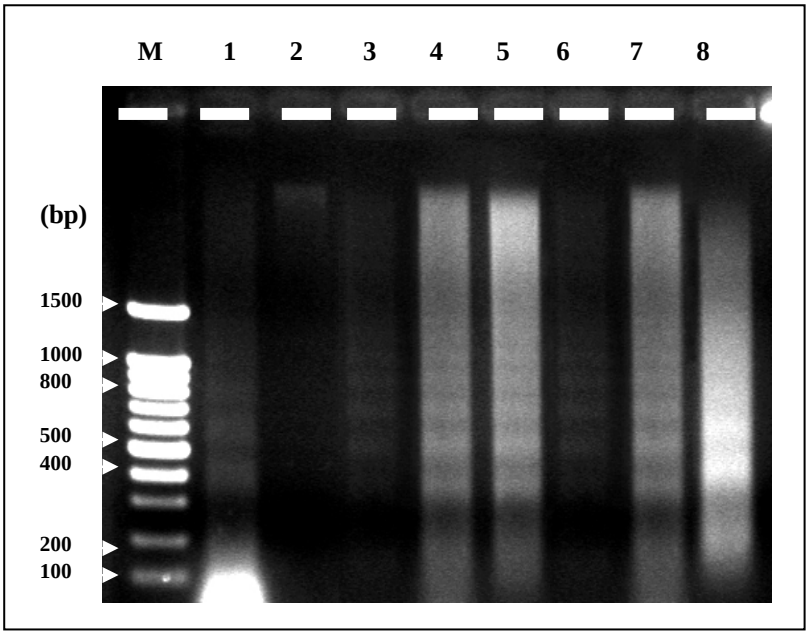


Figure 27 DNA fragmentation of Panc 2.03 cells after 48 h exposure to various concentrations of trans-resveratrol and ethanolic grape pomace extract. DNA fragmentation of cells treated with 10 µg/ml etposide a positive control (lane 1), negative control (lane 2), 20 µg/ml trans-resveratrol (lane 3), 40 µg/ml trans-resveratrol (lane 4), 80 µg/ml trans-resveratrol (lane 5), 100 µg/ml ethanolic grape pomace extract (lane 6), 200 µg/ml ethanolic grape pomace extract (lane 7), 400 µg/ml ethanolic grape pomace extract (lane 8) and Lane M represents 100 base-pair ladder marker used as molecular marker. DNA was analyzed by NP-40 lysis method using 1.6% agarose gel electrophoresis and staining with ethidium bromide.

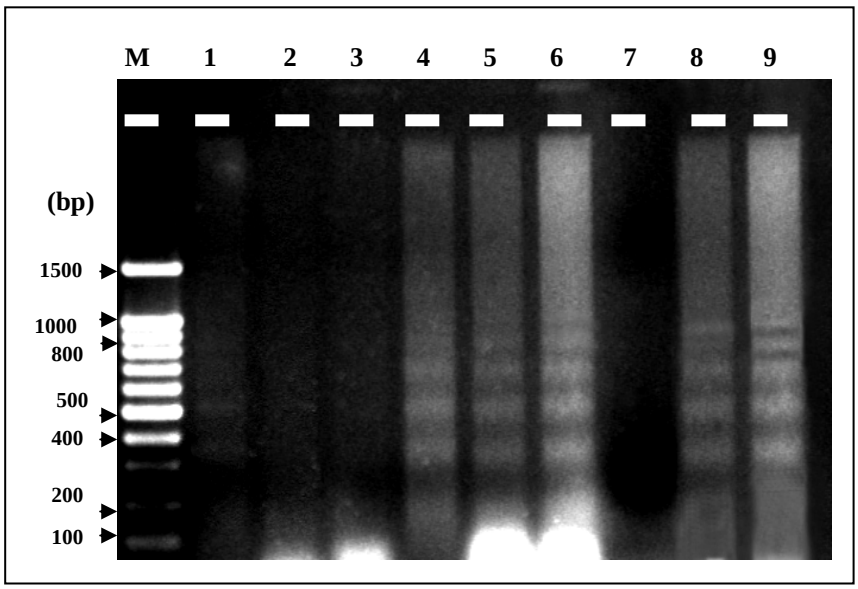
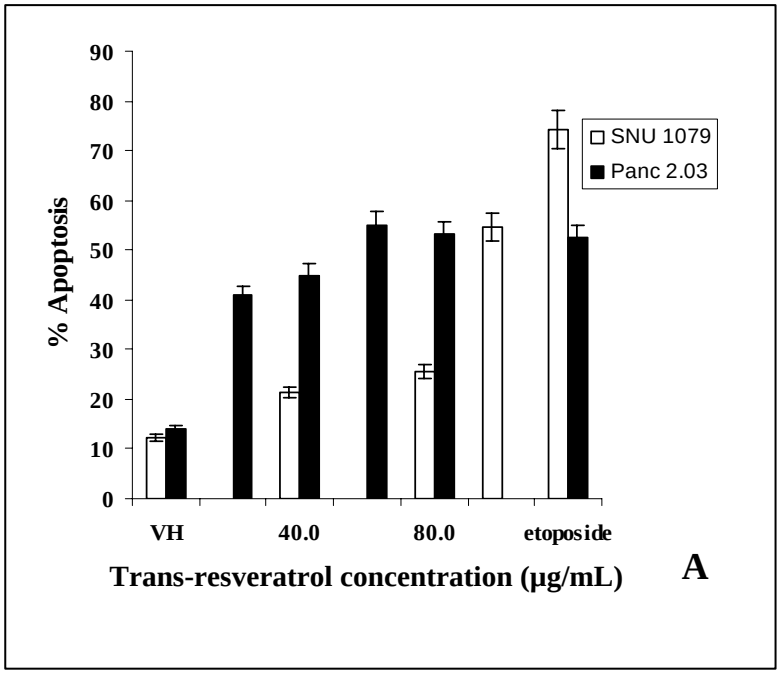


Figure 28 DNA fragmentation of SNU 1079 cells after 72 h exposure to various concentrations of trans-resveratrol and ethanolic grape pomace extract. DNA fragmentation of cells treated with 10 μg/ml etoposide a positive control (lane 1), negative control (lane 2), 5 μg/ml trans-resveratrol (lane 3), 20 μg/ml trans-resveratrol (lane 4), 40 μg/ml trans-resveratrol (lane 5), 80 μg/ml trans-resveratrol (lane 6), 200 μg/ml ethanolic grape pomace extract (lane 7), 400 μg/ml ethanolic grape pomace extract (lane 8), 800 μg/ml ethanolic grape pomace extract (lane 9) and Lane M represents 100 base-pair ladder marker used as molecular marker. DNA was analyzed by NP-40 lysis method using 1.6% agarose gel electrophoresis and staining with ethidium bromide.

4.8.4 Determination of trans-resveratrol and ethanolic grape pomace extract induced apoptosis in cancer cells using Annexin-V staining assay

Trans-resveratrol and ethanolic grape pomace extract induced apoptosis was determined by staining the cells with annexin V-FITC and propidium iodide (PI). Panc 2.03 cells were treated with various concentrations of trans-resveratrol (20, 40, 60 and 80 μg/ml) and ethanolic grape pomace extract (200, 400, 600 and 800 μg/ml) for 48 hours. Representative results of the Panc 2.03 cells are shown in Fig 31 and 32 Table 14. As shown in Fig 31 A, the percentage of apoptotic Panc 2.03 cells increased from 13% in control cells to 53% after treatment with 80 μg/ml trans-resveratrol. Similarly, the percentage of apoptotic Panc 2.03 cells increased from 13% in control cells to 64% after treatment with 800 μg/ml ethanolic grape pomace extract (Fig 29 B). Overall, the percentage of apoptotic Panc 2.03 cells were increased in dose dependent manner after trans-resveratrol or ethanolic grape pomace extract treatment (Fig 29 and Table 15).

After treatment of SNU 1079 cells with trans-resveratrol (40, 80, and 100 μg/ml) and ethanolic grape pomace extract (100, 200, 400 and 800 μg/ml) for 40 hours, the apoptotic SNU 1079 cells were determined using annexin V staining assay. Representative results of SNU 1079 cells are shown in Fig 29 and 31 Table 15. The percentage of apoptotic SNU 1079 cells increased from 12% in control cells to 54% after treatment with 100 μg/ml trans-resveratrol (Fig 29 A). The percentage of apoptotic SNU 1079 cells also increased from 14% in control cells to 43% after treatment with 800 μg/ml ethanolic grape pomace extract (Fig 29 B). Similar to trans-resveratrol treatment, ethanolic grape pomace extract also increased the percentage of apoptotic SNU 1079 cells in the dose dependent manner (Fig 29 and Table 15).



