การชักนำให้เกิดการยับยั้งวัฏจักรเซลล์และเกิดเอพอพโตซิส ในเซลล์มะเร็งเม็ดเลือดขาวของมนุษย์โดยซิตรอล

นาย ชาติกร บุญไกร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาเภสัชวิทยา (สหสาขาวิชา)
บัณฑิตวิทยาลัย จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2552

INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS IN HUMAN B-LYMPHOMA CELLS BY CITRAL

Mr. Chatikorn Bunkrai

A Thesis Submitted in Partial Fulfillment of the Requirements

for the Degree of Master of Science Program in Pharmacology

(Interdisciplinary Program)

Graduate School

Chulalongkorn University

Academic Year 2009

Thesis Title	INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS IN HUMAN B-LYMPHOMA CELLS BY CITRAL
By	Mr. Chatikorn Bunkrai
Field of Study	Pharmacology
Thesis Advisor	Assistant Professor Wacharee Limpanasithikul, Ph.D.
Thesis Co-Advisor	Professor Tada Sueblinvong, M.D.

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

…….. Dean of the Graduate School
(Associate Professor Pornpote Piumsomboon, Ph.D.)

THESIS COMMITTEE

…….. Chairman
(Assistant Professor Suree Jianmongkol, Ph.D.)

…….……. Thesis Advisor
(Assistant Professor Wacharee Limpanasithikul, Ph.D.)

…….. Thesis Co-Advisor
(Professor Tada Sueblinvong, M.D.)

…….. Examiner
(Assistant Professor Naowarat Suthamnatpong, Ph.D.)

…….. Examiner
(Associate Professor Suvara Wattanapitayakul, Ph.D.)

ชาติกร บุญไกร : การชักนำให้เกิดการยับยั้งวัฏจักรเซลล์และเกิดเอพอพโตซิส ในเซลล์มะเร็งเม็ดเลือดขาวของมนุษย์โดยซิตรอล. (INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS IN HUMAN B-LYMPHOMA CELLS BY CITRAL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : ผศ. ดร. วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : ศ. พญ. ธาดา สืบหลินวงศ์, 81 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ต้านมะเร็งของซิตรอลในเซลล์มะเร็งเม็ดเลือดขาวของมนุษย์ (เซลล์รามอส) โดยทำการศึกษาฤทธิ์ของซิตรอลในการเหนี่ยวนำให้เซลล์มะเร็งตายแบบเอพอพโตซิส การยับยั้งวัฏจักรเซลล์ และกลไกการการเหนี่ยวนำให้เซลล์ตายแบบเอพอพโตซิส การศึกษาฤทธิ์การเหนี่ยวนำให้เซลล์ตายแบบเอพอพโตซิส ด้วยวิธีการย้อมเซลล์ด้วย annexin V-FITC และ propidium iodide (PI) และวัดผลโดยใช้ฟลูออเรสเซนโฟลไซโตมิเตอร์ พบว่าซิตรอลที่ความเข้มข้น 37.5, 75 และ 150 ไมโครโมลาร์ เหนี่ยวนำให้เซลล์รามอสตายได้ตามความเข้มข้นของสารและตามระยะเวลาที่เซลล์ได้รับสาร ลักษณะการตายของเซลล์เป็นแบบเอพอพโตซิสเป็นหลัก พบลักษณะการตายแบบอื่นเมื่อเซลล์ได้รับซิตรอลที่ความเข้มข้น 150 ไมโครโมลาร์ ซิตรอลชักนำให้เซลล์ตายแบบเอพอพโตซิสหลังจากได้รับสาร 3 ชั่วโมง ฤทธิ์เหนี่ยวนำให้เซลล์ตายแบบเอพอพโตซิสของซิตรอล ยืนยันได้จากการวัดปริมาณเซลล์ที่ตายแบบเอพอพโตซิสซึ่งมีดีเอ็นเอน้อยกว่ากว่าเซลล์ปกติ (hypodiploid DNA) โดยการย้อมเซลล์ด้วย PI ซิตรอลออกฤทธิ์เหนี่ยวนำให้เซลล์เม็ดเลือดขาวปกติตายได้น้อยกว่าเซลล์มะเร็ง จากการศึกษาฤทธิ์ของสารต่อการเปลี่ยนแปลงลักษณะของวัฏจักรเซลล์โดยทำการล้างเซลล์ที่ได้รับซิตรอลแล้วนำไปเพาะเลี้ยงต่อเพื่อให้เซลล์แบ่งตัว ทำการศึกษาวัฏจักรเซลล์โดยการย้อมดูปริมาณดีเอนเอของเซลล์ในระยะต่างๆ ของวัฏจักรด้วย PI ไม่พบการเปลี่ยนแปลงลักษณะของวัฏจักรเซลล์ในเซลล์รามอสที่ได้รับสารซิตรอลทุกความเข้มข้น ในการศึกษากลไกการการออกฤทธิ์ของซิตรอล ได้ทำศึกษาวิถีการเหนี่ยวนำให้เซลล์ตายผ่านทางตัวรับบนผิวเซลล์ (Fas) โดยใช้แอนติบอดีต่อ FasL ยับยั้งการจับของ Fas และ Fas ligand พบว่าการยับยั้งดังกล่าวไม่มีผลต่อการออกฤทธิ์ของซิตรอล การศึกษาวิถีการเหนี่ยวนำให้เซลล์ตายผ่านทางไมโตคอนเดรียทำโดยวิเคราะห์การแสดงออกในระดับ mRNA ของโปรตีนกลุ่ม BCL-2 (BCL-2, BCL-XL, BAX, BAK) ซึ่งควบคุมการเกิดเอพอพโตซิสผ่านทางไมโตคอนเดรีย และ p53 พบว่าซิตรอลยับยั้งการแสดงออกของ BCL-2 mRNA ได้เพียงตัวเดียว ไม่มีผลต่อการแสดงออกของโปรตีนอื่นๆ และศึกษาการเหนี่ยวนำให้เซลล์ตายของสารเกิดจากการกระตุ้นการทำงานของเอนไซม์กลุ่มคาสเปสมากน้อยเพียงใดโดยใช้สาร Z-VAD-FMK ยับยั้งการทำงานของเอนไซม์ พบว่า Z-VAD-FMK ยับยั้งฤทธิ์ของซิตรอลได้ชัดเจนอย่างมีนัยทางสถิติ แสดงให้เห็นว่าการเหนี่ยวนำให้เซลล์ตายแบบเอพอพโตซิสโดยซิตรอลเกิดจากการกระตุ้นการทำงานของเอนไซม์ caspases ผลจากการศึกษาครั้งนี้แสดงให้เห็นว่าซิตรอลชักนำให้เซลล์มะเร็งเม็ดเลือดขาวตายแบบเอพอพโตซิส มีกลไกการออกฤทธิ์กระตุ้นผ่านทางไมโตคอนเดรียโดยลดการแสดงออกของ BCL-2 mRNA และกระตุ้นการทำงานของเอนไซม์ caspases เป็นหลัก

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

498 90809 20 : MAJOR PHARMACOLOGY

KEYWORDS : APOPTOSIS / CELL CYCLE / RAMOS CELLS / CITRAL

CHATIKORN BUNKRAI : INDUCTION OF CELL CYCLE ARREST AND APOPTOSIS IN HUMAN B-LYMPHOMA CELLS BY CITRAL. THESIS ADVISOR : ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., THESIS CO-ADVISOR : PROF. TADA SUEBLINVONG, M.D., 81 pp.

This study intended to evaluate anti-tumor activity of citral on human B-lymphoma cells, Ramos cells. The effects of citral on apoptotic induction, on cell cycle as well as its mechanism of action on apoptotic induction were studied. The apoptotic induction of citral on Ramos cells was examined by annexin V-FITC and propidium iodide (PI) staining monitored by fluorescence flow cytometer. The results demonstrated that 37.5, 75 and 150 μM citral induced Ramos cell death in a concentration- and time-dependent manner. It induced mainly apoptosis on Ramos cells. Other types of cell death were detected only when the cells were treated with 150 μM citral. It induced Ramos cell apoptosis after 3 h of exposure. The apoptotic activity of citral was confirmed by assessing apoptotic cells with hypodiploid DNA when compare to diploid DNA in viable cells by PI staining. The content of DNA in the cells was detected by flow cytometer. Citral, at 37.5, and 75 μM, induced normal PBMC death much less than Ramos cell death. The effect of citral on the cell cycle was investigated by washing citral-treated Ramos cells and allowed them to proliferate in fresh medium. Distribution of the treated cells in the cell cycle was detected by PI staining. There was no change in the cell cycle pattern in the citral treated cells, at all concentrations of citral. The mechanism of apoptotic induction of citral was also investigated. Apoptotic induction of citral via death receptor pathway was determined by using anti-FAS ligand antibody to inhibit Fas-Fas ligand binding. The results showed that anti-Fas ligand didn't inhibit citral activity. The effect of citral on the mitochondrial pathway was evaluated by analyzing the mRNA expression of the BCL-2 family proteins (BCL-2, BCL-XL, BAX, BAK) and p53, which control the mitochondrial pathway of apoptosis. It was revealed that citral only inhibited BCL-2 mRNA expression. It didn't have any effect on the mRNA expression of the other proteins in the study. To study the degree of dependency on caspase activation of citral activity, a pan caspase inhibitor, Z-VAD-FMK, was used. This inhibitor significantly decreased apoptotic induction activity of citral. The results in this study indicate that citral can induced human B-lymphoma cells apoptosis via the mitochondrial pathway by down regulating BCL-2 expression and depending mainly on caspases activation.

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

Academic Year : 2009 Advisor's Signature .....

Co-Advisor's Signature .....

ACKNOWLEDGMENTS

I wish to express my sincere gratitude to my advisor, Asst. Prof. Dr. Wacharee Limpanasithikul, Department of Pharmacology and my co-advisor Prof. Dr. Tada Sueblinvong, Department of Biochemistry, Faculty of Medicine whose encouraging guidance and open-mindedness to discuss anything anytime during the long working process, which enable me to accomplish this thesis.

I would also express my sincere appreciation to the committee of this thesis examination; Asst. Prof. Dr. Suree Jianmongkol, Department of Pharmacology and Physiology, Faculty of Pharmaceutical Sciences. Asst. Prof. Dr. Naowarat Suthamnatpong, Department of Pharmacology, Faculty of Veterinary Science and Assoc. Prof. Dr. Suvara Wattanapitayakul, Department of Pharmacology, Faculty of Medicine, Srinakharinwirot University for their constructive comment and suggestions.

I would like to thank the National Blood Bank, Thai Red Cross Society for providing whole blood samples from healthy donors.

I would like to give my special thanks to Mr. Atinop Pongpanich and Mrs. Panichaya Puripokai, Immunology unit, Department of Microbiology, Faculty of Medicine for advising FACs analysis technique.

I wish to thank all staff member of the Department of Pharmacology, Faculty of Medicine for their help.

This study was partly supported by the Graduate Research Funds from the Ministry of University Affairs and the Graduate School, Chulalongkorn University.

Finally, I would like to eternally thank to my family for their encouragement, love and caring which make everything possible.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF ABBREVIATIONS

AIF	apoptosis-inducing factor
Apaf-1	apoptosis protease-activating factor 1
APC	anaphasepromoting complex
ATCC	American Type Cell Culture
ATP	adenosine triphosphate
BAD	BCL-2 antagonist of cell death
BAK	BCL-2-antagonist/killer-1
BAX	BCL-2 associated x protein
BCL-2	B-cell CLL/Lymphoma 2
BCL-XL	BCL-2 related gene, long isoform
BH	BCL-2 homology domain
BH3	BCL-2 homology 3 domain
BID	BH3 interacting domain death agonist
BIK	BCL-2-interacting killer
BIM	BCL-2-like-11
BIR	baculovirus IAP repeat
BMF	BCL-2 modifying factor
CAD	caspase-activated DNase
CARD	caspase recruitment domains
CDK	cyclin-dependent kinases
Cip/Kip	CDK inhibitory Protein/Kinase Inhibitor protein
CKI	CDK inhibitors
CO_2	carbon dioxide
CTL	cytotoxic T lymphocytes
DD	death domain
DED	death effector domain
DEPC	diethyl pyrocarbonate
DISC	death inducing signaling complex
DNA	deoxyribonucleic acid

DRs	death receptors
EndoG	endonuclease G
ER	endoplasmic reticulum
FADD	Fas associated death domain
FasL	Fas ligand
FBS	fetal bovine serum
FLIP	FLICE-inhibitory protein
FLIPL	FLICE-inhibitory protein long form
FLIPS	FLICE-inhibitory protein short form
h	hour
HCI	hydrochloric acid
HMGB1	high mobility group protein B1
Htra2	high temperature requirement A2
IAPs	inhibitors of apoptosis protein
ICAD	inhibitor of caspase-activated DNase
INK4	inhibitors of CDK4
mg	milligram(s)
ml	milliliter(s)
M	molar (mole per liter)
MCL-1	Myeloid cell leukemia 1
MFG-E8	milk fat globule–EGF factor-8 protein
MMP	mitochondrial membrane permeabilization
MPF	mitosis promoting factor
ng	nanogram(s)
NaCl	sodium chloride
NAIP	neuronal apoptosis inhibitory protein
NF-ΚB	nuclear factor ΚB
NK	Natural killer cells
ox-LDL	oxidized low-density lipoprotein
PBS	phosphate buffer saline solution
PBMCs	peripheral blood mononuclear cells

PCNA	proliferating cell nuclear antigen
PS	phosphatidylserine
pH	the negative logarithm of hydrogen ion concentration
Rb	retinoblastoma protein
rpm	revolution per minute
S.E.	standard error
Smac	second mitochondria-derived activator of caspases
SR-A	scavenger receptor A
TGF-β	transforming growth factor β
TM	transmembrane domains
TNFR	tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-linked mammalian inhibitor of apoptosis protein
^{0}C	degree Celsius
μg	microgram(s)
$\Delta\psi_m$	mitochondrial membrane potential

CHAPTER I

INTRODUCTION

Background and Rationale

Cancer is still one of the leading life-threatenting diseases on people around the world as well as in Thailand. It has been estimated by the World Health Organization (WHO) that new cases of cancer per year will rise up to 16 million by the year 2020 **[1]**. Cancer cells are arisen from multistep processes of the progressive transformation of normal cells into highly malignant cells. It is generally accepted that the underlying etiology of cancer is the consequence of both dysregulated proliferation and evasion of programmed cell death by apoptosis. Chemotherapy with anticancer drugs is one of the recent modalities of cancer treatment. Many of the currently used anti-cancer drugs were developed on the fact that cancer is basically a disease of rapid or sustained cell proliferation. However, burden of cancer treatment with these anticancer drugs often comes from adverse drug reactions and cytotoxic effects on rapid dividing normal cells. Drug resistance is also the other critical problem leading to treatment failure. The phenomenon of drug resistance is in part due to the failure of apoptosis induction **[2]**. Therefore, new therapeutic strategies as well as new therapeutic agents for cancer are still required.

Apoptosis is a form of programmed cell death that enables the selective removal of unwanted cells from tissue without inflammatory response. It is essential for homeostasis in multicellular organisms **[3]**. Defective apoptosis of damaged and potentially harmful cells is considered to result to the development of cancer. Advanced knowledge on apoptosis not only provided the knowledge and understanding the development of cancer but also lead to the developments of therapeutic targets and strategies. To date, the molecular mechanisms of many currently use anticancer drugs have been clarified that they induce cell death by apoptotic induction. Several proteins that play role in the apoptosis process have been found to be mutated in cancer cells. These molecules may be new targets for cancer therapy or cancer prevention.

Drug discovery from medicinal plants has played an important role in cancer therapy. Many plant active constituents and their derivatives have been clinically used to treat cancer. Much effort has been made to search for new anticancer agents from plants. The candidates

should have apoptotic inducing properties on cancer cells, exhibit less toxicity to normal cells at therapeutic concentrations. These compounds may also be used as lead compounds for the synthesis and development of novel anticancer drugs.

Essential oils are one of the bioactive constituents which are found abundantly in plants. They are used worldwide in pharmaceuticals, cosmetics, perfume, food & beverages and aromatherapy. Many of them have been reported to be anti-infective agents against viruses, bacteria and fungi. In recent year, they becomes the focus as one of the novel plant products for the development of new anticancer agents.

Citral (3,7-dimethyl-2,6-octadienal) is a key component of essential oil found in several Thai medicinal and edible plants such as lemon grass (*Cymbopogon citratus*), lemon balm (*Melissa officinalis*), and *Litsea cubeba*. Essential oils containing citral from some medicinal plants have been investigated for possible anticancer potential in many cancer cell lines as well as *in vivo*. Citral was reported to induce apoptosis and arrest the cell cycle in human breast cancer cells MCF-7 **[4]**. This study intended to investigate anticancer potential of citral on human B cell lymphoma, Ramos cells, and explored its mechanisms of action as anticancer agent.

Objectives

1. To study the effects of citral on apoptosis induction and cell cycle arrest on human B-lymphoma cells.
2. To study the selectivity of citral on tumor cells by comparing to normal cells.
3. To investigate the mechanism of apoptosis induction and cell cycle arrest of citral on human B-lymphoma cells.

Hypothesis

Citral has ability to induce the cell cycle arrest and apoptosis in human B-lymphoma cells.

Keywords

Apoptosis / Cell cycle / Ramos cells / Citral

CHAPTER II

LITERATURE REVIEWS

Under normal conditions, cellular homeostasis is regulated by the balance between cell proliferation and cell death. When this balance is disturbed it may result in hyperplasia and progress to cancer **[5]**. Cancer is a cellular disorder characterized by progressive accumulation of a mass of cells as a result of excessive cell proliferation over cell death. Cancer cells arise when normal cells lose the normal regulatory mechanisms that control their growth and proliferation. They lose differentiation and become immortal. Many currently used anticancer agents were developed to overcome excessive cellular proliferation by causing cytostasis or cytotoxicity. These cytotoxic agents kill cancer cells by either necrosis or apoptosis.

Necrosis is an accidental cell death. It typically occurs as a result of cell injury or exposure to cytotoxic chemicals. The characteristics of necrotic cells are a rapid loss of membrane integrity, swelling of intracellular organelles including endoplastic reticulum, Golgi apparatus, or mitochondria and the release of cellular contents into the extracellular space. These cellular contents collectively called danger-associated molecular patterns (DAMPs) or alarmins, include the high mobility group protein B1, uric acid, certain heat shock proteins, single-stranded RNA and genomic DNA. These molecules trigger inflammation by stimulating one or more pattern-recognition receptors on neutrophils, macrophages, dendritic cells and natural killer cells **[6-7]** and cause the information of an inflammatory process that damages the cells and surrounding tissues.

In contrast, apoptosis is a most common physiological process of cell death. It is the programmed cell death which is extremely well organized. Apoptosis process involves DNA fragmentation, membrane blebbing, cell shrinkage and disassembly into membrane-enclosed vesicles known as “apoptotic bodies”. These apoptotic bodies are rapidly eliminated by phagocytosis without triggering inflammation at the surrounding areas **[8]**. Many currently used cytotoxic drugs kill cancer cells by apoptotic induction. Not only apoptotic induction, the induction of cell cycle arrest is also the common activity shared by many anticancer drugs. Many stimuli such as radiation, mitotic inhibitors and topoisomerase inhibitors have been found to arrest cancer cells at specific phase of the cell cycle. Inhibition of the cell cycle can result in severe cell

damage and provoke apoptosis **[9]**. The detailed of apoptosis and cell cycle arrest are described below.

Programmed cell death by apoptosis

Apoptosis is a process of cell death usually utilized in multicellular organisms to eliminate unwanted cells in a diversity of settings. It involves a series of dramatic changes in the cellular components which lead not only to cell death, but also to prepare apoptotic cells for removal by phagocytes and prevent unwanted immune responses. Apoptosis is characterized by cellular biochemical and morphological changes. These include cell shrinkage, chromatin condensation, multiple of 200 bp DNA fragmentation, plasma membrane convolutes or blebbing, and the formation of membrane bound vesicles named as apoptotic bodies, but most organelles remain intact. These apoptotic bodies are recognized and engulfed by phagocytes without initiating any inflammatory response (Fig. 1) **[10]**.

Many events during the apoptotic process are initiated and executed by cysteine aspartate-specific proteases called caspases. These caspases specifically target several hundred proteins for proteolysis in a controlled manner that minimizes damage neighboring cells and avoids the release of alarmins **[11]**.

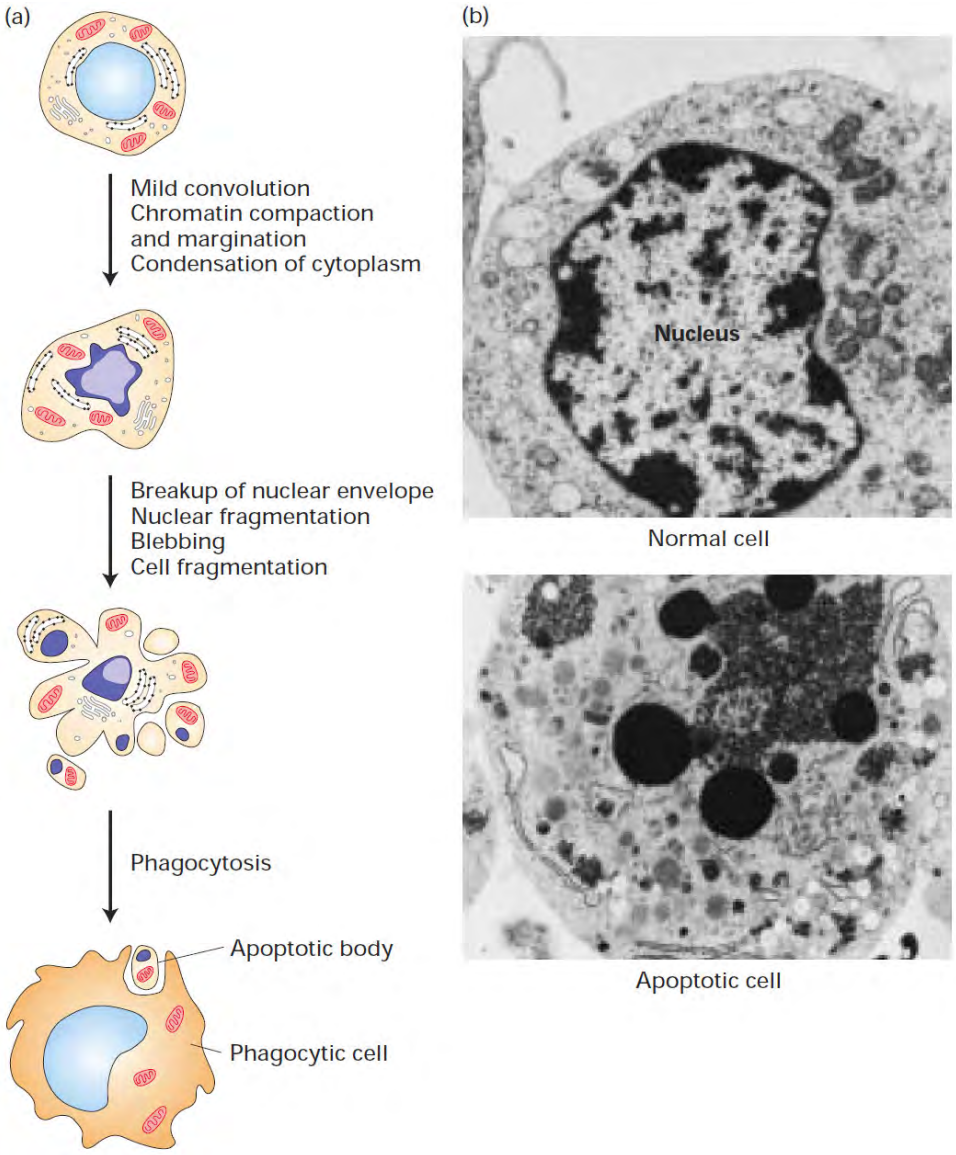


Figure 1: Ultrastructural features of cell death by apoptosis **[12]**.

a. Schematic drawings illustrating the progression of morphologic changes observed in apoptotic cells.

b. Photomicrographs comparing a normal cell (top) and apoptotic cell (bottom).

Caspases

Caspases (cysteine aspartate-specific proteases) belong to a highly conserved cysteine-dependent aspartate-specific acid protease family. These enzymes contain a cysteine residue at their catalytic site and specifically cleave their protein substrates after aspartic acid residues **[13]**. Twelve human caspases (caspases 1–10, caspase-12 and caspase-14) have been identified to date **[14]**.

All caspases are synthesized and exist within the cell as pro-enzymes or inactive zymogens. These zymogen are single polypeptide chains of 32–55 kDa containing 3 domains. There are a 17–21 kDa central large internal domain (p20) containing an active site, a 10–13 kDa small C-terminal domain (p10) called small catalytic subunit, and a variable length (3–24 kDa) NH_2-terminus called prodomain. The p20 and p10 subunits are separated by a small linker sequence in many pro-caspases. The active form of a caspase consists of p20 and p10 subunits which each contain residues that are essential for catalysis and substrate recognition. The longer prodomain contain distinct domains that are required for the activation of the caspases while the function of the short prodomian remains unknown. There are 2 different types of these domains known as the death effector domain (DED) and the caspase-recruitment domain (CARD) **[15-16]**. Selective proteolytic cleavages at 2 specific aspartic acid residues [Asp(P1)–X(P1) bonds] of these procaspases results in the release of large and small subunits. Two large and two small subunits form an active heterotetramer protease to initiate or execute apoptosis (Fig. 2) **[17]**.

Based on the structure and function of prodomians, mammalian caspases are classified into 3 major groups (Fig. 3). Inflammatory caspases are the group I caspases with a long prodomains (caspase-1, caspase-4, caspase-5, caspase-12, caspase-13 and caspase-14) that play a role in cytokine maturation and inflammatory responses. The group II caspases are initiator caspase containing a long prodomain with either DED domain(s) (caspase-8 and -10) or a CARD (caspase-2 and -9). These enzymes play role as initiator caspase in apoptosis. The group III caspases are effector caspases (caspase-3, caspase-6, and caspase-7) containing a short prodomain (20–30 amino acids) **[13, 18]**. They play role as executioner caspases during apoptotic process.

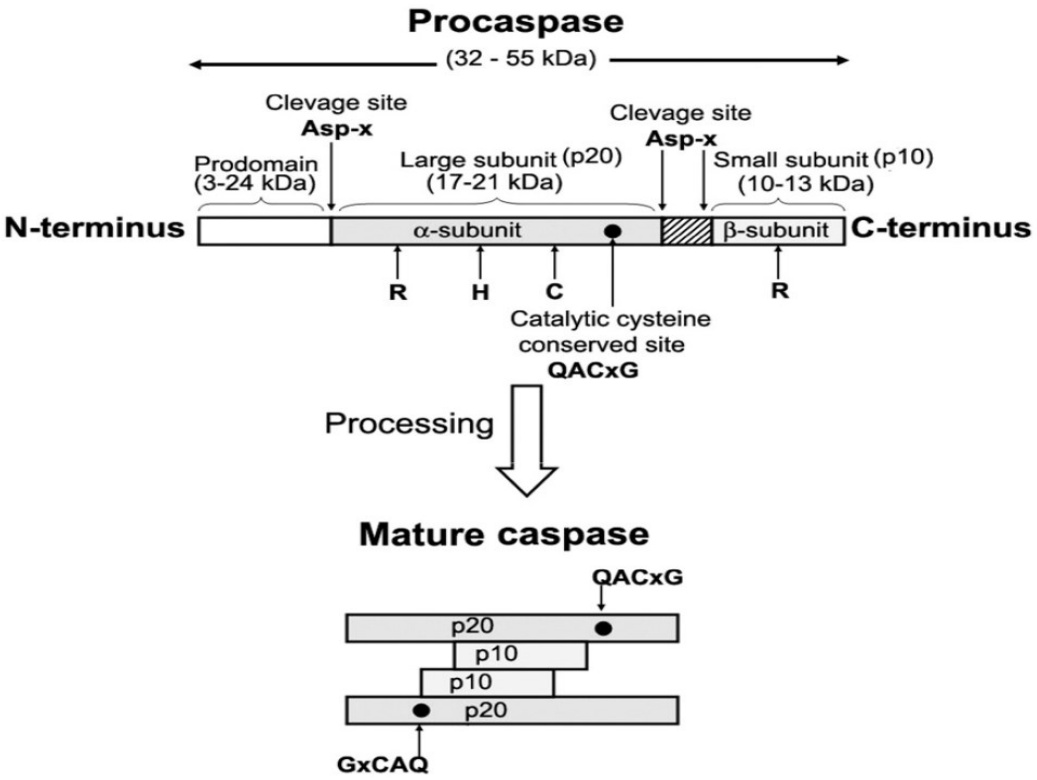


Figure 2: A schematic representation of structural features of mammalian caspases **[13]**. C, H and R represent the active site residues.