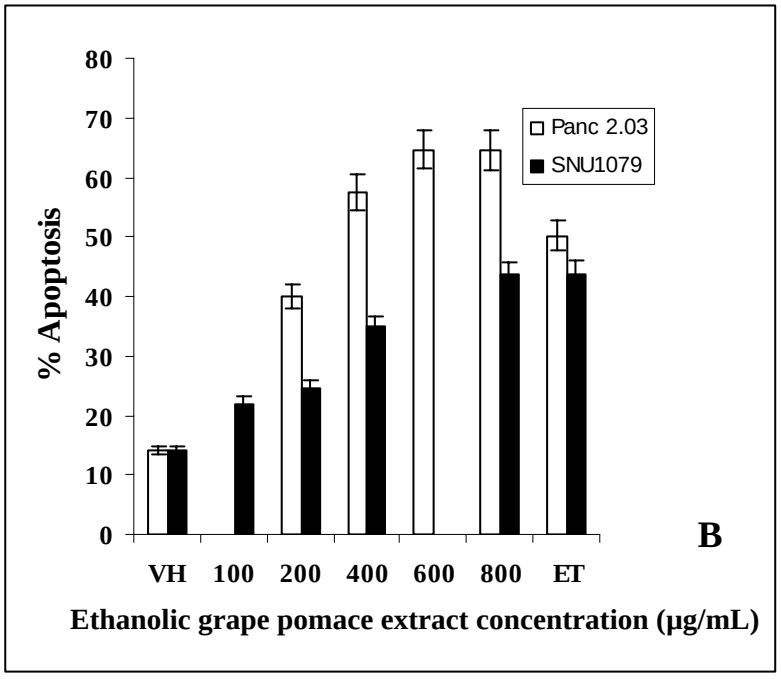


Figure 29 Apoptosis induction. Panc 2.03 and SNU 1079 cell lines were treated with 0.1% DMSO or various concentrations of trans-resveratrol and ethanolic grape pomace extract for 24 - 48 hours. Data indicates the percentage of annexin V-FITC positive cells (apoptosis).

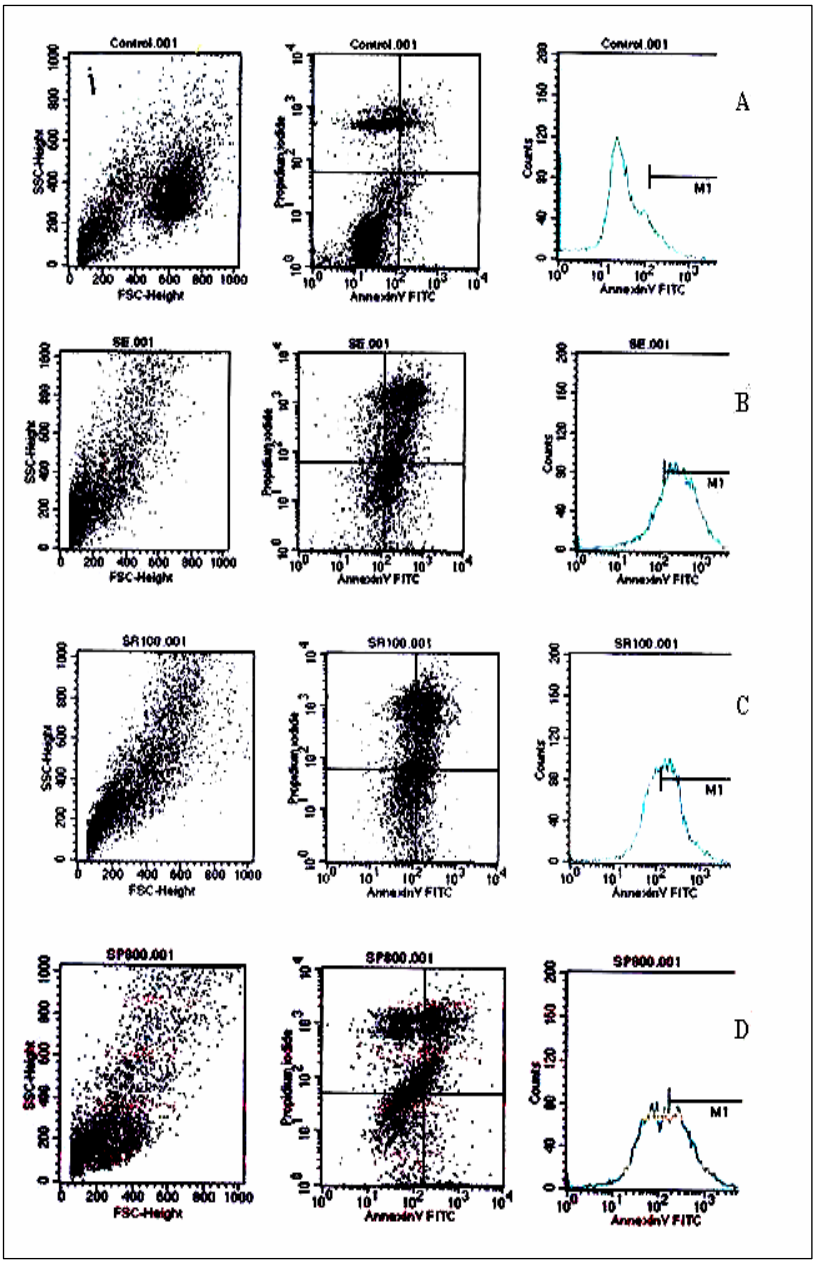


Figure 30 Trans-resveratrol and ethanolic grape pomace extract induced apoptosis in Panc 2.03 cells in culture. After 48 hours, cells were harvested and stained with annexin V-FITC and propidium iodide (PI) according to the manufacturer's instructions (appendix E). Ten thousand events were collected and analyzed for each sample using flowcytometer (Becton Dickinson FACScalibur cell analyzer). Cells were cultured in medium (A), 20 µg/mL etoposide (B). 80 µg/mL trans-resveratrol (C) and 800 µg/mL ethanolic grape pomace extract (D).

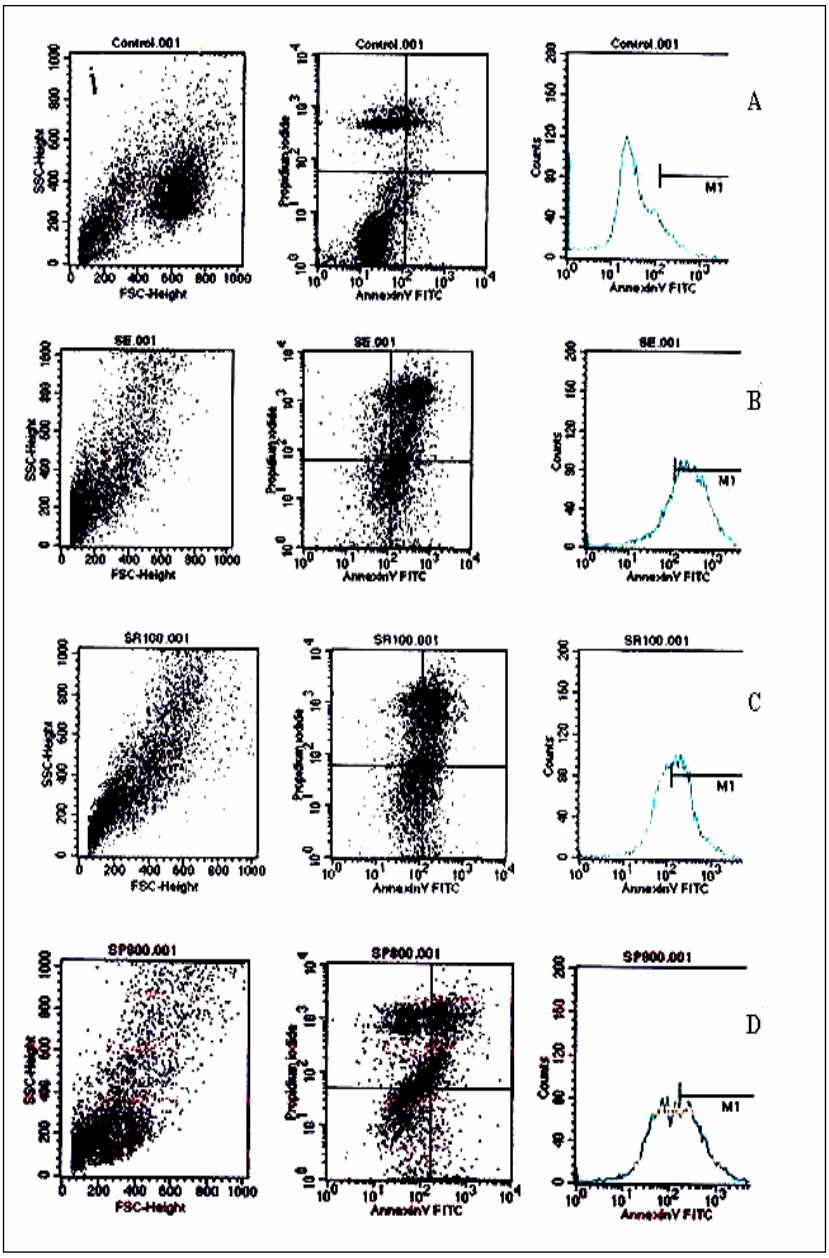


Figure 31 Trans-resveratrol and ethanolic grape pomace extract induced apoptosis in SNU 1079 cells in culture. After 48 hours, cells were harvested and stained with annexin V-FITC and PI according to the manufacturers' instructions. Ten thousand events were collected and analyzed for each sample using flowcytometer (Becton Dickinson FACScalibur cell analyzer model). Cells were cultured in medium (A). 20 µg/mL etoposide (B), 100 µg/mL trans-resveratrol (C) and 800 µg/mL ethanolic grape pomace extract (D).

Table 15 Apoptosis induction in Panc 2.03 cells after treatment for 24 - 48 hours with vehicle control, various concentrations of trans-resveratrol and ethanolic grape pomace extract.

Exposure/ Cell lines	% apoptosis [a]											
	Trans-resveratrol (µg/mL)						Ethanolic grape pomace extract (µg/mL)					
	DMSO	20	40	60	80	etoposide	DMSO	200	400	600	800	etoposide
Panc 2.03	13.98	40.84	44.98	54.93	53.10	52.38	13.98	39.90	57.48	64.59	64.52	50.13
Time of exposure	48h	48h	48h	48h	48h	48h	24h	24h	24h	24h	24h	24h
IC_{50} values	**5.09±0.04**						**274.46±0.82**					

n=2 duplicate independent experiment.

+ = positive control, 20 ug/mL

[a] apoptosis was determined using an annexin V-FITC assay and flowcytometry. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results.

Table 16 Apoptosis induction in SNU 079 cells after treatment for 40 hours with vehicle control, various concentrations of trans-resveratrol and ethanolic grape pomace extract.

Exposure /Cell line	**% apoptosis [a]**											
	Trans-resveratrol (µg/mL)						**Ethanolic grape pomace extract (µg/mL)**					
	DMSO	**40**	**60**	**80**	**100**	**etoposide**	**DMSO**	**100**	**200**	**400**	**800**	**etoposide**
SNU 1079	12.28	21.37	-	25.60	54.62	74.22	14.24	21.98	24.67	35.05	43.65	43.71
Time of exposure	40h	40h	-	40h	40h	40h	40h	40h	40h	40h	40h	40h
IC_{50} values	**18.10±0.11**						**716.42±0.05**					

n=2 duplicate independent experiment.

+ = positive control, 20 ug/mL

[a] apoptosis was determined using an annexin V-FITC assay and flowcytometry. The data indicate the percentage of cells undergoing apoptosis in each sample. All experiments were conducted in duplicate and gave similar results.

4.8.5 Effect trans-resveratrol and ethanolic grape pomace extract on the levels of Bcl-2 and caspase-3 proteins

According to our previous results, *in vitro* exposure to various concentrations of trans-resveratrol and ethanolic grape pomace extract for 48 hours induced apoptosis in both Panc 2.03 and SNU 1079 cells in a dose dependent manner. Since Bcl-2 family protein and caspase-3 play a crucial role in apoptosis (Kim et al., 2004), we determined whether the apoptosis observed was associated with the Bcl-2 and caspase-3 proteins. The levels of Bcl-2 and caspase 3 in Panc 2.03 and SNU 1079 cells that had been exposed to various concentrations of trans-resveratrol or ethanolic grape pomace extract in cultures for 48 hours were investigated. Immunoblot analysis of proteins extracted from treated cells showed a decrease in the level of Bcl-2 proteins in Panc 2.03 cells (Fig 32) and SNU 1079 cells (Fig 33) in a dose dependent manner as compared with untreated control cells. While the level of caspase-3 was shown to be increased in both Panc 2.03 cells (Fig 32) and SNU 1079 cells (Fig 33) in a dose dependent manner as compared with control cells. The same membrane was probed with β-actin (Fig 32 and 33) for loading correction, which did not show any significant different in the protein levels of β-actin. Therefore, trans-resveratrol and ethanolic grape pomace extract induced a dose dependent reduction of Bcl-2 and increasing caspase-3 level in both cell lines, the alterations which may partly explain the induction of apoptosis observed in our studies.

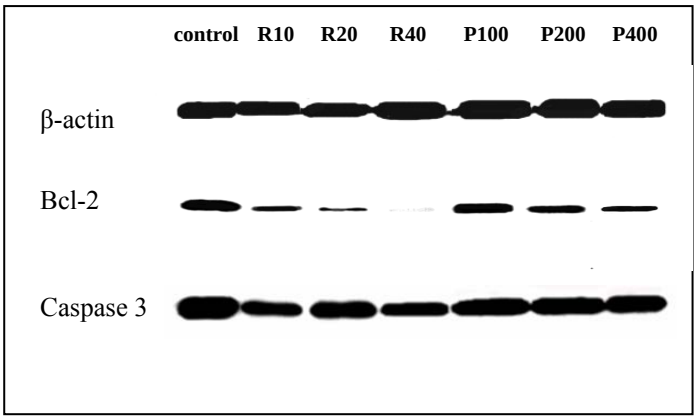


Figure 32 Bcl-2, caspase 3 and β-actin protein expressions in Panc 2.03 cells. Cells were treated with a series of concentrations of 10, 20 and 40 μg/mL trans-resveratrol and 100, 200 and 400 μg/mL ethanolic grape pomace extract for 48 hours, then cell lysates (90 μg) were prepared and evaluated for the levels of Bcl-2, caspase 3 and β-actin expression by western blotting analysis.

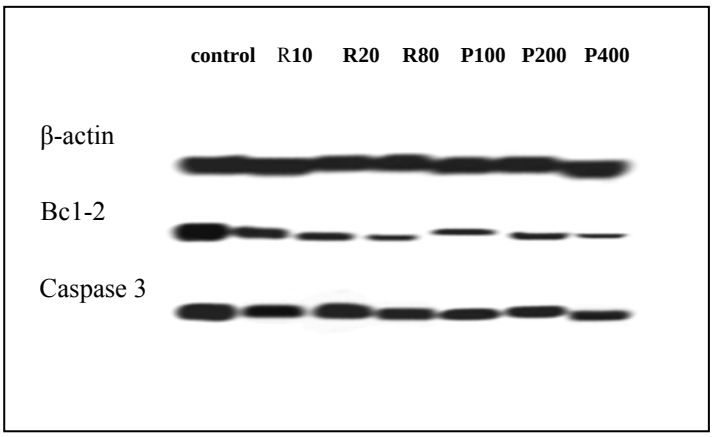


Figure 33 Bcl-2, caspase 3 and β-actin protein expressions in SNU 1079 cells. Cells were treated with a series of concentrations of 10, 20 and 80 μg/mL trans-resveratrol and 100, 200 and 400 μg/mL ethanolic grape pomace extract for 48 hours, then cell lysates (90 μg) were prepared and evaluated for levels of Bcl-2, caspase 3 and β-actin expression by western blotting analysis.

CHAPTER V

DISCUSSION

The search of anticancer agents from natural sources has been successful worldwide, active constituents from several plants have been isolated and are nowadays used to treat human tumors The important consideration of the present study is to investigate certain grape products as important sources of food supplement for anticancer and a candidate for the treatment of human cancer cells in the future. The grape products were selected because of their known antioxidant, antimutagenic, and chemopreventive properties (Vang and Bonnesen, 2005).

Determination of TPC content and trans-resveratrol of Zinfandel grape products

Zinfandel grape products from SUT farm were used in the present study to evaluate the properties of TPC contents and effects of these products *in vitro* and *in vivo*. The contents of phenolic compounds found in ethanolic grape pomace extract, wine and juice were 4,407.33±13.65, 3,613.00±15.13 and 1,102.67±21.96 mg/L, respectively. The results were similar to those presented by Teissedre and Landrault (1996) who reported the variability in the levels of TPC ranged from 1,847 – 2,600 mg/L for red wine. Jang et al. (1997) reported about 50 - 100 µg of trans-resveratrol per gram in grape skin. In last few years, several methods to measure phenolic compounds and trans-resveratrol have been presented by Folin-Ciocalteu's method using pure gallic acid as standard.

Our results revealed that grape products derived from SUT farm contain high levels of TPC and different methods of extraction are important for the yield. The results suggested that ethanolic grape pomace extract extracted with maceration had higher amount of TPC contents than extraction by soxhlet. This finding was similar to the result of Jeandet, Bessis and Gautheron (1991) who reported that trans-resveratrol, one of the important polyphenol, was found only in grape skin. Okuda and Yokotsuka (1996) confirmed that trans-resveratrol is found in grape skin and McMurtrey (1997) reported that Zinfandel wine had 1.38±0.18 mg GAE /L trans-resveratrol.

Absorption of TPC *in vivo* is rapid. After oral administration in ICR mice, the highest concentrations of TPC in plasma was around 30 min. Our result is similar to previous studies. By investigating with radioactivity assay, Goldberg, Yan and Soleas (2003) reported the highest adsorption levels of all three polyphenol (resveratrol, catechin, and Quercetin) in the serum occurring around 30 min post oral administration in human. Soleas et al (2001) reported that 50 - 75 % of oral administered trans-resveratrol appeared to be absorbed. Bertelli, Giovannini, Stradi, Urien, Tillement (1996a) and Bertelli et al. (1996b) reported resveratrol measured as free polyphenol occurred in rat plasma 60 min after gavage. There are interaction of TPC absorption between ethanolic grape pomace extract and juice at 30 min time point. Because of the highest TPC absorption of ethanolic grape pomace extract than juice, we selected ethanolic grape pomace extract to do subsequent *in vitro* studies.