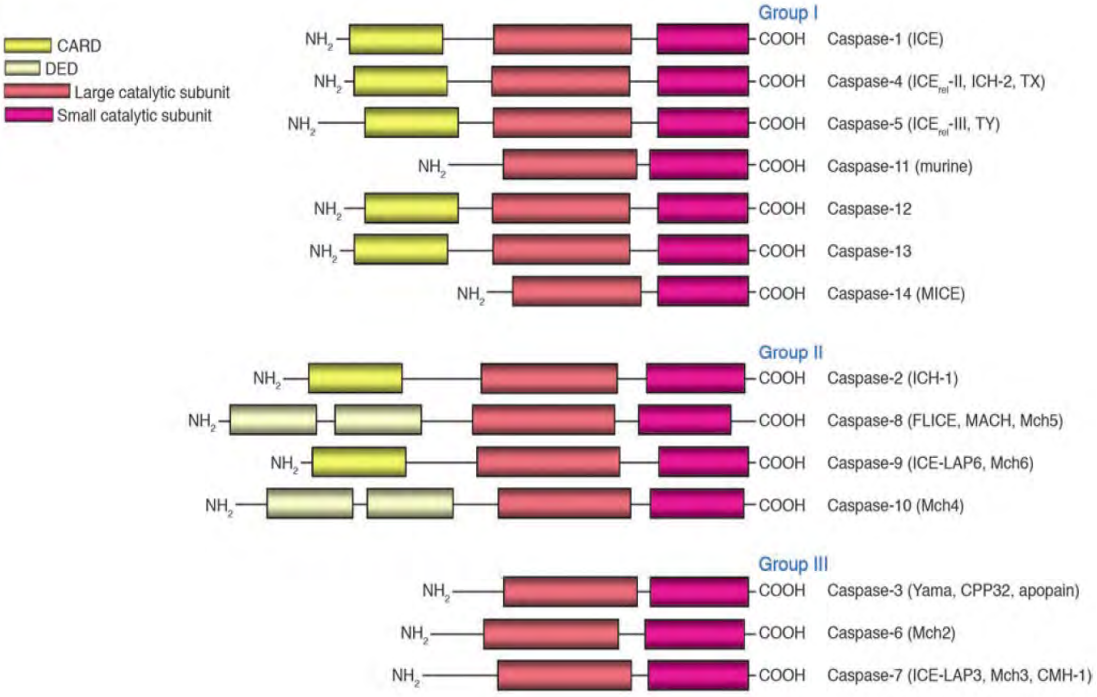


Figure 3: The caspase family. Three major groups of caspases **[18]**.

Caspase signaling

Caspases involved in apoptosis are the initiator caspases including caspase-2, -8, -9, and -10, and the effector caspases which are caspase-3, -6, and -7. By their prodomains, the initiator caspases are recruited to and activated at death inducing signaling complexes in response to either the ligation of cell surface death receptors (DRs), the extrinsic apoptosis pathway or the intracellular signals, the intrinsic apoptosis pathway **[19]**. The induction of apoptosis through both pathways results in the activation of initiator caspases. DRs, through adaptor molecules, recruit initiator caspases-2, -8, or -10, while intrinsic death signals result in the activation of caspase-9. Activation of initiator caspases is the first step of a highly regulated, irreversible, selfamplifying proteolytic pathway. Initiator caspases are able to cleave procaspases to become activate effector caspases (caspases-3, -6, and -7). Both the extrinsic and intrinsic death pathways use the common effector caspases to induce apoptosis **[20]**.

Once activated, the effector caspases are in charge for the proteolytic cleavage of a numerous cellular targets, leading to cell death. These enzymes cause perturbation of various cell architectures by cleavage of specific substrates. These proteolytic cleavage induce the cellular phenotypic changes to apoptotic cell (Fig. 4), for example. Caspases cleave inhibitor of caspase-activated DNase (ICAD) to release caspase-activated DNase (CAD), which can then catalyze DNA cleavage **[21-22]**. They mediate the cleavage of lamins at the nuclear lamina, causing nuclear fragmentation, and proteolysis of nuclear envelope proteins **[23]**. Proteolysis of proteins at focal adhesion sites and cell–cell adhesion sites causes cell detachment and retraction. Proteolysis of the Rho effector, ROCK1, leads to actin contraction and plasma membrane blebbing as well as DNA fragmentation **[24].** The cleavage of tubulin and microtubule-associated proteins leads to changes in the microtubule cytoskeleton and contribute to apoptotic body formation **[25-26]**. Caspases cleave the Golgi-stacking proteins causing the Golgi apparatus fragmentation **[27-28]**. They cleave the mammalian sterile-20 kinase, MST1, to an active kinase. This kinase then translocates to the nucleus and phosphorylates histone H2B to initiate chromatin condensation **[29]**. Moreover, caspase-mediated proteolysis of multiple translation initiation factors (eIFs) causing translation disruption **[30]**.

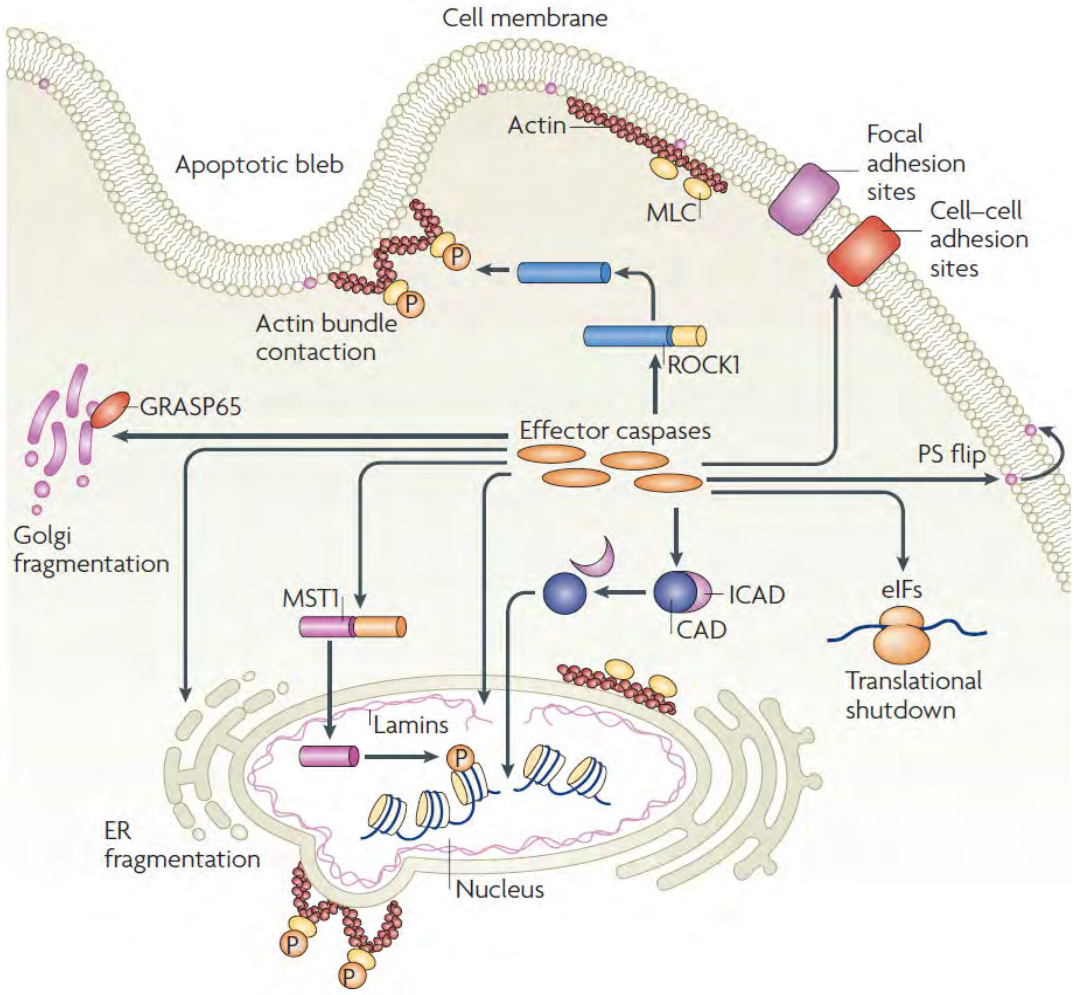


Figure 4: Caspases coordinate demolition of key cellular structures and organelles **[31]**.

Phagocytosis is the terminal phase of apoptosis in most tissues. Several molecules have been proposed to act as signals for the engulfment of apoptotic cells by phagocytes such as macrophages and dendritic cells (Fig. 5). Proteolytic cleavage by caspases leads to the exposure of phosphatidylserine (PS) as well as other phagocytic signals on the surface of apoptotic cell. When PS is exposed on the outer plasma membrane leaflet **[32-33]** it is recognized by a specific receptor on phagocytes, possibly thrombospondin (TSP), milk fat globule–EGF factor-8 protein (MFG-E8) or CD36 **[34-35]**. The PS exposure can also induce opsonization of apoptotic cells by the complement factor, iC3b. This may promote the uptake of apoptotic cells by integrins (complement receptors CR3 and CR4) **[36]**. Calreticulin that is exposed on the apoptotic cell is bound by CD91 **[37]**, whereas oxidized low-density lipoprotein (ox-LDL)-like sites is recognized by scavenger receptors, including scavenger receptor A (SR-A) and oxidized low-density lipoprotein-1 (LOX1) **[38]**.

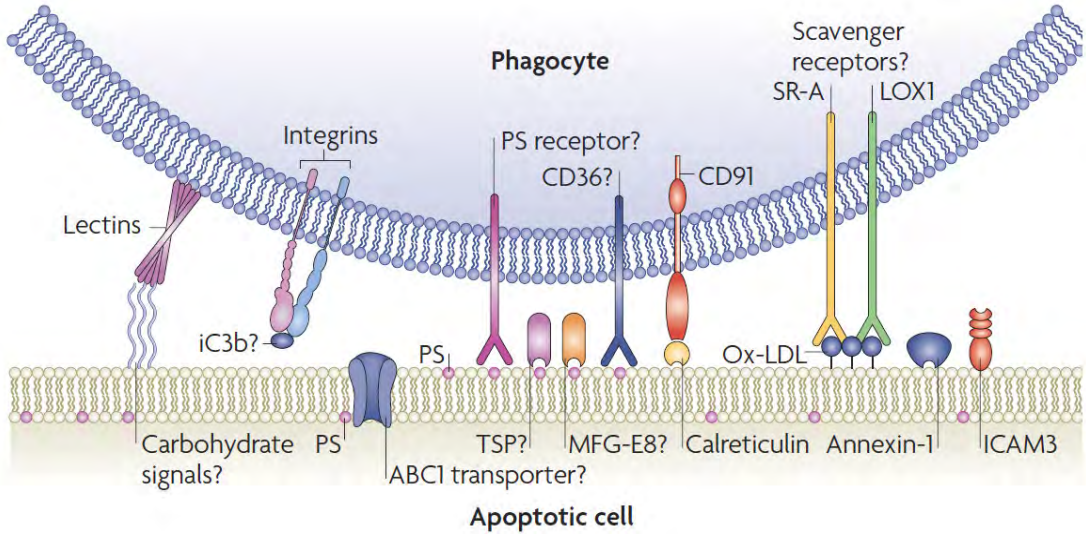


Figure 5: Apoptotic cells display ligands that promote their engulfment by phagocytes **[31]**.

Caspase activation pathways

There are three major pathways to caspase activation (Fig. 6); the extrinsic pathway or the death receptor-mediated pathway, the intrinsic pathway or the mitochondria-mediated pathway, and the granzyme B-mediated pathway **[31]**.

Caspase activation by the extrinsic pathway involves the binding of extracellular death ligands to transmembrane death receptors, recruitment of adaptor proteins, activation of initiator and effector caspases, proteolysis of caspase substrates, and cell death. Signals from the extrinsic pathway can crosstalk with the intrinsic pathway through caspase-8-mediated proteolysis of the BH3-only protein, BID, which can activate the intrinsic pathway **[39]**.

For the intrinsic pathway, diverse non-receptor mediated stimuli that induce cell stress or damage initiate the assembly of BCL-2 protein family members, BAK–BAX oligomers, within mitochondrial outer membranes. These oligomers cause changes in the inner mitochondrial membrane that result in loss of the mitochondrial transmembrane potential and release of intermembrane space proteins, such as cytochrome c, into the cytosol. Cytochrome c interacts with adaptor proteins causing and activation of initiator caspases, subsequently activation of effector caspases, resulting in apoptosis **[40]**.

The granzyme B-dependent pathway of caspase activation is one of the killing mechanisms of cytotoxic T lymphocytes (CTL) and natural killer (NK) cells. It involves secretion of the transmembrane pore-forming molecule, perforin, with a subsequent permit entry of cytoplasmic granules, especially granzymes B, through the pore and into the target cell. Granzyme B has similar substrate specificity to caspases. It cleaves its substrates after aspartic acid residues of BID as well as caspase-3, -6 and -7 to trigger apoptosis **[31]**.