Determination of trans-resveratrol concentration in plasma by CE

Absorption of trans-resveratrol (pKa = 9.14±0.20) in mice was conducted and

analyzed by CE analysis. To evaluate the optimum condition of CE for separation of trans-resveratrol, the following parameters were investigated: pH, concentration of buffer, organic modifier, and appied voltage. pH has the most important influence on the separation because pH not only has a significant impact on the ionization of acidic silanol group of capillary wall but also affects the overall charge of the analytes, electroosmotic flow and electophoretic mobilities of the analytes. In this experiment we used pH = 9.32 because this pH provided the best resolution and peak shape. Increasing of pH decreases the apparent mobility which results in slow migration in capillary. Besides pH, the concentration of electrolyte buffer also affected the resolution of trans-resveratrol peak. 40 mM sodium tetraborate was selected as the buffer used in this study. Although increasing of borate concentration results in the increasing of resolution, but high current in capillary were also observed and it leads to overheat and peak broadening. To enhance the resolution, 20% acetonitrile (ACN) was used since the resolution of trans-resveratrol was the best at this concentration. In addition, long analysis time was observed at high content of ACN, and the lower the time of analysis, the better solution was achieved. Because increasing of applied voltage results in increasing of apparent mobility and electroosmosis which give sharper peak. Whereas, too low applied voltage (lower than 20 kV) results in peak broadening and prolong migration time. In order to obtain high efficiency, the applied voltages of 25 kV at 25ºC was used in this study.

The concentration of trans-resveratrol in plasma was performed by CE. The calibration curves of trans-resveratrol in running electrolyte and plasma were constructed by increasing amounts of trans-resveratrol in the range of 1.0 – 100.0 mg/L and analyzed under the optimum condition. The correlation coefficient (R^2) are

0.99931 and 0.99922, respectively. The percentage recoveries of trans-resveratrol in running electrolyte and plasma were 92.49±1.46 and 110.57±2.99, respectively.

After ingestion of trans-resveratrol, the recovery of trans-resveratrol in plasma was not detected by CE. Confirmation of the identity of the peak seen in the plasma was achieved by both migration time and spectra analysis with stored spectra libraries. Probability of peak identity in the electropherogram was done by using automated comparison to match the peaks. Therefore, the present of trans-resveratrol in plasma can be certainly identified. However, the LOD and LOQ, calculated by measuring for eight time injections, were 2 and 4 mg/L, respectively. Therefore, it was possible that the inability to detect trans-resveratrol in plasma might be due to the contents of trans-resveratrol in samples were lower than the LOD.

Beside the problem of matching spectra, some trans-resveratrol dimer had also been changed to other metabolites, such as resveratrol bound to pallido mono and di-glucosides (Vitrac, Monti, Vercauteren, Deffieux and Merillon, 2002). This finding was similar to the result of Meng et al. (2004) who reported that trans-resveratrol was not found in CF-1 mice after being dosed with grape juice. The trans-resveratrol in this experiment was detected by LC/MS/MS analysis. However, they reported that 10-11% of total resveratrol was presented in free form at the initial time point (0.5 and 1.5 h) and declined to 5-7% at 4 h (Meng et al., 2004). Besides, stilbene concentration depends on multiple factors such as climate, geographical origin, fungal pressure and virification procedures. The variation of their concentrations in relation to each parameter remain poorly understood (Vitrac et al., 2002). However, the absorption of trans-resveratrol depend on enzymatic sulfation and glucoronidation within the

intestinal mucosa before entry into the portal blood (Soleas, Grass, Jiseohy, Goldberg and Diamandis, 2002).

Besides the CE method, HPLC was a commonly used procedure to determine trans-resveratrol in plasma. However, HPLC method lacks the ability to separate the isomer of trans-resveratrol and results in a concentration of 0.175 mg/L at 15 min after administration (Juan, Torre-Boronat and Planas (1999). This experiment used CE for analysis because CE was faster than HPLC and GC method for determination of both isomer of resveratrol (Nevado, Contento Salcedo and Castaneda Penalvo, 1999). However, CE has disadvantage of low sensitivity. According to Juan et al. (1999) the amount of trans-resveratrol found in plasma was lower than the LOD of CE that was proposed in this experiment. In order to detect low concentration of trans-resveratrol in plasma, samples preparation is very necessitated to be cleaned up before experiment, especially in solid phase extraction (SPE) and it should be done in further study.

Ultrastructeral pathologic changes study of liver cells morphology

Grape product and trans-resveratrol induced ultrastructural changes of liver cells which led to pathologic condition was conducted. However, it appeared that there might be a cytoprotective changes. The pathological conditions accompanied by ultrastructural alterations of the hepatocytes included glycogen and fat accumulation, organelle abnormality, and focal cytoplasmic degeneration. The effects observed in our study were similar to previously reported (Zhou, Sun and Kang, 2001). However, the VH control alcohol induced hepatotoxicity was in liver cells of mice. However, other studies have indicated that increased resistance to the disruption of

Mitochondrial membrane. (Gordon, Rochman, Arai and Lieber, 1982).

Cytotoxic activity of cancer cells

For the study of direct cytotoxicity in cancer cells ethanolic grape pomace extract and trans-resveratrol exhibited selective cytotoxicity towards human cancer cell lines. Cholangiocarcinoma(CCA) is a malignant tumor arising from bile duct epithelium. It is the most common primary liver cancer in Northeast Thailand and is one of the most common fatal cancers in the world. CCA is an aggressive malignancy for which effective treatment is lacking. This cancer is characterized by a poor prognosis and there is an urgent need for novel and effective treatment for CCA. Pancreatic cancer is known as a cancer with poor prognosis. Surgical resection of pancreatic cancer is avialable only in 15 - 20% of all patients. This malignant tumor is highly fatal and poor prognosis because there is no method for early detection and lack of effective treatments.

The search for anticancer agents from natural sources has been successful worldwide. Active constituents have been isolated and are nowadays used to treat human tumors. The ethnopharmacological knowledge is helpful in the searching for plants with potential cytotoxic activity. In the present study, plants which are known as an important source in the search for new anticancer agents are evaluated as candidates for the treatment of human CCA and pancreatic cancer.

In the present study, trans-resveratrol and ethanolic grape pomace extract are evaluated as candidates for the treatment of human CCA and pancreatic cancer. The MTS assay was used to assess the *in vitro* cytotoxic activity of these substances on Panc 2.03 and SNU 1079 cell lines.

Cytotoxic activity of trans-resveratrol on normal human fibroblast cancer Panc 2.03 and SNU 1079 cells

Trans-resveratrol has been reported to exert a variety of biological effects including antioxidant, anti-proliferative and cancer chemopreventive activities (Clement et al., 1998 and Jang et al., 1997). It has previously been shown to have anticancer activities in both cell culture and animal carcinogenesis models of both hematological and solid tumors (Joe et al., 2002).

The present study demonstrated that trans-resveratrol exerted cytotoxic activity in normal human fibroblast, Panc 2.03 and SNU 1079 cells. The data revealed that trans-resveratrol exerted a greater cytotoxic effect on Panc 2.03 cells than normal human fibroblast and SNU 1079 cells. Joe et al. (2002) reported that there is limited information on the toxicity of resveratrol in experimental animals, and there are, apparently, no clinical toxicity data on the use of pure resveratrol in human. Clement et al. (1998) demonstrated that resveratrol is minimally toxic to human peripheral blood cells. The different susceptibility of these cell lines are likely due to the different in genetic background. The results in this study agree with several previous reports which showed the growth inhibitory activity of resveratrol in various human cancer cell lines including epidermoid carcinoma A 431 cells (Ahmad et al., 2001), human SW480 colorectal tumor cells (Delmas et al., 2002), melanoma cells (Niles et al., 2003), Seg-1 esophageal adenocarcinoma cells, MCF7 breast carcinoma cells, HL60 promyelocytic leukemia cells (Joe et al., 2002). These results supported our hypothesis that trans-resveratrol or ethanolic grape pomace extract could inhibit cancer cell growth or enhance the molecular mechanism of the chemopreventive effects on cancer.

Induction of apoptosis by trans-resveratrol

Apoptosis occurs in both normal and neoplastic tissues. Defects in apoptosis mechanisms can extend cell lifespan. Deficiencies in apoptosis also contribute to carcinogenesis by creating a permissive environment for genetic instability and accumulation of gene mutations conferring resistance to cytotoxic anticancer drugs and radiation. Recently, natural medicinal herbs with both antitumor and apoptosis-inducing properties have been described. The present study focus on the search for medicinal plant that induce apoptosis for CCA and pancreatic cancer cells. In this study, the potent cytotoxic activity of trans-resveratrol and ethanolic grape pomace extract on Panc 2.03 and SNU 1079 cells were a result of apoptosis induction. Both cell lines were chosen for apoptosis study because their aggressiveness, poor diagnosis, and higher incidence than other cancer cell lines.

The chemopreventive property and molecular mechanism of trans-resveratrol and ethanolic grape pomace extract induced apoptosis in cells are not well defined but has shown to involved cell cycle arrest. This is the first study of the program cell death and cell cycle induction in cancer cells by ethanolic grape pomace extract.

Trans-resveratrol, through different regulatory mechanisms, has been shown to induce apoptosis in a varity of other human cancer cell types from different origins (Joe et al., 2002; Ferr-Dumazet et al, 2002, Surh et al., 1999 and Clement et al., 1998). To further characterize whether the cytotoxic activity of resveratrol on Panc 2.03 and SNU 1079 cells was related to the induction of apoptosis, we analyzed the cell morphology by microscopic examination, the nuclear morphology by fluorescent microscopy using DAPI staining, and annexin-V staining assay by Flow cytometry. Our results demonstrated that the death of Panc 2.03 and SNU 1079 cells induced by

resveratrol were mediated via apoptosis. After treatment of resveratrol, we observed the typical morphological characteristics of apoptosis, including chromatin condensation and widespread formation of apoptotic bodies. In addition, internucleosomal DNA fragmentation as determined by agarose gel electrophoresis and annexin-V staining assay also clearly revealed that trans-resveratrol induced apoptotic cell death. These results indicate that the cytotoxic effect of resveratrol on Panc 2.03 and SNU 1079 cells are associated with induction of apoptosis. The induction of apoptosis by trans-resveratrol is important because apoptosis is a physiological process that functions as an essential mechanism of tissue homeostasis and is regarded as the preferred way to eliminate unwanted cells (Mukhtar and Ahmad, 1999). The results in this study are in accordance with previous reports which showed the apoptotic effects of resveratrol in a variety of other human cancer cell lines, including HL-60 cells (Surh et al., 1999 and Joe et al., 2002), melanoma cells (Niles et al., 2003), human promyelocytic leukemia cells (Kang et al., 2003) and MCF-7 breast carcinoma A431 cells (Ahmad et al., 2001).

The Bcl-2 family protein plays a crucial role in apoptosis, an increased expression of box can induce apoptosis by suppressing the activity of Bcl-2 (Hockenberry, Giedt, O' NEIL, Manion and Banker, 2002; MacCarthy-Morrogh, Mouzakiti, Townsend, Brimmell and Packham, 1999). Thus, we asked whether the constitutive expression levels of the Bcl-2 family were modified in Panc 2.03 and SNU 1079 cells by resveratrol treatment. Using Western blot analysis, we found that resveratrol reduced the level of Bcl-2 in a dose-dependent manner. Our result is similar to that of Kim et al. (2004) who reported that resveratrol reduced the level of Bcl-2, Bcl-X_L and increased the Bax in breast carcinoma MCF-7 cells.

Caspases are crucial components of the apoptosis pathway (Cohen, 1997). Among them, caspase-3 was reported to be a key protease in apoptosis. Wolter et al. (2001) have suggested the increase of caspase-3 activity in resveratrol induced apoptosis of colon cancer Caco-2 cell line. We therefore determined the involvement of caspase-3 during resveratrol induced apoptosis of Panc 2.03 and SNU 1079 cells. Using Western blot analysis, we found that the level of caspase-3 protein was increased in resveratrol treated Panc 2.03 and SNU 1079 cells in a dose-dependent manner.

Taken together, the decreased level of Bcl-2 protein and increased level of caspase-3 protein might contribute to the induction of apoptosis in resveratrol treated Panc 2.03 and SNU 1079 cells.

Effect of trans-resveratrol on cell cycle distribution

Cell cycle check points can be targeted for cancer therapy either by activating checkpoint-mediated apoptosis or by exploiting chemical sensitivity because of loss of checkpoint function (Stewart, Westfall and Pietenpol, 2003). Trans-resveratrol has been previously shown to induce cell cycle arrest in several human cancer cell lines including Seg-1, Bic-1, esophageal adenomacarcinoma, SW 480 colon carcinoma, and MCF 7 breast cancer cell lines (Joe et al., 2002). The present study was designed to investigate the involvement of cell cycle dysregulation during cytotoxic effect of trans-resveratrol on Panc 2.03 and SNU 1079 cells. Our data demonstrated that the untreated cells showed the expected pattern for continuously growing cells. Whereas trans-resveratrol-treated Panc 2.03 showed dose dependent accumulation in the S phase of cell cycle which correlated with decreased number of cells in the G_1 and

G_2/M phase. Our results are consistant with the results obtained from several other investigators. Previously, the trans-resveratrol was shown to induce S phase arrest of the cell cycle in various human cancer cell lines including colonic cancer cells (Schnider et al., 2000), SW 480 colorectal tumor cells (Delmas et al., 2002), breast cancer MCF 7 cells (Kim et al., 2004) and histiocytic lymphoma U937 cells (Park et al., 2001). Whereas trans-resveratrol-treated SNU 1079 showed cells accumulation in G_o/G_1 phase which correlated with decreased number of cells in S and G_2 phase. Our results are consistent with several previous reports. Ahmad et al. (2001) reported that trans-resveratrol caused a G_1 phase arrest of the cell cycle followed by apoptosis of human epidermoid carcinoma A 431 cells, Zhang et al. (2000) reported that the tea polyphenolic compounds significantly inhibited the proliferation, induced apoptosis, and induced cell cycle arrest at the G1 phase on a rat hepatoma cell line. Sahelian (2005) reported that the ellagic acid, a dietary phenolic compound, significantly reduced the viable cells, induced G_0/G_1 phase arrest of the cell cycle and induced apoptosis in T 24 human bladder cancer cells.

Taken together, these results suggest that trans-resveratrol appears to affect process that are essential to allow cells to progress through the S phase of the cell cycle, presumably by perturbation of the cell entrance into the G2 phase in both Panc 2.03 and SNU 1079 cell lines.

Cytotoxicity and induction of apoptosis by ethanolic grape pomace extract on normal human fibroblast cancer, Panc 2.03 and SNU 1079 cells

There is considerable evidence that consumption of green and yellow vegetables and fruits reduces the risk of human cancer (Block et al., 1992). A wide

variety of naturally occurring substances have been shown to have marked cancer chemopreventive properties (Surh, 1998). Phenolic compounds have widespread occurrence in nature and are consumed by humans through diet containing fruits, vegetables and beverages. These substances also modify critical cellular and molecular events relevant to carcinogenesis. Apoptosis plays an essential role as a protective mechanism against carcinogenesis by eliminating genetically damaged cells, initiated cells or cells progressed to malignancy (Hickman, 1992). Induction of apoptosis thus is a highly desirable mode as a chemotherapeutic as well as a chemopreventive strategy for cancer control. Indeed, many chemopreventive agents of natural origin act through the induction of apoptosis as a mechanism to suppress carcinogenesis (Kelloff et al., 1996).

In the present work, our results showed the cytotoxic effect of ethanolic grape pomace extract, containing high phenolic compound, on normal human fibroblast cancer, Panc 2.03 and SNU 1079 cells in dose dependent manner. The data revealed that ethanolic grape pomace extract exerted a higher cytotoxic effect on Panc 2.03 cells than normal human fibroblast cancer and SNU 1079 cells. The different susceptibility of these cell lines are likely due to a different in genetic background of the cells. The results in our study are supported by Roy et al. (2002) who reported the cytotoxic effects of four natural phenolic compounds including curcumin, yakuchinone B, resveratrol, and capsaicin on acute myeloblastic leukemia (HL-60), chronic myelogenic leukemia (K-562), breast adenocarcinoma (MCF-7) and cervical epithelial carcinoma (HeLa) cells lines.

It has long been known that trans-resveratrol is present in red grape and red wine. Ethanolic grape pomace extract in this study is a crude extract from red grape

and it was proven to contain high concentration of phenolic compounds. Roy et al. (2002) reported that some phenolic compounds such as curcumin, yakuchinone B, resveratrol and capsaicin did not elicit any cytotoxicity towards lymphocytes purified from normal human blood. Sgambato et al. (2001) suggested that trans-resveratrol suggesting that trans-resveratrol can inhibit the activity of DNA polymerase. Our results showed that ethanolic grape pomace extract exhibit higher cytotoxic effects against normal human fibroblast cells than SNU 1079 cells. It is noteworthy that the normal human fibroblast cells displayed a higher sensitivity to ethanolic grape pomace extract is probably related to the high proliferation rate of these cells which have a doubling time (about 48 hours) shorter than those of SNU 1079 cells (about 96 hours). Our results was consistent with Sgambato et al. (2001) who reported that trans-resveratrol showed the high growth inhibition of the HC11 normal mouse mammary cells than human breast, colon and prostate cancer cells. Taken together, the cytotoxic effect of ethanolic grape pomace extract on normal and cancer cells in this study could be partly induced by trans-resveratrol.

Morphological characterization of treated cells revealed that the mode of action of cell death induced by ethanolic grape pomace extract was mediated through apoptosis. Thus, chromatin condensation and nuclear fragmentation of treated cells were clearly evident. Cleavage of DNA at the internucleosomal linker sites yielding DNA fragments is regarded as a biochemical hallmark of apoptosis (Compton, 1992). Appearance of such fragments resulting in a ladder formation was evident when fragmented DNA free of genomic DNA was isolated from Panc 2.03 and SNU 1079 cells treated with ethanolic grape pomace extract and subjected to agarose gel electrophoresis. Our results support the previous study of Roy et al. (2002) which

demonstrated that chemopreventive phenolics curcumin, yakuchinone B and resveratrol were capable of inducing apoptosis in diverse neoplastic cells of human origin.

The Bcl-2 family protein plays a crucial role in apoptosis. Using Western blot analysis, we found that ethanolic grape pomace extract reduced the level of Bcl-2 in a dose-dependent manner. Our result supports the previous work of Kim et al. (2004) who showed that resveratrol reduced the level of Bcl-2, Bcl-X_L and increased the level of Bax in breast carcinoma MCF-7 cells.