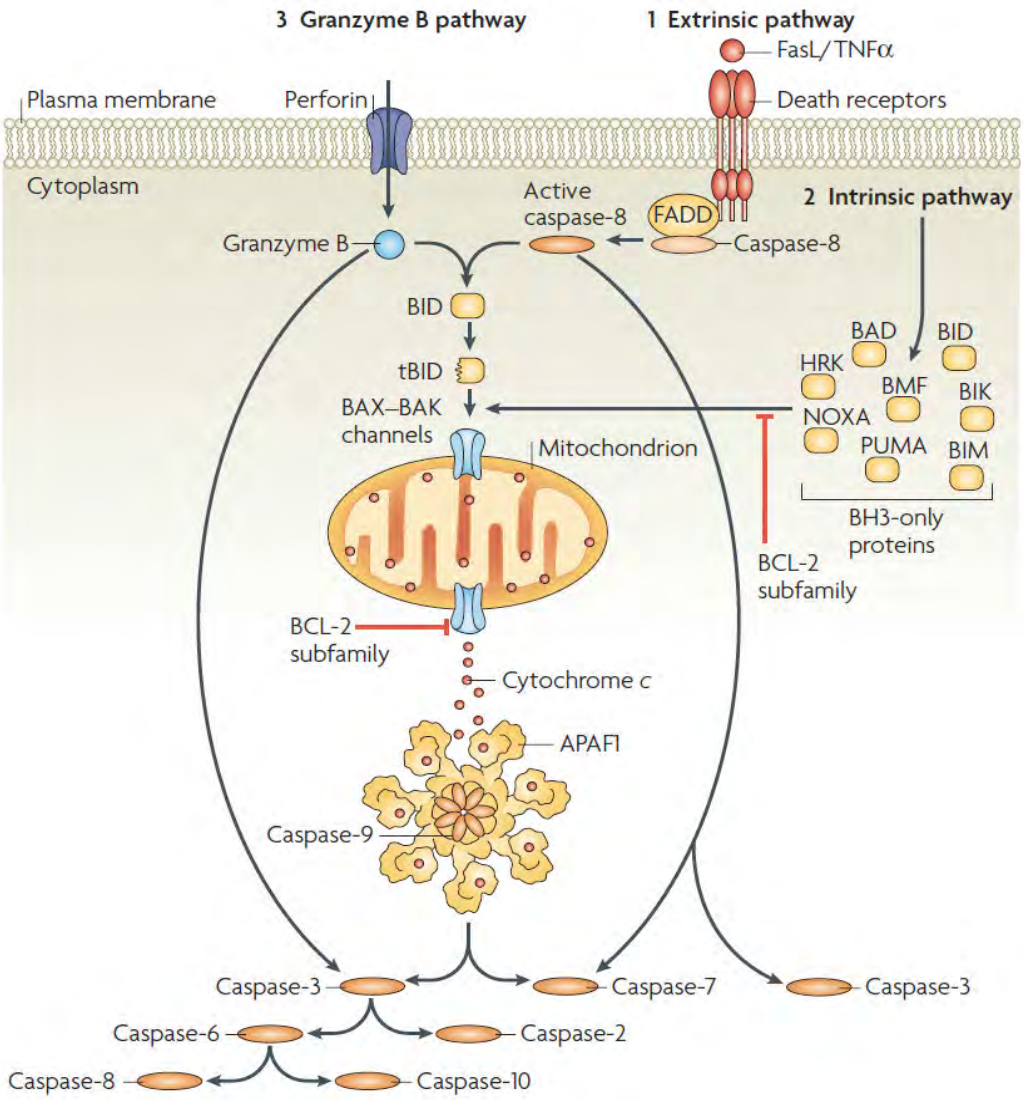


Figure 6: Caspase activation pathways **[31]**.

The extrinsic pathway of apoptosis

The extrinsic pathway is initiated by the activation of cell surface death receptors (DRs) after binding to their specific ligands. These DRs are the members of the tumor necrosis factor receptor (TNFR) superfamily. The well known members and their specific ligands include FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 **[41-43]**. Members of the TNF receptor superfamily share similar extracellular cyteine-rich domains (CRD) and intracellular death domains (DDs) of about 80 amino acids at the cytoplasmic tail. The CRD are critical for receptor self-association (CRD1) and receptor–ligand interactions (CRD2 and CRD3) **[44]**. The DD plays an important role in transmitting the death signal from the cell surface to the intracellular signaling cascades. These receptors initiate apoptotic signaling cascades within seconds after binding to their specific death ligands and result in apoptosis within hours. Upon ligand binding, they undergo oligomerization and/or conformational change. They then recruit both an adaptor molecule, either Fas-associated death domain (FADD) or TNF receptor-associated death domain (TNFADD), and procaspase-8 or procaspase-10 to form the death inducing signaling complex (DISC). At the DISC, the auto-catalytic activation of procaspase-8 occurs and becomes active caspase-8. This caspase then triggers execution phase of apoptosis **[45]**.

Signaling by Fas/FasL

Fas (apo-1 or CD95) is a 45–52 kDa glycoprotein. It is a transmembrane receptor widely expressed in various tissues and constitutively present on activated T and B lymphocytes. It binds specifically to Fas ligand (FasL) which is a transmembrane protein expressed mainly in T lymphocytes and natural killer cells. FasL is also expressed on many tumor cells, including melanomas, astrocytomas, lymphomas and various carcinomas **[46]**. It expression is induced in activated lymphocytes and can be cleaved from the cell surface by metalloprotease to become soluble FasL. This soluble FasL is found *in vivo* and can trigger apoptosis **[47]**. The Fas-FasL signaling pathway of apoptosis is shown in Fig. 7.

Once ligation with FasL, Fas undergoes trimerization and recruits an adapter protein called the Fas-associated death domain (FADD), which interacts with the homologous death

domain (DD) of Fas. FADD contains not only DD but also a death effector domain (DED) which can associate with other DED-containing proteins such as pro-caspase-8 or procaspase-10. The oligomerization of Fas, FADD and procaspase-8 is termed the death-inducing signaling complex (DISC), which plays role as a platform to initiate cascade of the caspase activation. At the DISC, pro-caspase-8 undergoes autoproteolytic cleavage to generate active caspase-8. Active caspase-8 is released from the DISC and subsequently cleaves downstream pro-caspases, such procaspase-3, -6 or -7, to produce active effector caspases **[48],** which results in the cleavage and activation of a vast number of downstream substrates eliciting the morphological and biochemical characteristics of apoptosis.

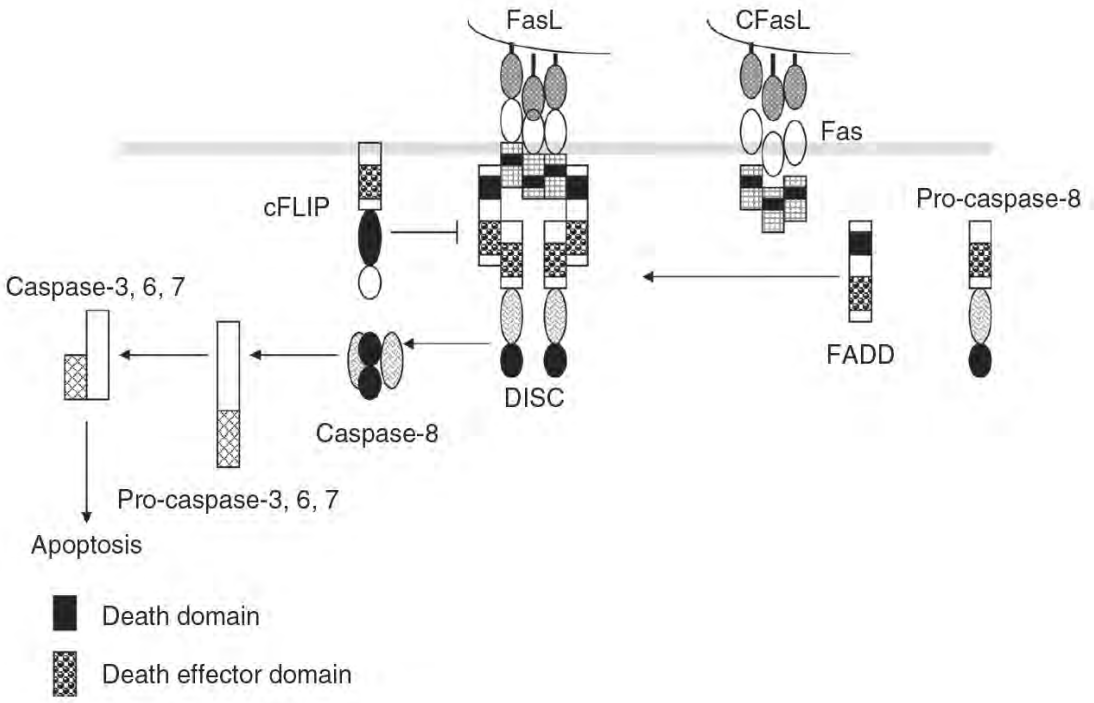


Figure 7: The Fas-FasL pathway of apoptosis **[49]**.

Cells using Fas signaling pathway of apoptosis have been identified into 2 types **[50]**. In type I cell, there is sufficient active caspase 8 for directly activating effector caspases including caspase-3, -6, and -7. In type II cell, the activation of effector caspases cell need to be amplified by the mitochondrial pathway. The type II cell has limited activation of caspase-8 at the DISC due to either insufficient procaspase-8 expression or high levels of caspase-8 inhibitor FLICE Inhibitory Protein (FLIP). Limited active caspase-8 cannot proceed apoptotic signaling through the direct pathway. Therefore, the caspase cascade needs to be amplified via the mitochondrial pathway **[51]**. The mitochondrial amplification loop is depends on the cleavage of Bid, a cytoplasmic pro-apoptotic BCL-2 family protein, to a truncated protein, tBid, by active caspase-8 (Fig. 6). tBid translocates to the mitochondria to efficiently activates BAX, the other pro-apoptotic BCL-2 family member. BAX initiate the release of cytochrome c and second mitochondria-derived activator of caspases (Smac/DIABLO) into the cytosol to drive the formation of the caspase-9 –activating apoptosome in the mitochondria pathway of apoptosis **[52]**.

The mitochondrial pathway of apoptosis

The mitochondria or intrinsic pathway originates from apoptotic signals resulting in a perturbation of intracellular homeostasis. A vast number of stimuli, such as ultraviolet radiation, reactive oxygen and reactive nitrogen species, chemotherapeutic agents, heat shock, growth factor withdrawal and DNA damage, mediate apoptosis via this pathway **[53]**. These stimuli cause changes in the inner mitochondrial membrane permeabilization which leads to an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential ($\Delta\psi m$) and release of two main groups of pro-apoptotic proteins normally sequestered in the intermembrane space into the cytosol. The first group composed of cytochrome c, second mitochondria-derived activator of caspase/direct IAP-binding protein with low isoelectric point (Smac/Diablo), and the high temperature requiment protein HtrA2/Omi, which is the mitochondrial serine protease **[49, 54]**. All of them activate the caspase dependent mitochondrial pathway. The second group consists of apoptosis-inducing factor (AIF), endonuclease G (EndoG) and caspase-activated DNase (CAD), which are released from the mitochondria at a late phase of

apoptosis after the cell has committed to die. They play role in apoptosis in either a caspase independent or dependent manner.

Cytochrome c interacts with apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP, and procaspase-9 forming an apoptosome **[55]**. Apaf-1 contains a CARD, which involves in its interaction with caspase-9, and a WD-40 repeat domain that may play role to maintain protein inactivity in the absence of cytochrome c. Interaction of cytochrome c and Apaf-1 in the presence of ATP/dATP, Apaf-1 undergoes a conformational change by self aggregation **[56]**. The CARD of Apaf-1 is exposed and induces the recruitment of procaspase-9 which is subsequent autoproteolytic cleaved to active caspase-9. Caspase-9 then directly activate effector caspases, caspases-3, -6 and -7 (Fig. 8), which results in the sequentially cell death by proteolytic clevage of numberous downstream targets. Smac/DIABLO and HtraA2/Omi which are also released from the mitochondria into the cytosol **[57]**, can binds to the inhibitor of apoptosis proteins (IAPs) and disrupting its association with active caspase-9, thus allowing caspase-9 to activate caspase-3, resulting in apoptosis.

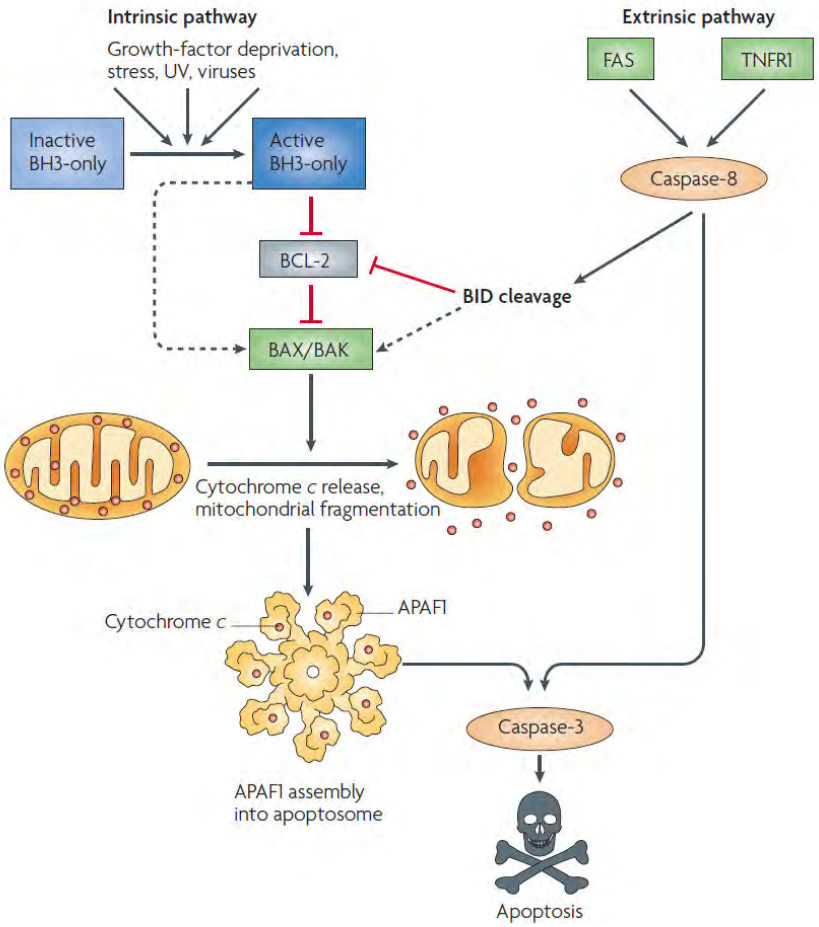


Figure 8: The intrinsic pathway of apoptosis signaling **[58]**.