Caspases are crucial components of the apoptosis pathway (Cohen, 1997). Caspase-3 was reported to be a key protease in apoptosis. Using Western blot analysis, we found that the level of caspase-3 protein was increased in ethanolic grape pomace extract treated Panc 2.03 and SNU 1079 cells in a dose-dependent manner.

Taken together, the decreased level of Bcl-2 protein and increased level of caspase-3 protein might contribute to the induction of apoptosis in ethanolic grape pomace extract treated Panc 2.03 and SNU 1079 cells.

Effect of ethanolic grape pomace extract on cell cycle distribution

Trans-resveratrol is a natural polyphenolic compound produced by a number of plants and found in high amount in peanuts, seed, grapes or berries as source of human nutrition. Epidermiological studies strongly suggest that trans-resveratrol may act as a cancer chemopreventive compound (Delmas et al., 2002). Trans-resveratrol has been previously shown to induce cell cycle arrest in several human cancer cell lines (Niles et al., 2003). Curcumin is also a natural phenolic compound, and has been

demonstrated to be a potent inhibitor of proliferation and differentiation of endothelial cells by mainly G_0/G_1 and partially G_2/M phase arrest of the cell cycle (Park et al., 2002).

Ethanolic grape pomace extract used in this study was extracted from grapes and proved to have high phenolic substance. In this study, we investigated the involvement of cell cycle dysregulation during cytotoxic effect of ethanolic grape pomace extract in Panc 2.03 and SNU 1079 cells. Our data showed that the Panc 2.03 cells treated with increasing concentratiuon of ethanolic grape pomace extract showed S phase arrest (84.09% as corresponding to control) whereas SNU 1079 cells treated with 800 μg/mL ethanolic grape pomace extract showed a reduction of cells in G_0/G_1 phase of cell cycle and induced a dose dependent increased of cells distribution both S and G_2.phases by 31.94 and 58.33%, respectively. This result was similar to the previous studies. Park et al. (2001) showed that resveratrol inhibits progression through S and G2 phases of the cell cycle in human histiocytic lymphoma U937 cells. Trans-resveratrol-treated HepG2 cells were accumulated in both S and G2/M phases. So, the effect of the polyphenol depends on tissue type, and trans- resveratrol seems to cause a preferential accumulation in S and G2/M. These results suggest that resveratrol treatment induces a specific response in a tissue-dependent manner and this polyphenol may act as cell synchronizating agent (Delmas et al., 2002). The different effect of ethanolic grape pomace extract on phase of the cell cycle arrest between Panc 2.03 and SNU 1079 cells are likely due to a different genetic background of the cells.

Taken together, these results suggest that ethanolic grape pomace extract appears to affect process that are essential to allow cells to progress through the S and

G_2 phase of the cell cycle in SNU 1079 cells, presumably by perturbation of the cell entrance into the S phase or G_2 phase in Panc 2.03 or SNU 1079 cells, respectively.

The active oxidant could mediate an apoptosis-inducing signal and apoptosis is a possible explanation for the anti-cancer effect of trans-resveratrol and grape products such as juice, pomace and red wine. Therefore, trans-resveratrol and ethanolic grape pomace extract are capable of inducing apoptosis in Panc 2.03 and SNU 1079 cells. These effects, together with observations of other investigators, suggest that trans-resveratrol and ethanolic grape pomace extract possess chemotherapeutic property and further development as anticarcinogens along with elucidation of their molecular mechanism are highly warranted.

CHAPTER VI

CONCLUSION

This study demonstrates that the use of Zinfandel red grape products and trans-resveratrol possess anticancer and antiproliferative activities. The characterization of grape products is depended on the concentrations of total phenolic compound (TPC) contents and activities. The TPC contents of ethanolic grape pomace extract, red wine and juice measured by Folin Ciocalteu's method are 4,407.33±13.65, 3,613.00±15.13 and 1,102.67±21.96 mg/L, respectively. Using capillary electrophoresis, the recoveries of trans-resveratrol absorption are not found in plasma when compared with standard by CE because the amount of its recovery might be lower than LOD of capillary electrophoresis.

Trans-resveratrol and grape product treatment caused ultrastructural changes of liver cell morphology including glycogen, fat accumulation and organelle abnormality. The study also suggests that trans-resveratrol and ethanolic grape pomace extract exhibit the potent cytotoxic and apoptotic activities towards cancer cells. Our results demonstrated that trans-resveratrol and ethanolic grape pomace extract exhibited cytotoxic effect on Panc 2.03 and SNU 1079 cell lines in a dose dependent manner as assessed by MTS assay.

Trans-resveratrol and ethanolic grape pomace extract were found to induce S phase arrest of the cell cycle in Panc 2.03 cells, whereas trans-resveratrol was found to induce G_1 phase arrest of the cell cycle in SNU 1079 cells, and ethanolic grape -

pomace extract perturbed cell cycle progression from the S and G_2 phase in SNU 1079 cells. In addition, induction of apoptosis by trans-resveratrol and ethanolic grape pomace extract was confirmed by cellular morphology, nuclear morphology, DNA ladder formation and annexin V-FITC staining. At the same time, the apoptosis related proteins such as a Bcl-2 and pro-caspase 3 were determined by western blot analysis. A decrease in expression of the anti-apoptotic protein Bcl-2 was observed in both treated cells in a dose dependent manner. Trans-resveratrol and ethanolic grape pomace extract could also up-regulate the expression of pro-caspase 3 in a dose dependent manner. These data suggest a possible underlying molecular mechanism whereby trans-resveratrol and ethanolic grape pomace extract could induce the apoptosis signaling pathway in Panc 2.03 and SNU 1079 cells by the regulation of apoptosis related proteins and/or cell cycle dysregulation. Studies are ongoing to identify the molecular mechanisms of these effects. These properties of trans-resveratrol and ethanolic grape pomace extract suggest that it could have a possible therapeutic in pancreatic and cholangiocarcinoma patients. Therefore, trans-resveratrol and ethanolic grape pomace extract possess antiproliferative properties towards cancer cells and could be promising anticarcinogens. Further explanation in the development of both compounds as chemopreventive agents should be highly warranty.

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APPENDICES

APPENDIX A

PREPARATION OF SOLUTION

APPENDIX A

Preparation of solutions

1. Phosphate Buffer Solution pH 7.4

   Solution A

Monobasic sodium phosphate ($NaH_2PO_4.2H_2O$)	31.2	gm
Distilled water	1,000	ml

   Solution B

Dibasic sodium phosphate ($NaHPO_4$ anhydrous)	28.6	gm
Distilled water	1,000	ml

   Working buffer

Solution A	23	ml
Solution B	77	ml
Distilled water	100	ml

   Adjust the pH with 1 N HCl or 1 N NaOH

2. 2.5% Glutaraldehyde

25% glutaraldehyde	5	ml
Working buffer	45	ml

3. Modified Karnovsky's fixative

   0.2 M Phosphate buffer (pH 7.4)

0.2 M Dibasic sodium phosphate	40.5	ml
0.2 M Monobasic sodium phosphate	9.5	ml

10% Paraformaldehyde solution

Paraformaldehyde (powder)	2.0	gm
Distilled water	20	ml

Mix well and warm at 60-65ºC and add 1 N NaOH about 2-3 drops until solution is clear

Modified Karnovsky's fixative

0.2 M phosphate buffer	50.0	ml
25% glutaraldehyde	20.0	ml
10% paraformaldehyde	10.0	ml
Distilled water	20.0	ml

4. Embedding media

Araldite 502	30	gm
DDSA	23	gm
DMP-30	42	drops

Mix well and add 1.3 ml of acetone and again mix well

5. 4% Uranyl acetate

Uranyl acetate	0.2	gm
Stain water	5	ml

6. 0.5% Lead citrate

Lead citrate	0.025	gm
Stain water	5	ml

Add 2-3 pellets of NaOH until solution is clear

7. 1% Toluidine blue		
Toluidine blue	1.0	gm
Borax ($Na_2B_4O_7.10H_2O$)	1.0	ml
Distilled water	99.0	ml
8. 0.85% NaCl		
NaCl	0.85	gm
Distilled water	100.0	ml
9. Developer D-19		
Warm Distilled water (52ºC)	600	ml
Elon	9	gm
Sodium sulfite (Na_2SO_3)	90	gm
Hydroquinone	8	gm
Sodium carbonate ($NaCO_3$)	52.5	gm
Potassium bromide (KBr)	5	gm
10.Developer D-163		
Warm distilled water (52ºC)	600	ml
Elon	2.2	gm
Sodium sulfite (Na_2SO_3)	75	gm
Hydroquinone	17	gm
Sodium carbonate ($NaCO_3$)	65	gm
Potassium bromide (KBr)	15	gm
11.Fixer		
Warm distilled water (52ºC)	600	ml
Sodium thiosulphate ($Na_2S_2O_3.5H_2O$)	240	gm

Sodium sulfite (Na_2SO_3)	15	gm
Acetic acid (CH_3COOH) 28%	48	ml
Borax	7.5	gm
Potassium bromide (KBr)	15	gm

APPENDIX B

TEM PREPARATION TECHNIQUES

APPENDIX B

TEM preparation techniques

I. Negative film processing for TEM

1. The negative film was placed in developer D-19 and agitated about 1 minute in the dark room.

2. The negative film was placed in a stop bath (the mixer of tab water and acetic acid) for 10 second to stop the reaction.

3. The negative film was placed in fixer I for 10 second.

4. The negative film was placed in fixer II for 20-30 minutes.

5. The developed negative film could now be visualized under room light.

6. The negative film was washed in running tap water for 20-30 minutes.

7. The negative film was placed in photo-flo for 10 second.

8. The negative film was dried in the film dryer.

II. Enlargement and printing

1. The negative was placed on the negative holder with the emulsion upside down.

2. The negative holder was placed in enlarger.

3. The enlarger’s light was turned on.

4. Set sliding bars and paper stops on easel to give desired size of print and margins of desired width.

5. Turn switch on timer to focus.

6. The appropriate aperture was set.

7. The enlarger's light was turned off.

8. The photographic paper emulsion upside down was placed on the easel.

9. The light was turned on with appropriate exposure time.

10. The exposed paper was placed in developer D-163 (D-163: water = 1:3).

11. The developed paper was placed in a stop bath for 2-3 second.

12. The photograph was placed in fixer I for 10 second.

13. The photograph was placed in fixer II for 20-30 minutes.

14. The photograph was washed in running water for 20-30 minutes.

15. The photograph was dried in the print dryer.

III. Transmission electron microscopy (TEM)

1. Hepatocytes to be studied by TEM were cut into small pieces and were prepared as the followings.

2. Fix the specimen in modified Karnovsky's fixative in 0.1 M phosphate buffer at 4ºC overnight.

3. Wash several times in the same buffer at room temperature.

4. Post fixed in 1% osmium tetroxide in 0.1 M phosphate buffer at 4ºC for 2-4 hours.

5. Wash several times in the same buffer at room temperature.

6. Dehydrate in increasing concentration of acetone (50, 70, 80, 90, 95, 100, 100 and 100%) 5 minutes each concentration.

7. Infiltrate the specimen with mixture of araldite 502 and acetone at the ratio of 1:1 for 1 hour then change to pure araldite 502 overnight.

8. Embed in pure araldite 502 in flat mold and leave at 60ºC in the oven for 2-3 days. Each block of specimens was trimmed by a single edge razor blade under a

stereoscopic microscope. Thick section were cut by ultramicrotome (Reichert ultracut S, Austria) using a new glass knife and picked up on a glass slide, stained with 1.0% toluidine blue and examined under a light microscope to find the affected area for thin sectioning. The affected area were then cut and the sections showing silver to gold interference colors (60-90 ηm) were picked up by an uncoated 200-300 mesh copper grids. After drying, the sections were double stained with 4% uranyl acetate for 45 minutes and 0.5% lead citrate for 8-15 minutes. The specimens on the grid were examined under transmission electron microscope, micrographs taken, developed and printed.

APPENDIX C

REAGENTS FOR CELL CULTURE

APPENDIX C

Reagents for cell culture

I. Reagents for cell culture

1. Unsupplemented RPMI 1640 Medium

   RPMI 1640 powder (Catalog No,Gibco BRL, NY, USA)

$NaHCO_3$	2	g
Hepes	2.86	g
Penicillin G (Dumex)	5	units/ml
Streptomycin (Dumex)	50	µg/ml
Bioresearch grade distilled water up to	1	liter

   Sterilized by 0.22 µm Millipore membrane filtration

2. Supplemented RPMI 1640 Medium

Unsupplemented RPMI 1640 Medium	100	ml
FBS	10	ml
Penicillin (200,000 U/ml)	50	µl
Streptomicin (200,000 µg/ml)	50	µl
Fungizone (amphotericin B) (5,000 µg/ml)	20	µl

   Sterilized by 0.22 µm Millipore membrane filtration, store at 4ºC

3. 0.25% Trypsin-EDTA

2.5% Trypsin solution (Gibco BRL)	2	ml
Versine EDTA(Gibco BRL)	0.1	g

Filter through 0.22 micron Millipore filter membrane and store at 4ºC.

4. EDTA

EDTA (Gibco BRL)	0.1	g
PBS	500	ml

Sterilize by autoclaving at 121ºC, 15 pounds per square inch for 15 minutes and store at room temperature.

5. Phosphate Buffered Saline (PBS), 0.15 M

NaCl	8.0	g
KCl	0.2	g
KH_2PO_4	0.2	g
Na_2HPO_4	1.15	g
Bioresearch grade distilled water	90	ml

Sterilize by autoclave at 121ºC, 15 pounds per square inch for 15 minutes and store at room temperature.

6. 1M HEPES pH 7.5

HEPES	23.53	g
Add distilled water to final volume of	100	ml.

Adjust to pH 7.5 with NaOH store at 4ºC.

7. 7.5% $NaHCO_3$

$NaHCO_3$	7.5	g
Add distilled water to final volume of	100	ml

Sterilize (121ºC, 15 lb for 15 min) store at 4ºC.

8. Antibiotics

a. Penicillin 200,000 U/ml

Penicillin	100,000	U

Add Distilled water to final volume of	5	ml
Store at -20ºC.		

b. Streptomicin (200,000 µg/ml)

Streptomicin	1	g
Add distilled water to final volume of	5	ml
Store at -20ºC.		

c. Fungizone (5,000 µg/ml)

Fungizone	50	mg
Add distilled water to final volume of	10	ml
Store at -20ºC.		

9. 0.25% Trypsin-EDTA

2.5% Trypsin solution (Gibco BRL)	2	ml
Versine EDTA(Gibco BRL)	0.1	g

Filter through 0.22 micron Millipore filter membrane and store at 4ºC.

APPENDIX D

REAGENTS FOR STAINING & WESTERN BLOT

APPENDIX D

Reagents for staining & western blot

I. Reagents for staining

1. DAPI stock solution (1 mg/ml)

DAPI	1	mg
Distilled water	1	ml

2. DAPI working solution (1 µg/ml)

DAPI stock solution (1 mg/ml)	1	µl
Methanol (CH_3OH)	999	µl

Store at 4ºC.

3. 10% Glycerol in PBS

Glycerin 99%	100	µl
PBS	890	µl

Store at 4ºC.

4. 0.1% Typan Blue

Typan Blue	0.1	g
NaCl	0.82	g
NaN_3	0.40	g
6% acetic acid	100	ml

5. Comassie Blilliant Blue

Comassie Blilliant Blue	2.5	g
Methanol	500	ml

Acetic acid	100	ml
Distilled water	400	ml

6. 0.2% Coomassie blilliant-blue R 250 solution

Coomassie blilliant - blue R 250	2	g
Absolute methanol	500	ml
Acetic acid, glacial	100	ml
Distilled water	1,000	ml

7. Destain solution (5% methanol, 7%acetic acid)

Acetic acid, glacial	140	ml
Absolute methanol	100	ml
Distilled water	2,000	

8. Ethidium bromide

Ethidium bromide	0.2	g
Distilled water	20	ml

II. Reagents for gel electrophoresis & SDS-PAGE preparation

1. 10x TBE buffer (pH 8.0)

Tris-base	54	g
Boric acid	27.5	g
0.5 M Na EDTA (pH 8.0)	20	ml
Distilled water to final volume of	500	ml

Store at 4ºC.

2. 1x TBE buffer (pH 8.0)

10x TBE buffer (pH 8.0)	10	ml
Distilled water	90	ml

Store at room temperature.

3.	1x TE buffer (pH 8.0)		
	1 M Tris-HCl	10	ml
	0.5 M EDTA	2	ml
	Distilled water to final volume of	1,000	ml
	Store at 4ºC.		
4.	5x Tris-Borate Buffered		
	Tris base	54	g
	Boric acid	27.5	g
	EDTA, pH 8.0	20	ml
	Distilled water (analytical grade)	1,000	ml
5.	6x Gel – Loading Buffer		
	Bromophenol blue	0.25	%
	Xylene cyanol	0.25	%
	Glycerol	40	%
6.	Stock Acryamide (30%)		
	Acryamide (Sigma)	30	g
	N, N-bis-methylene acrylamide	0.8	g
	Analytical grade distilled water to	100	ml
	Filter through filter paper and store at 4^{o}C		
7.	Eletrophoresis Buffer pH 8.0		
	Trisma base	3.0	g
	Glycine	14.4	g
	SDS	1	g
	Distilled water (analytical grade)	1,000	ml
	Adjust pH with HCl.		

8. Sample Buffer 5x, pH 6.8

Trisma base	0.3784	g
SDS	0.5	g
Glycerol	5	ml
2-mercaptoethanol	2.5	ml
Bromphenol blue	0.005	g
Distilled water (analytical grade)	10	ml

9. Towbin's Buffer

Trisma base	3.035	g
Glycine	14.413	g
Methanol	200	ml
Distilled water (analytical grade)	1,000	ml

10. 20% Sodium dodecyl sulphate solution

Sodium dodecyl sulphate	20	g
Distilled water to a final volume	70	ml

Adjust volume to 100 ml

11. 4x stacking gel buffer (0.5 M Tris – HCl , pH 6.8, 0.4% SDS)

Tris base	15.1	g
20% SDS solution	5	ml
Distilled water	200	ml

Adjust to pH to 6.8 with Conc. HCl

N, N, N', N' tetramethylenediamine, TEMED	0.5	ml

Adjust volume to 250 ml with distilled water and store at $4^{o}C$

12. 4X separating gel buffer (1.5 M Tris – HCl , pH 8.8, 0.4% SDS)

Tris base	90.83	g
20% SDS solution	10	ml
Distilled water	300	ml

Adjust to pH to 8.8 with Conc. HCl

N, N, N', N' tetramethylenediamine, TEMED	0.5	ml

Adjust volume to 250 ml with distilled water and store at 4^{o}C

13. 10X Running buffer (0.25 M tris , 1.92 M glycine)

Tris base	30.28	g
Glycine	144.13	g
Distilled water to a final volume	1,000	ml

Adjust to pH to 8.3 with Conc. HCl , store at 4 ^{o}C

14. 1X Running buffer (0.025 M tris, 0.192 M glycine 0.1% SDS)

10X Running buffer	200	ml
20% SDS	10	ml
Distilled water to a final volume	2,000	ml

Store at 4^{o}C

15. 1% Ammonium persulphate solution

Ammonium persulphate	50	mg
Distilled water	4	ml

Adjust volume to 5 ml.