The group II pro-apoptotic proteins from mitochondria intermembrane space, including AIF and EndoG, can induce apoptosis in a caspase-independent manner. Once release from the mitochondria, AIF translocates and causes chromatin condensation and large-scale DNA fragmentation to fragments of approximately 50-300 kb pieces **[59]**. EndoG also translocates to the nucleus and cleaves nuclear chromatin to nuclear DNA fragments **[60]**.

The mitochondrial membrane permeabilization (MMP) is regulated by a various BCL-2 (B-cell lymphoma 2) family proteins. These proteins are classified based on their BCL-2 homology domains (BH1–4 domains) into three groups, the anti-apoptotic BCL-2, the pro-apoptotic BCL-2 and the BH3-only proteins (Fig. 9) **[61-62]**. The anti-apoptotic BCL-2 proteins, including BCL-2, BCL-w, BCL-XL, A1 and MCL-1, contain BH domains 1- 4. Most of them contain transmembrane domains (TM) and typically associate with membranes. They are integrated in the endoplasmic reticulum (ER), the nuclear envelope and the outer mitochondrial membrane. These proteins block apoptosis, their functions at the ER and the nuclear envelope are less clear than those on mitochondria. They prevent cytochrome c release from mitochondria via directly bind to and inhibit the pro-apoptotic BCL-2 proteins. This leads to preserve cell survival **[63]**. The pro-apoptotic BCL-2 proteins, BAK and BAX, contain BH 1-3 domains. They are critical for inducing permeabilization of the outer mitochondrial membrane by forming a dimer or high-order oligomers to creating the proteolipid pore. This pore is responsible for pro-apoptotic proteins, cytochrome c and DIABLO/SMAC, release which leads to caspase activation **[64].** The pro-apoptotic BH3-only proteins, including BAD, BID, BIK, BIM, BMF, Harakiri (HRK), Noxa and PUMA (p53-upregulated modulator of apoptosis), contain a conserved BH3 domain that can bind and regulate the anti-apoptotic BCL-2 proteins to induce apoptosis. These proteins are pro-apoptotic and play role as initial sensors of apoptotic signals. BH3-only proteins act as pathway-specific sensors for various types of stimuli that provoke cell stress or damage. Activation of these proteins is a crucial for overcoming the effect anti-apoptotic BCL-2 proteins **[58]** and promoting the assembly of BAK–BAX oligomers within mitochondrial outer membranes. These oligomers permit the efflux of intermembrane space proteins into the cytosol and result in apoptotic induction.

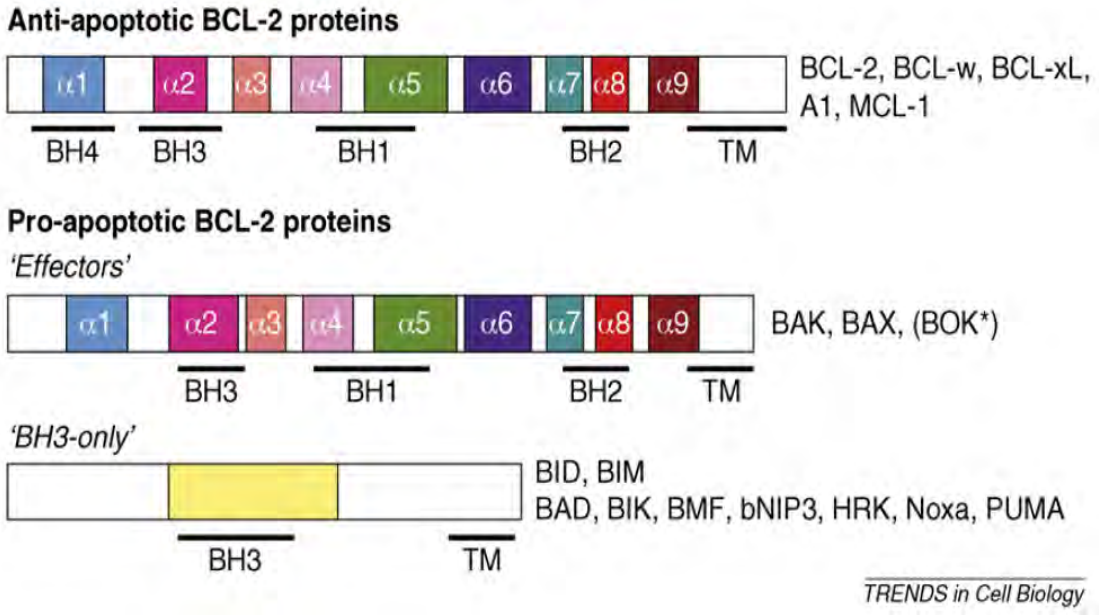


Figure 9: The BCL-2 family of proteins **[63]**.

In addition to the regulatory roles of the BCL-2 family proteins on the apoptosis pathway described above, the tumor suppressor gene, p53, has also been proven to play an important role in the regulation of apoptosis. A numerous stimuli that induce DNA damage result in the p53 activation and subsequent result in apoptosis **[65]**. p53 plays a principal role in cell cycle arrest or apoptosis when DNA damage. It is a sequence-specific DNA binding protein and activates expression of numerous genes involve in growth arrest and apoptosis. Relating to apoptosis, many pro-apoptotic genes containing a p53-responsive element have been reported. These include BAX, the BH3-only, Noxa and PUMA genes **[66-67]**. Apaf-1 has also been identified as a direct target of p53 activation **[68]**.

The granzyme B-mediated pathway of apoptosis

Grandzyme B is a serine protease that plays a pivotal role in natural killer (NK) cell and cytotoxic T lymphocyte (CTLs) functions to clear viral infected cell or tumor cells **[69]**. Both NK cells and CTLs use 2 major mechanisms to kill target cells including the death receptor, Fas-FasL, pathway and the granule exocytosis pathway **[70]**. There are 2 important proteins involve in the granule exocytosis pathway. One is a pore-forming protein, perforin, that form pore by polymerization on target cell to facilitate the delivery of the other granule components inside the

cell. The other is granzyme B, a serine protease that shares substrate specificity with many caspases **[71]**. Granzyme B enters target cells by the help from perforin as described above. In the cytosol it cleaves and activates procaspase-3 (Fig. 6). However, it cannot fully process procaspase-3. Other pro-apoptotic mediators such as cytochrome c, Smac/Diablo, and Htra2/omi, from mitochondria may involve in the full activation may involve **[72-73]**. Granzyme B is also signals apoptosis through to the mitochondria via the cytosolic, pro-apoptotic protein Bid. It has been reported that granzyme B cleaves Bid to truncated Bid (tBid) within the first few minutes of exposure to target cells **[74]**. tBid translocates to mitochondria and recruits BAX to mitochondria through a caspase-independent mechanism. This leads to the release of cytochrome c and other pro-apoptotic proteins results in apoptotic induction **[74-75]**.

Inhibitor of apoptosis proteins

Inhibitor of apoptosis proteins (IAP) is a family of structurally related anti-apoptotic proteins originally identified in baculoviruses. IAPs inhibit apoptosis through their ability to bind to specific caspases. To date, 8 mammalian as well as 6 human IAPs are identified. The human IAPs are NAIP (neuronal apoptosis inhibitory protein), c-IAP1/HIAP-2, c-IAP2/HIAP-1, XIAP/hILP (X-linked mammalian inhibitor of apoptosis protein), survivin and BRUCE **[76]**. Their molecules contain one or more repeated of a highly conserved 70 amino acids domain called baculovirus IAP repeat (BIR) at the N-terminus and one conserved RING domain at the C-terminus **[77-78]**. The BIR domains are zinc finger like domains which can chelate zinc ions. These zinc fingers bind to specific caspases resulting in blocking the catalyzing grooves of the caspases. XIAPs is the best characterized human IAPs. It contains 3 BIR domains (BIR1, BIR2 and BIR3) which are functionally different. It specifically inhibits the activity of the initiator caspase-9 and effector caspases-3 and -7 (Fig. 10). Its BIR 3 inhibits caspase-9 by binding to caspase-9, while its BIR 1 and BIR 2 selectively target caspases-3 and -7. The IAPs inhibit the extrinsic pathway of apoptosis via inhibiting procaspase-3 **[79]**. XIAP, c-IAP1, and c-IAP2 regulate the intrinsic pathway of apoptosis by inactivating caspases via binding directly to procaspase-9. The expression and the activity of IAPs are inhibited by BAX and other pro-apoptotic proteins Smac/DIABLO and Omi/HtrA2 **[80-83]**.

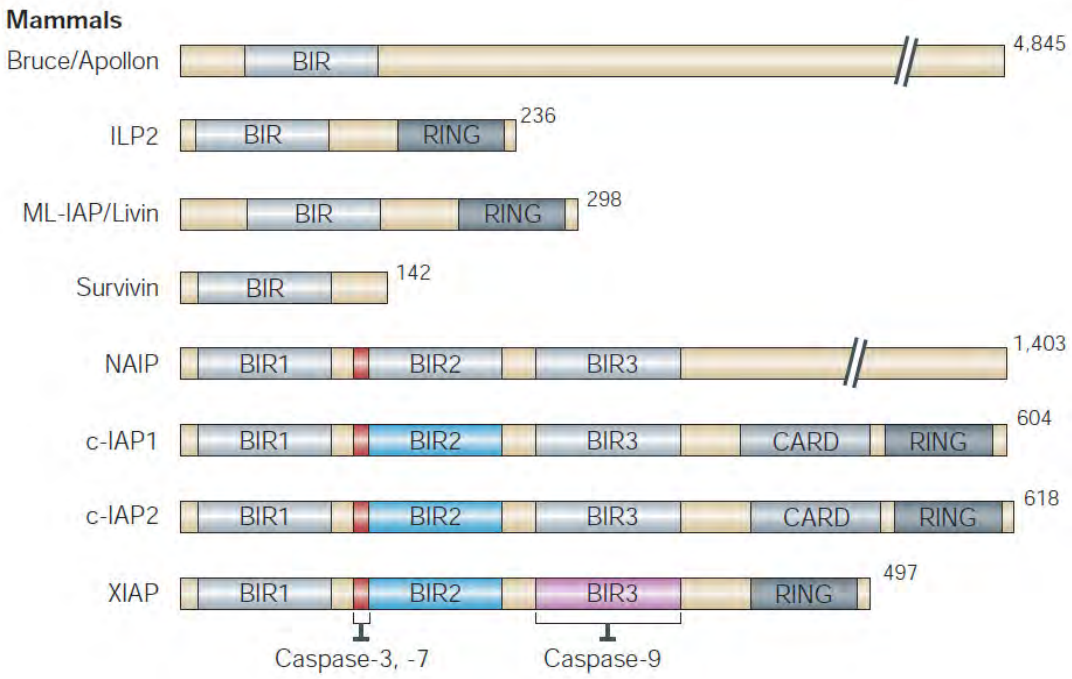


Figure 10: Proteins of the inhibitor of apoptosis (IAP) family **[54]**.

The cell cycle and cancer

Human tumorigenesis is a multistep and age-dependent process **[84]**. Cancer consists of somatic mutant cells which result from multiple genetic mutations accumulated over time. Uncontrolled cell proliferation, one of the main hallmarks of cancer, is directly involved in mutations of either oncogenes and/or tumor suppressor genes. Mutation of oncogenes usually results in the increase activity of their gene products which are proteins function in cell growth and survival. In contrast, mutations in tumor suppressor genes causing a recessive loss of function of their gene products which are proteins involve in growth arrest or apoptosis **[85]**. Mutations of oncogenes and/or tumor suppressor genes commonly affect the cell cycle.

The cell cycle is a sequence of events which leads to cell growth and replication. It is tightly controlled by many regulatory mechanisms that either allow or arrest its progression. There are 4 major phases in the cell cycle including G1, S, G2, and M phases. (Fig.11). In the cell cycle, cells in the G1 phase produce RNAs and proteins required for DNA synthesis and chromosome replication during the S phase. After complete DNA synthesis, the cells progressing to the G2 phase in which cells synthesize RNAs and proteins essential for cell duplication during

mitosis or M phase **[86]**. The M phase is divided into prophase, metaphase, anaphase and telophase. The cytokinesis after the M phase produces two identical daughter somatic cells.

The cell progression through each phase of the cell cycle in an orderly manner is controlled at the points referred to as the cell cycle checkpoints. The regulation at these checkpoints ensure that each phase of the cell cycle takes place only once in the right sequence and the cell undergoes completed transition in each phase before progression into the next phase **[87]**. There are 3 checkpoints in the cell cycle including the G1/S checkpoint at the onset of S phase, the G2/M checkpoint at the entry of mitosis, and the metaphase/anaphase or spindle checkpoint at the exit of mitosis (Fig. 11). The G1/S and G2/M checkpoints are the DNA damage checkpoints. The G1/S checkpoint functions in response to DNA damage to prevent entry into S phase until the damage is repaired. The G2/M checkpoint regulates during G2 phase to prevent entry into M phase before the DNA synthesis is complete. The spindle checkpoint controls during early mitosis to prevent the initiation of anaphase until the mitotic spindle is completely formed and all chromosome kinetochores are properly attached to spindle fibers **[88]**.

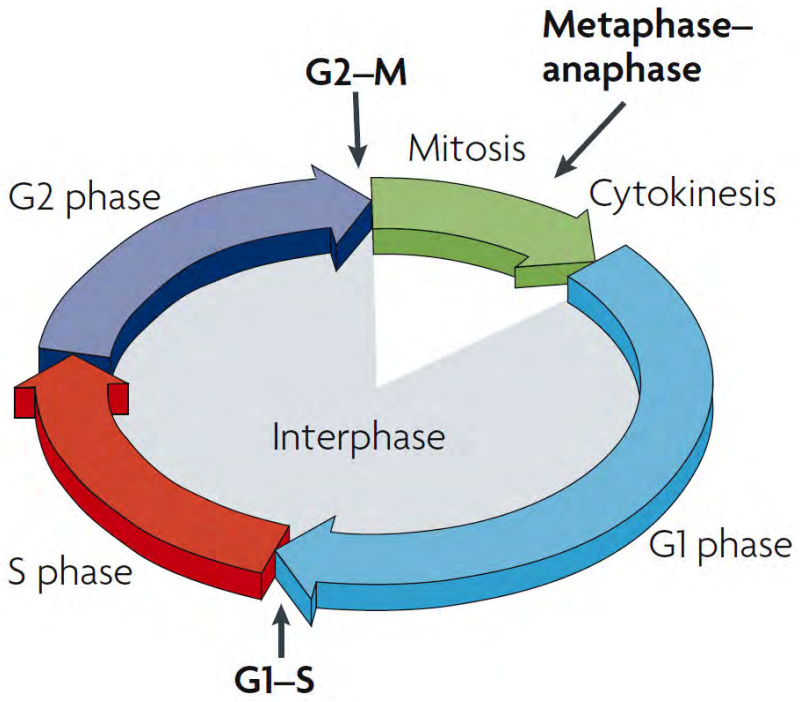


Figure 11: The stages of the cell cycle and checkpoints **[86]**.

Key regulatory proteins in the cell cycle checkpoints are the cyclin-dependent kinases (CDK). CDKs are proteins in a family of serine and threonine protein kinases. There are 4 CDKs function during the cell cycle, CDK1 at G2 and M phases, CDK2 at G1 and S phase, and CDK4 and CDK6 at G1 phase. CDKs are active at specific points in the cell cycle. They become active kinases after association with their regulatory subunits named cyclins to be CDK-cyclin complexes. Each CDK associates with its specific cyclin. During the cell cycle, the CDK proteins remain stable, but cyclins are cyclically synthesized and destroyed at specific times in the cycle. Thus the CDK kinase activity is regulated in a timely manner **[89]**. Different CDK-cyclins are required at different phases of the cell cycle (Fig. 12).

The kinase ctivity of CDKs is not only regulated by cyclin association but also by phosphorylation or dephosphorylation on conserved threonine and tyrosine residues. Full activation of CDKs requires phosphorylation of Thr161 in CDK1, Thr160 in CDK2, and Thr172 in CDK4 by the CDK7-cyclin H complex, also called CAK. These phosphorylations induce conformational changes of CDKs and enhance the binding of cyclins **[90]**. The Wee1 and Myt1 kinases phosphorylate CDK1 at Tyr15 and/ or Thr14 results in inactivating the kinase. Dephosphorylation at these residues by the phosphatase enzyme Cdc25 is essential for activation of CDK1 **[91]**.

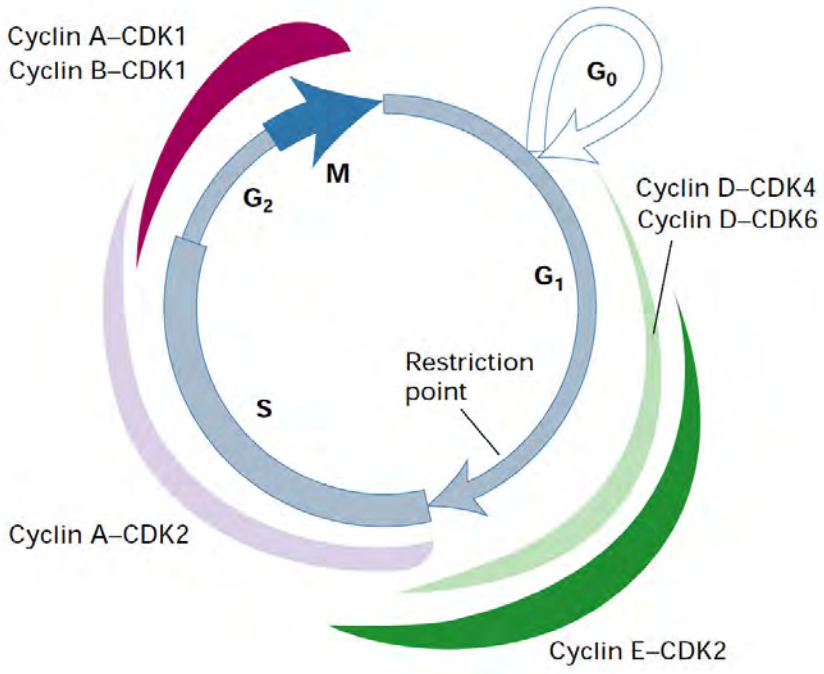


Figure 12: Activity of mammalian cyclin-CDK complexes through the course of the cell cycle **[92]**.

A cell is committed to enter the cell cycle when it is activated by growth factors. These factors bind to specific receptors on the cell surface and relay activated cascade signals into cytosol and nucleus to push the cell through the cell cycle **[93]**. D cyclin is the first cyclin to be induced when G0 cells are stimulated to enter the cell cycle. Unlike other cyclins, D cyclin does not oscillate during the cell cycle, but its level is regulated by the presence of growth stimuli. This cyclin associates with and activates CDK4 and CDK6. The primary substrate for CDK4-cyclin D and CDK6-cyclin D kinases is the retinoblastoma protein (Rb). Rb is a tumor suppressor protein that plays a critical role in regulating G1 progression. It is a key regulator at the restriction point. It binds to and regulates many cellular proteins, including proteins in the E2F family of transcription factors **[94]**. E2F transcription factors regulate the expression of many proteins involved in cell cycle progression and DNA synthesis such as CDK1, cyclin E and A, DNA polymerase α, thymidine kinase, and dihydrofolate reductase. These transcription factors are inactive when they bind to Rb. Phosphorylation of Rb by D cyclin kinases causes the dissociation of Rb from E2F **[95].** This results in the expression of the E2F-regulated genes. The activation of E2F leads to the expression of cyclin E which is the next cyclin needed during the progression of cells through G1. Cyclin E associates with CDK2 to be CDK2-cyclin E complex which is required for the transition of cells from G1 to S phase **[96]**.

Expression of cyclin A is also regulated in part by E2F. This cyclin accumulates at the G1/S phase transition and persists through S phase. It associates with CDK2 in G1/S phase and then with CDK1 in late S phase. Cyclin A-associated kinases required for entry into S phase, completion of S phase, and entry into M phase **[97]**. This kinases are found to co-localize with the sites of DNA replication, suggesting that they may participate in or prevent DNA replication. Some E2F transcription factors are negatively regulated by this kinase. Cyclin A-associated kinases phosphorylate the E2F heterodimerization partner called DP1, resulting in an inhibition of E2F DNA-binding activity **[97]**.

The M phase is regulated by CDK1 associated with cyclins A, B1, and B2 **[98]**. The CDK1/cyclin complexes phosphorylate multiple proteins required for chromosome condensation, retraction of the nuclear envelope, assembly of the mitotic spindle apparatus, and alignment of condensed chromosomes at the metaphase plate. These kinase complexes activate the anaphase promoting complex (APC). This complex induces the ubiquitin-mediated proteolysis of anaphase inhibitor called securin, resulting in the degradation of the cross-linking proteins connecting sister

chromatids **[99]**. This leads to the initiation of anaphase by allowing free sister chromatids to segregate to opposite spindle poles. In late anaphase, the APC also induces the ubiquitin-mediated proteolysis of the mitotic cyclins, leads to inactivation of CDK1 **[100]**. As a result, the cells enter telophase characterized by separated chromosome decondensation, the nuclear envelope reformation around daughter-cell nuclei, and the Golgi apparatus reassembly. Finally, the cytoplasm divides at cytokinesis, producing the two daughter cells.

CDK inhibitors

CDK inhibitors (CKI) are the other regulators of CDK activity. CDK activity can be counteracted by cell cycle inhibitory proteins termed as CDK inhibitors (CKI). These CKIs bind to CDK or to the CDK-cyclin complex and inhibit CDK activity. They are classified to two families according to their substrate specificity, including the inhibitors of CDK4 (INK4) family and the CDK inhibitory Protein/Kinase Inhibitor protein (Cip/Kip) family (Fig. 13) **[101]**. The INK4 family includes p15 $^{(INK4b)}$, p16 $^{(INK4a)}$, p18 $^{(INK4c)}$, p19 $^{(INK4d)}$. These CKIs specifically inactivate G1-CDK (CDK4 and CDK6) by forming stable complexes with the CDK enzymes before associating with cyclin D **[102]**. The Cip/Kip family includes p21$^{(Waf1,\ Cip1)}$, p27 $^{(Cip2)}$, p57 $^{(Kip2)}$. These CKIs inactivate CDK-cyclin complexes. They inhibit the activity the G1 CDK-cyclin complexes, and to a lesser extent, CDK1-cyclin B complexes **[103]**. p21 inhibits DNA synthesis by binding to and inhibiting the proliferating cell nuclear antigen (PCNA). CKIs are regulated both by internal and external signals. The expression of p21 is transcriptional regulated by the p53 **[104]**. The expression and activation of p15 and p27 is regulated by transforming growth factor β (TGF-β), resulting in growth arrest **[105]**. CKIs mediate cell cycle arrest in response to several anti-proliferative signals. Dysfunction of CKIs has been identified in many human cancers **[106]**, suggesting a role of CKIs in preventing abnormal cell proliferation and tumor formation.

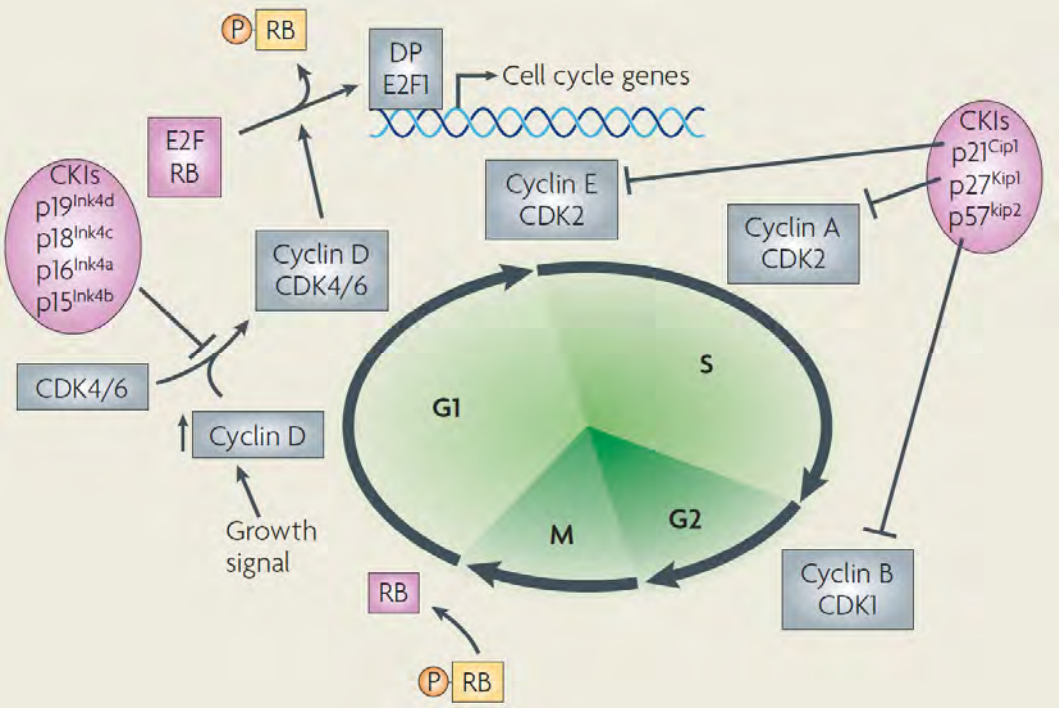


Figure 13: The cell cycle inhibitory proteins (CKI) **[107]**.

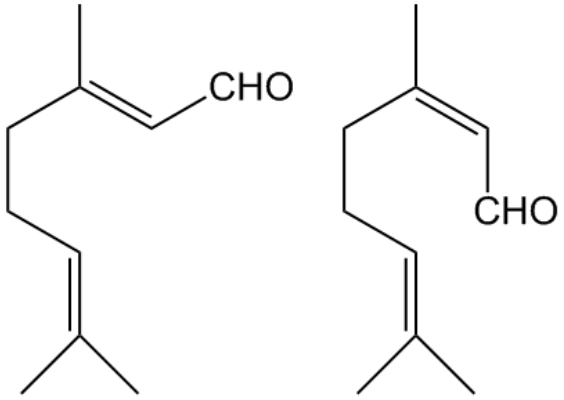
Essential oils as anticancer agents

Several plant-derived anticancer drugs including vincristine, vinblastine, taxanes, epipodophyllotoxin derivatives and camptothecin derivatives have been approved for clinical use. Many compounds isolated from medicinal plants have been widely investigated for cancer treatment. These include essential oils which are bioactive constituents found abundantly in plants as secondary metabolites. They are widely used in medicine, cosmetics, food and beverages and aromatherapy. Many essential oils or their constituents are known as potent anti-infective agents against viruses, bacteria and fungi. In recent year, essential oils from Guacatonga (*Casearia sylvestris* Sw.), Sweet fern (*Comptonia peregrine* L.), Guava (*Psidium guajava* L.), Sweet basil (*Ocimum basilicum* L.), *Tanacetum gracile* and lemon grass (*Cymbopogon flexuosus*) have been reported to exhibit cytotoxicity to many human cancer cells **[108-110]**. Several Thai edible plants such as Lemongrass (*Cymbopogon citratus* D.C.), Holy Basil (*Ocimum sanctum*) and Sweet Basil (*O. basilicum*) also have been shown to possess antitumor activity in mice **[111]**. Many of these plants contain citral in their essential oils.