Store -20^{o}C

16. 12.5 % separating acrylamide solution

30 % Acrylamide solution	8.8	ml

4X separating gel buffer	6	ml
Distilled water	8.6	ml

17. 10% SDS

SDS	1	g
Distilled water	10	ml

18. 10% Ammonium persulphate

Ammonium persulphate	0.1	g
Distilled water	1,000	ml

19. 50x TAE

Tris	242	g
Acetic acid	57.1	ml
0.5 M EDTA pH 8.0	100	ml
Distilled water	1,000	ml

III. Reagents for Immunoblot (Bolt & Mahoney, 1997)

1. 10x Blotting buffer (400 mM Tris, 200 mM sodium acetate, and 20 mM EDTA)

Tris	48.44	g
Sodium acetate	16.40	g
EDTA	7.44	g
Distilled water	1,000	ml

Adjust pH with glacial acetic acid to 7.4

2. Working blotting (40 mM Tris, 20 mM sodium acetate, and 2 mM EDTA, pH 7.4, 20% (v/v) methanol, 0.05% (w/v) SDS)

10x blotting buffer	100	ml

Methanol	200	ml
20% SDS	2.5	ml
Distilled water	1,000	ml

Store 4ºC

3. 0.1% (v/v) Tween-20 in PBS pH 7.5

Tween-20	100	µl
PBS	100	ml

4. Blocking solution (5% skim milk , 0.1% tween-20 in PBS, pH 7.5)

Non – fat skim milk powder	5	g
PBS	500	ml
Tween-20	0.5	ml

Store 4ºC

5. Antibodies diluent (1% skim milk , 0.1% tween-20 in PBS, pH 7.5)

Non – fat skim milk powder	1	g
PBS	500	ml
Tween-20	0.5	ml

Store 4ºC

6. 0.1 M Phosphate buffered saline (PBS) pH 7.5 (20X)

$Na_2HPO_4.2H_2O$	14.42	g
KH_2PO_4	2.96	g
NaCl	8.77	g
Distilled water	1,000	ml

Adjust pH to 7.5 with 1 N NaOH

7. 50 mM Tris-HCL pH 7.5

Trisma base	3.028	g
Dissolve in Distilled water	400	ml.
Adjust pH to 7.5 with 1 N HCl		
Adjust final volume to	500	ml.

8. Lysis Buffer (RIPA buffer)

1 mM EDTA	10	µl
1 mM DTT	1	µl
1% NP 40	1	ml
2 mM Leupeptin	10	µl
1 mM E-64	1	µl
200 mM PMSF pH 7.4	10	µl
PBS 1X	100	ml

APPENDIX E

DETECTION KITS

APPENDIX E

Detecion kits

I. Genomic DNA detection kit (Mini) (Blood/Culture cell). (Real Biotech Corperation, Taiwan. catalog number YGB 100).

II. Annexin V-FITC apoptosis detection kit II. (BD Bioscience, USA. catalog number 556570).

III. ECL chemiluminescence detection kit. (Pierce Biotechnology, Inc., Rockford, USA).

I. Genomic DNA extraction

1.Wash cell pellets with PBS and then centrifuge for 5minutes.

2. Add GB lysis 100 µl at a concentration of 1 x 10^6 cells/ml., incubate for 10 minutes and then add 1% NP-40.

3. After centrifugation for 5 minutes at 1600 X g. Supernatant was collected and the extraction was repeated with the same amount of lysis buffer.

4. Treated with RNase A (final concentration 5 µg/µL) for 90 minutes at 56ºC.

5. Digested with proteinase K (final concentration 2.5 µg/µL) for 60 minutes at 37ºC.

6. DNA is precipitated with 2.5 volume ethanol and then centrifugation for 10 minutes at 10,000 X g.

7. Air dry and resuspended in 50 µL TE buffer.

8. Separated by electrophoresis in 1.6% agarose.

II. Annexin V-FITC Staining

1. Wash cells twice with cold PBS and then resuspend cells in 1X binding buffer.

2. Transfer 100 µl of the sample solution to a 5 ml culture tube.

3. Add 5 µl of Annexin V-FITC and 5 µl of PI.

4. Vortex the cells and incubate for 15 min at RT (25°C) in the dark.

5. Add 400 µl of 1X binding buffer to each tube. Analyze by flow cytometry within one hour.

III. ECL chemiluminescence detection kit

1. After transferred protein binding site onto blotted nitrocellulose membrane.
2. Mixed solution A and B and incubated on membrane for 5 minutes.
3. Protein was detected and visualized by using ECL chemiluminescence system as described in the manufacturing instruction (Pierce Biotechnology, Inc., Rockford, USA).
4. The protein bands were quantified by autoluminographs.

APPENDIX F

DATA ANALYSIS

APPENDIX F

Data analysis

Table 1 Standard Gallic acid (GAE) concentration (mg/mL) measured by Folin-Ciocalteau's phenol reagent as modified from the method of Matthaus (2002), n = 3.

GAE concentration (mg/mL)	OD 750 nm
0.00	0.009
0.05	0.140
0.10	0.262
0.15	0.403
0.20	0.529
0.25	0.633

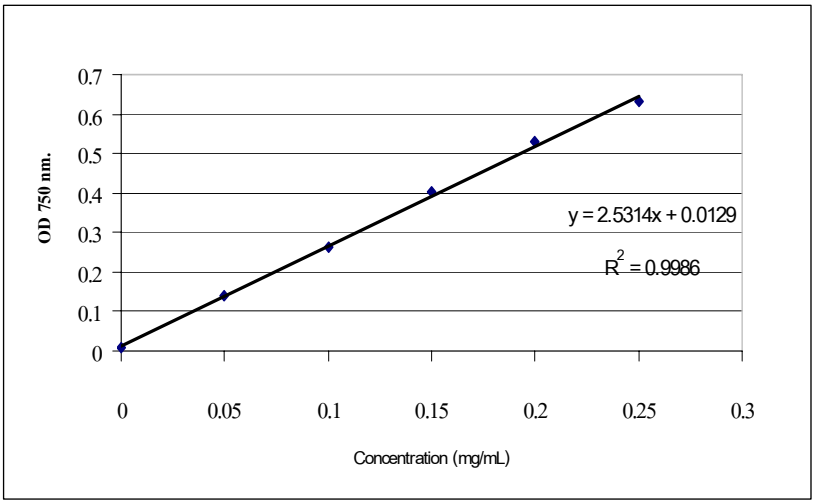

Figure 1 Calibration curve of standard gallic acid concentration (mg/mL) measured by Folin-Ciocalteau's method with slightly modification.

Table 2 Standard bovine serum albumin (BSA) concentration (µg/mL) measured by Bradford protein assay using coomassie protein assay kit reagent (Pierce, Biotechnology), n = 3.

Final BSA (µg/ml)	BSA Stock (µl)	Lysis Buffer (µl)	Milli Q Water (µl)	Total volume (µl)	OD 595 nm
0	0	10	30	40	0
100	16	6	18	40	0.102
125	20	5	15	40	0.149
150	24	4	12	40	0.181
175	28	3	9	40	0.207
200	32	2	6	40	0.238
225	36	1	3	40	0.272
250	40	0	0	40	0.299

Method for measuring

1. add 10 µl of BSA or protein concentration in 96 well.

2. add 150 µl of Bradford coomassie protein assay reagent in 96 well and Mix,

3. Incubate 10 min at room temperature and measure by ELISA READER at detection wavelength 595 nm.

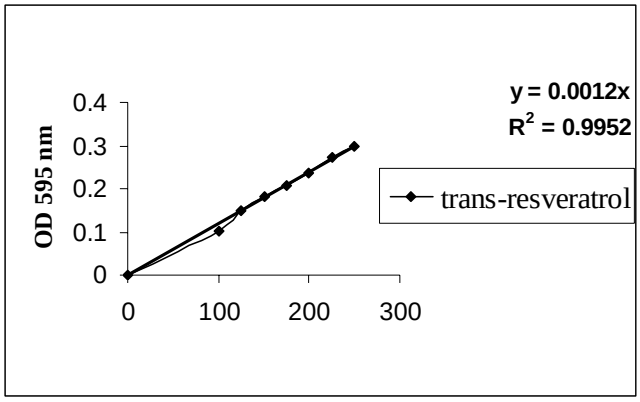

Figure 2 Calibration curve of standard BSA concentration (µg/mL) measured by ELISA plate reader.

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