Citral

Citral (3,7-dimethyl-2,6-octadienal) is a key component of essential oil found in several Thai medicinal and edible plants such as lemon grass (*Cymbopogon citratus*), lemon balm (*Melissa officinalis*), and *Litsea cubeba*. It is the mixture of two isomeric acyclic monoterpene aldehydes, the *trans*-isomer referred to geranial or citral and the *cis*-isomer called neral or citral B **[112]**. Both isomers have the same molecular formula, $C_{10}H_{16}O$, but different structures, as shown in Fig.14.

The physical and chemical properties of citral are; colorless to light yellow liquid, lemon-like odor, MW 162.24 g/mole, boiling point: 229°C, specific gravity 0.893, vapor pressure 0.2 mm of Hg at 20°C and vapor density: 5.2.



Geranial (*trans*-citral) Neral (*cis*-citral)

Figure 14: Chemical structure of the citral **[113]**.

Citral is used as a food additive and as a fragrance material in cosmetics because of its strong lemon flavor and odor and bitter–sweet taste **[114]**. It is also an important material in the manufacture of some chemicals, such as vitamin A and (methyl)ionone **[115]**. It has a long history of acceptance by US and European regulatory bodies and has been accorded GRAS (generally recognized as safe) status. The average daily intake of citral in humans was estimated to be 5 mg/kg.

The pharmacokinetic properties of citral were studied in rats and mice. It had rapid metabolism and complete within 72 h mainly in the urine but also via the lungs and in the feces. There were 2 major metabolites of citral had been detected including 2,6-dimethyl-2,6-octadiendioic acid and 2,6-dimethyl-2-octendioic acid **[116]**.

The toxicity studies demonstrate that citral has no major toxicity and carcinogenic potential in both rats and mice. It has low acute toxicity, with variation in the LD_{50} values depending on the strains and species of animals used and the route of administration. The oral LD_{50} in rats is 4.96 g/kg body weight and the dermal LD_{50} in rabbits is 2.25 g/kg body weight. There was no macroscopic changes in rats after administration of citral to rats at levels of 10,000, 2500 or 1000 ppm in the diet for 13 week **[117]**. The short-term (28-day) studies on citral given by gavage to rats revealed a maximum non-lethal daily dose of 2.4 g/kg body weight. At this dose, citral caused hepatomegaly, microsomal enzyme induction and peroxisome proliferation **[118]**. It was also reported that citral had no mutagenic effect in *in vitro* studies **[119]**.

The pharmacological properties of citral were evaluated in many aspects. The examples of pharmacological effects of citral are;

Anti-infective effects

Citral has *in vitro* broad spectrum activities against many microorganism including gram-positive and gram-negative bacteria, fungi, and some protozoa. It has been reported to have antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, methicillin-resistant *S. aureus* (MRSA), *Aspergillus niger*, *Klebsiella pneumonia, Propionibacterium acnes* **[120],** *Bacillus sp.*, *L. monocytogenes*, *S. aureus*, *A. hydrophila*, and *E. faecalis* **[121]**. It also had potent *in vitro* antifungal activities against several *Candida* species (*C. albicans*, *C. glabrata*, *C. krusei*, *C. parapsilosis* and *C. tropicalis*) **[113],** *Colletotrichum coccodes*, *Botrytis cinerea*, *Cladosporium herbarum*, *Rhizopus stolonifer* and *Aspergillus niger* **[122]**. The antiviral activity of citral against *Herpes simplex* virus-1 was also reported **[123]**. Further more, the antiparasitic effects of citral on different organisms have been suggested in several studies **[124]**.

Anti-cancer effect

Citral was found to possess anticancer effect against several cancer cell lines. In several hematopoietic cancer cells, treatment with citral induced 58-90% cell death with the IC_{50} = 47 µg/ml. Treatment of human U937 and HL60 and mouse RL12 and BS-24-1 leukemic cell lines for 4-24 h with citral 45 µg/ml, resulted in activation of caspase-3 activity and induces apoptosis. Citral induced DNA ladder in BS-24-1cells after 70 min exposure. Additionally, treatment of leukemic cells with citral metabolism products, geranic acid or geraniol, or the citral derivatives citronellal and citronellol, did not activate procaspase-3 or induce DNA ladder, indicated that the α,β-unsaturated aldehyde group of citral is crucial for procaspase-3 activation **[125]**. Citral inhibited MCF-7 cell growth with the IC_{50} at 48 h = 180 µM. It arrested the cell cycle in G2/M phase and induced apoptosis. It also inhibited cyclo-oxygenase activity, resulted in decrease in prostaglandin E_2 synthesis 48 h after citral treatment **[4]**.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Test compounds

Citral and mint oil were purchased from Sigma-Aldrich (USA).

1.2 Cell culture

- Human peripheral blood mononuclear cells (human PBMCs)

Heparinized blood was obtained from healthy male blood donors, age between 20 to 35 years old, with informed consent at the National Blood Bank, Thai Red Cross Society. Human PBMCs were isolated from the whole blood by ficoll gradient centrifugation. The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin; at 37^{o}C in a humidified atmosphere of 5% CO_2.

- Human B-Lymphoma cells (Ramos cells)

The human B-Lymphoma cells, Ramos cells, were purchased from the American Type Cell Culture (ATCC) (Rockville, MD). The cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 0.5% L-glutamine, 100 µg/ml streptomycin and 100 units/ml penicillin at 37^{o}C in a humidified atmosphere of 5% CO_2.

1.3 Equipments and Instruments

The following instruments were used in this study; autoclave (Hirayama, Japan), fluorescence flow cytometer (Coulter, USA), biohazard lamina-flow hood (Science, Germany), Incubator (Thermo, USA), Light microscope (Nikon, Japan), refrigerator 4^{o}C and -20^{o}C (Sanyo, Japan), pipette (Falcon, USA), pH meter (Mettler tuledo, Switzerland), autopipette (Gilson, France), centrifuge (Eppendorf, Germany), gel documentation (Bio-Rad, USA), PCR thermal cycler (Eppendorf, Germany), gel electrophoresis (Bio-Rad, USA), T25 tissue culture flasks (Corning, USA), 24-well microplates (Corning, USA).

1.4 Reagents

The following reagents and reagent kits were used in this study; annexin V apoptosis detection kit (Santa Cruz Biotechnology, USA), caspase inhibitor Z-VAD-FMK (Promega, USA), anti-Fas ligand antibodies (Biolegend, USA), fetal bovine serum (Gibco, USA), RPMI 1640 medium (Gibco, USA), L-glutamine (Gibco, USA), penicillin/streptomycin (Gibco, USA), etoposide (Ebewe Pharma, Austria), heparin (Leo, Denmark), Histopaque®-1077 (Sigma, USA), 0.4% trypan blue dye (Sigma, USA), Platinum® Taq DNA polymerase (Invitrogen, USA), Improme-II™ reverse transcription system (Promega, USA), agarose (Bio-Rad, USA), dNTP mix (Vivantis, Malaysia), absolute ethanol (Merck, Germany), TRIzol® Reagent (Invitrogen, USA), and diethyl pyrocarbonate (DEPC) (Molekula, UK)

2. Methods

2.1 Preparation stock solutions of citral and mint oil

The stock solutions of citral and mint oil were prepared by dissolving in 70% ethanol. The solutions were stored at 4^{o}C. The working solutions of both oils were prepared by diluting the stock solutions with sterile double distilled water to 5% ethanol solutions. The final concentration of ethanol in treated cells would be 0.5%.

2.2 Preparation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from heparinized blood by Ficoll (Histopaque-1077) density gradient centrifugation as the followings:

1. Equally mix the blood sample with RPMI 1640 medium containing 2 µl/ml heparin at room temperature.

2. Slowly layer 9 ml of the mixture on 5 ml Ficoll solution in a 15 ml sterile polypropylene tube at room temperature.

3. Centrifuge the tube at 3,200 rpm for 30 min at room temperature.

4. Carefully remove the top layer solution without disturbing the interface.

5. Collect cells from the interface into a new 15 ml sterile polypropylene tube.

6. Wash the cells twice with 12.5 ml RPMI 1640 medium (+ 2 µl/ml heparin) by centrifugation at 1,200 rpm for 10 min at room temperature.

9. Discard the supernatant and resuspend the pellet with 5 ml the completed RPMI medium (RPMI 1640 medium with 10% fetal bovine serum, 0.5% L-glutamine, 100 µg/ml streptomycin, and 100 units/ml penicillin).

10. Determine viable cells by staining with 0.4% trypan blue dye solution at the ratio 1:1 and counting the number of the cells on a hemocytometer. The cells were adjusted to required density with the completed RPMI 1640 medium.

In this study, PBMCs were used at the density of $1x10^6$ cells/ml, with cell viability more than 90%.

2.3 Determination of apoptotic induction by citral

Apoptotic cells were determined by detecting the exposure of phosphatidylserine (PS) on the outer cell membrane with annexin-V-FITC staining and by analyzing the decrease of DNA content with PI staining.

2.3.1 Analysis the exposure of phosphatidylserine

Citral at the final concentrations of 37.5, 75 and 150 µM in 0.5% ethanol was used in this experiment. 0.5% ethanol, 10 µg/ml etoposide, and 0.5% v/v mint oils in 0.5% ethanol were used as the negative, the positive and the oil controls, respectively. The assay was performed in duplication according to the following procedures (n=3).

1. Incubate 900 µl of Ramos cells in the completed RPMI 1640 medium, at the density of $1x10^6$ cells/ml, with 100 µl of each sample in a well of 24-well plates at 37^oC in a humidified atmosphere of 5% CO_2 for 3, 6 and 12 h.

2. Collect the cells in each well to a micro-centrifuge tube and centrifuge at 12,000 rpm 25^oC for 1 min.

3. Removed the supernatant, wash the cells twice with 500 µl cold PBS, and separate the cells by centrifugation at 12,000 rpm, 25^oC for 1 min.

4. Discard the supernatant, resuspend the cell pellet in 100 µl of the assay buffer and transferred into a flow cytometer tube.

5. Add 1 µl of 0.05 µg/ml PI and 0.5 µl of annexin V-FIT C in each tube.

6. Incubate the tubes in the dark condition at room temperature for 15 min.

7. Subsequently add 400 µl of the assay buffer into each tube and immediately analyze the cells (10,000 cells/sample) by fluorescence flow cytometer.

8. Assess types of death cells as follow; the annexin V-$FITC^-$/PI^- cells as viable cells, the annexin V-$FITC^+$ cells as apoptotic cells, the PI^+ cells as nonapoptotic cells, and the annexin v-$FITC^+$/PI^+ cells as late apoptotic or secondary necrotic cells.

2.3.2 Analysis of cellular DNA content

1. Incubate 900 µl of Ramos cells in the completed RPMI 1640 medium, at the density of $1x10^6$ cells/ml, with 100 µl of each sample in a well of 24-well plates at 37^oC in a humidified atmosphere of 5% CO_2 for 6 and 12 h.

2. Collect the cells in each well to a flow cytometer tube and centrifuged at 1,500 rpm 4^oC for 5 min.

3. Removed the supernatant, wash the cells twice with 500 µl of cold PBS, and separate the cells by centrifugation at 1,500 rpm 4^{o}C for 5 min.

4. Discard the supernatant and resuspend the cell pellet in 150 µl cold PBS.

5. Slowly add 350 µl absolute ethanol while continuously shaking the tube.

6. Incubate at -20^{o}C for 15 min, then separate the cells by centrifugation at 1,500 rpm 4^{o}C for 5 min.

6. Removed the supernatant, wash the cells twice with 500 µl of cold PBS and separate the cells by centrifugation at 1,500 rpm 4^{o}C for 5 min.

7. Discard the supernatant and resuspend the cells pellet in 500 µl of the assay buffer.

8. Add 5 µl of 4 mg/ml RNase and incubate at room temperature for 30 min.

9. Add 5 µl of 0.05 µg/ml PI and incubate the tube in dark condition at room temperature for 15 min.

10. Analyze the cells (100,000 cells/sample) by fluorescence flow cytometer.

2.4 Determination of mechanisms of citral on apoptotic induction

The effect of citral on the extrinsic pathway of apoptosis was determined by using an antibody against the death receptor ligan, Fas ligan (FasL), to inhibit Fas and FasL interaction. The activity of citral on the intrinsic pathway of apoptosis was investigated by studying the mRNA expression of various anti-apoptotic and pro-apoptotic proteins. A pan caspase inhibitor, Z-VAD-FMK, was also used to analyze the caspase-dependent activity of citral.

2.4.1 Fas-Fas ligand dependent pathway

1. Incubate 900 µl of Ramos cells in the completed RPMI 1640 medium, at the density of 1x10^{6} cells/ml, with 20 µl of FasL antibody in a well of 24-well plates at 37^{o}C in humidified atmosphere of 5% CO_2 for 1 h.

2. Add 100 µl of each sample solution into each well.

3. Incubated the cell at 37^{o}C in humidified atmosphere of 5% CO_2 for 6 h.

4. Performed the next procedures as in the step 2nd -10th of 2.3.1.

2.4.2 Mitochondria dependent pathway

The mRNA expressions of *p53* gene, pro-apoptotic genes (*BAX and BAK*) and anti-apoptotic genes (*BCL-XL and BCL-2*) were determined as in the following procedures;

2.4.2.1 Preparation of total RNA

1. Incubate 900 µl of Ramos cells in the completed RPMI 1640 medium, at the density of $1x10^6$ cells/ml, with 100 µl of each sample in a well of 24-well plates at 37°C in a humidified atmosphere of 5% CO_2 for 6 h.

2. Collect the cells into centrifuge tube 15 ml and separate the cells by centrifugation at 1,200 rpm 25°C for 10 min.

3. Removed the supernatant, lyse and homogenize in 1 ml of TRIzol® reagent by passing the cell up and down through a pipette, transfer and incubate the lysate to a microcentrifuge tube for 5 min at room temperature.

5. Add 0.2 ml of chloroform, vigorously shake the tube by hand for 15 sec., and incubate at room temperature for 2-3 min.

6. Centrifuged at 12,000 rpm 4°C for 15 min. and transfer the aqueous phase to a fresh tube.

7. Add 0.5 ml of isopropyl alcohol, incubate at room temperature for 10 min, and centrifuge 12,000 rpm at 4°C for 10 min.

8. Removed the supernatant, wash the RNA pellet with 1 ml of 75% ethanol, mix by vortexing, separate the pellet by centrifugation 7,500 rpm at 4°C for 5 min.

9. Removed the supernatant and air-dry the RNA pellet (for 5-10 min).

10. Dissolve the pellet in DEPC-treated water, incubate at 55-60°C for 10 min, and stored at -70°C until use.

The amount of RNA in the sample was determined by spectrophotometer at 260 nm. The protein contamination in it was checked by the ratio of optical densities of the RNA sample at 260 nm. and 280 nm. which must be > 1.8.

2.4.2.2 Preparation of complementary DNA (cDNA) by reverse transcription-polymerase chain reaction

One µg of total RNA was reverse transcribed to cDNA synthesis using Improme II™ reverse transcription system reagent with oligo$(dT)_{15}$ primer following the manufacturer protocol.

2.4.2.3 Determine mRNA expression of pro- and anti-apoptotic genes by PCR

PCR was performed using gene-specific primers (table 1) for p53, BCL-2, BCL-Xl, BAX and BAK. The GAPDH gene was used as the internal control. PCR was carried out in a 50 µl reaction mixture containing PCR buffer, 0.5 µl cDNA of p53, GAPDH, BAK and BCL-Xl or 1 µl cDNA of BAX and BCL-2, 1.5 mM $MgCl_2$, 0.2 mM mixed dNTP, 0.4 µM of each primers and 1 unit of platinum® Taq DNA polymerase. The PCR conditions were: 94°C for 2 min., followed by 35 cycle of 30 sec. denaturation at 94°C, 30 sec. annealing at appropriate Tm, 1 min extension at 72°C, and finally 10 min extension at 72°C. PCR products were analyzed by electrophoresis in 1.5% agarose gel at 100 V. The gel was stained with ethidium bromide in 1xTBE buffer. PCR products were imaged and determined its density by gel documentation. The density of the PCR products were expressed as % of internal control (ratio of the band density divided by that of the housekeeping gene (GAPDH) x 100)

Table 1: The gene-specific primers used for RT-PCR

Gene	Primer sequences	Tm ^{o}C	Production length (bp)
GAPDH	Forward: 5'-AAG GTC GGA GTC AAC GGA TTT GGT-3' Reverse: 5'-ATG GCA TGG ACT GTG GTC ATG AGT-3'	60	530
p53	Forward: 5'-CAT GAG CGC TGC TCA GAT AG-3' Reverse: 5'-CTG AGT CAG GCC CTT CTG TC-3'	56	643
BAK	Forward: 5'-TGA AAA ATG GCT TCG GGG CAA GGC-3' Reverse: 5'-TCA TGA TTT GAA GAA TCT TCG TAC C-3'	55	642
BAX	Forward: 5'- ATG GAC GGG TCC GGG GAG -3' Reverse: 5'- TCA GAA AAC ATG TCA GCT GCC -3'	58	315
BCL-Xl	Forward: 5'- CAA TGG ACT GGT GAG CCC A -3' Reverse: 5'- AGT TCA AAC TCG TCG CCT G -3'	55	307
BCL-2	Forward: 5'-GGT GCC ACC TGT GGT CCA CCT-3' Reverse: 5'-CTT CAC TTG TGG CCC AGA TAG G-3'	58	458

2.4.3 Caspase dependent pathway

Ramos cells were pretreated with 50 μM Z-VAD-FMK, a pan caspase inhibitor, 1 h. before adding the sample solution. The assay was performed in duplication as in 2.4.1 (n=3).

2.5 Determination of the effect of citral on cell cycle

1. Incubate 900 μl of Ramos cells in the completed RPMI 1640 medium, at the density of $1x10^{6}$ cells/ml, with 100 μl of each sample in a well of 24-well plates at 37^{o}C in a humidified atmosphere of 5% CO_2 for 3 h.

2. Removed the supernatant, wash the cells twice with 2 ml of RPMI 1640 medium and separate the cells by centrifugation at 1,200 rpm 25^{o}C for 10 min.

5. Discard the supernatant and resuspend the cells pellet in 1ml of the completed RPMI 1640 medium.

6. Transfer the cells suspension into each well of 24-well plates and incubated the cells at 37^{o}C in humidified atmosphere of 5% CO_2 for 48 h.

8. Performed the next procedures as in the step 4th-12th of 2.3.2.

2.6 Statistical analysis

Data were presented as mean plus or minus standard error (mean ± S.E.). Statistical comparisons were made by one-way ANOVA followed by Tukey's post hoc test. All statistical analysis was performed according to the statistic program, SPSS version 17. Any p-value < 0.01 was considered statistically significant.

CHAPTER IV

RESULTS

1. Citral induced apoptotic cell death in human B-lymphoma cells

To investigate the apoptotic induction effect of citral on lymphoma cells, Ramos cells were treated with 37.5, 75 and 150 μM citral for 3, 6 and 12 h. The types of cell death were determined by annexin V-FITC/PI staining assay with fluorescent flow cytometer. Fig. 15 represents a histogram from the assay, which demonstrates the profiles of cell death by 150 μM citral as in the following: annexin V-FITC positive cells are early apoptotic cells, PI positive cells are necrotic cells, double annexin V-FITC/PI positive cells are necrotic and late apoptotic cells, and double annexin V-FITC/PI negative cells are viable cells. The results demonstrated that citral induced cell death as early as 3 h, at the concentration of 75 and 150 μM. It statistically increased Ramos cells death in a concentration- and time-dependent manner, as shown in Fig. 16,17 and Table 2. It induced the cell death mostly by apoptosis after 3 and 6 h exposure. After 12 h of exposure, the increased in late apoptosis and necrosis were detected but the death cells were still mainly apoptotic cells (Fig. 16 and Table 2). In this study, the solvent control (0.5% ethanol), the oil control (0.5% v/v mint oil), and the positive control (10 μg/ml etoposide) had very little cytotoxic effect on Ramos cells. Even though etoposide did not cause Ramos cells death after 3 to 12 h exposure, it induced Ramos cells death after 24 h of treatment. Etoposide induced Ramos cells death mainly by both apoptosis and necrosis/late apoptosis. These results suggest that citral induces Ramos cells death mainly by apoptosis in a short time after treatment.

The apoptotic effect of citral was confirmed by analyzing sub-diploid cells with PI staining assay. Ramos cells were treated with 37.5, 75 and 150 μM citral for 6 and 12 h. The cells were fixed, stained with PI, and then analyzed for cellular DNA content by fluorescent flow cytometer (Fig. 18). The population of apoptotic cells was defined as a sub-diploid or sub-G1 population in a histogram of the cell cycle. After 6 and 12 h of citral exposure, sub-diploid population of Ramos cells statistically increased in a concentration- and time-dependent manner, when compared to the solvent (0.5% ethanol) and the mint oil control (Fig. 19 and 20). These results correlated with the study by detecting phosphatidyl serine exposure above.

The harmful effect of citral on normal cells was also determined in human PBMCs from normal subjects. The cells were treated with citral in the same procedure as the treated Ramos cells for 3, 6, and 12 h. The apoptotic cells were determined by annexin V-FITC/PI staining assay. Citral had no cytotoxic effect on PBMCs after 3 h of treatment, when compared to the solvent and the mint oil controls. It induced apoptosis, in a concentration- and time-dependent manner, after 6 and 12 h of exposure (Fig. 21). Its effect on PBMCs was much less than on Ramos cells after 3 and 6 h of treatment. It had higher cytotoxic to the normal cells at the longer time of exposure, as 12 h (Fig. 22), but this effect was still less than its effect on the lymphoma cells. These results demonstrate that within a short time citral can induce apoptosis in lymphoma cells with much higher potency than its apoptotic effect on normal cells.

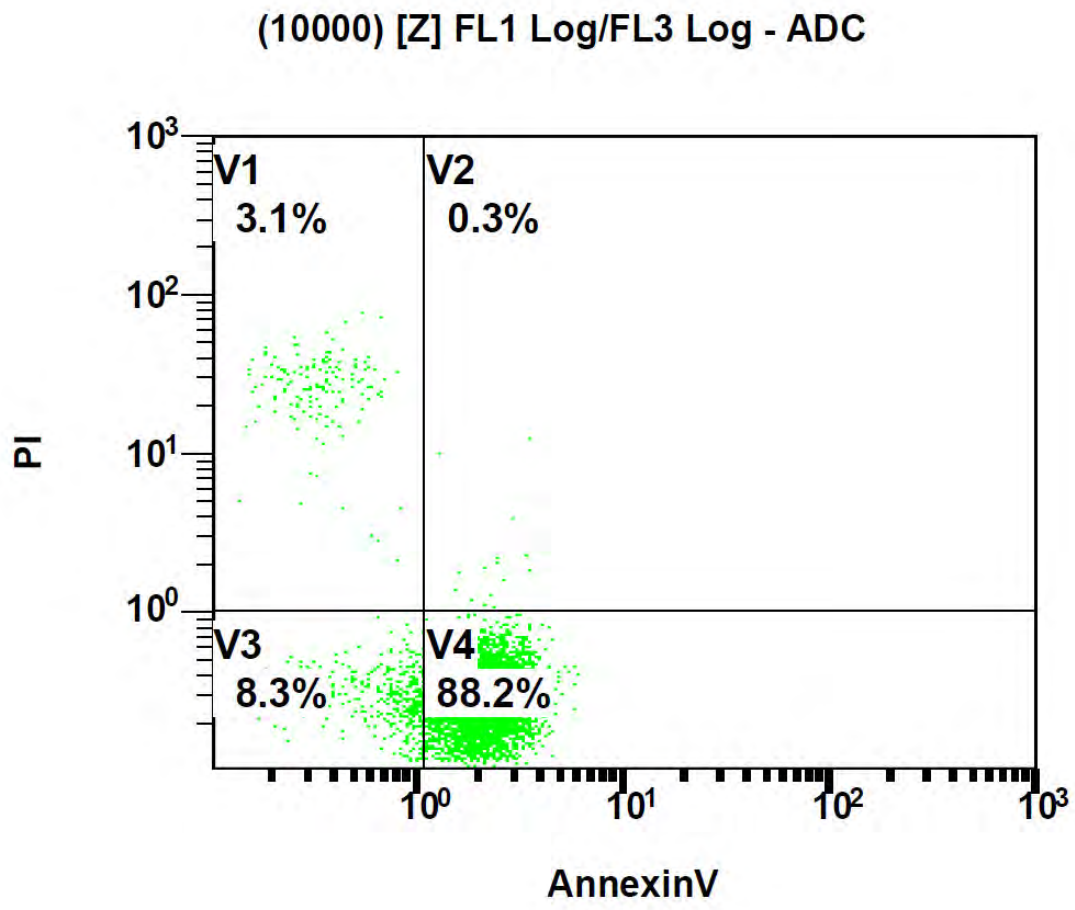


Figure 15: A representative dot plot histogram from annexin V-FITC/PI staining assay using fluorescent flow cytometer of 150 µM citral treated Ramos cells for 6 h. Cells in each quadrant (V1-V4) were interpreted as in the following; V1: necrotic cells (annexin V^- /PI $^+$), V2: necrotic and late apoptotic cells (annexin V^+/PI $^+$), V3: viable cells (annexin V^- /PI $^-$), and V4: early apoptotic cells (annexin V^+/PI $^-$).

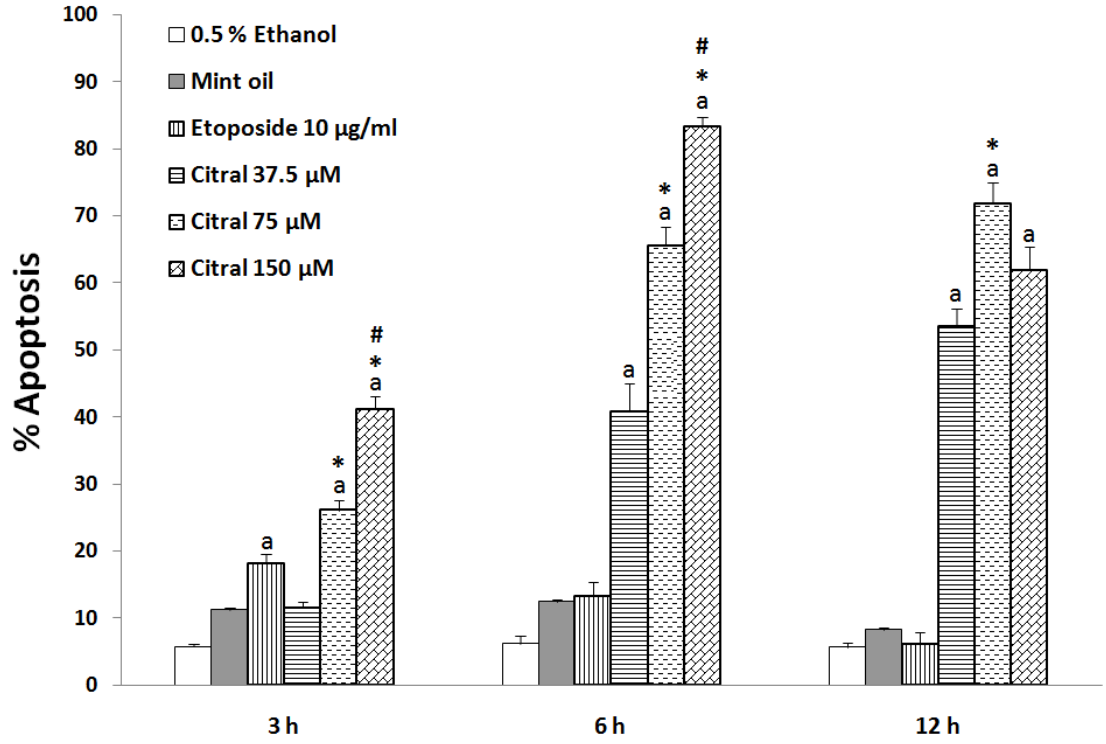


Figure 16: The concentration-dependent effect of citral on Ramos cells apoptosis. The cells were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. The percentage of apoptotic cells (annexin V^+/PI^-) were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

a $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

* $p < 0.01$ denotes statistically significant difference between 75 and 150 µM citral compared with 37.5 µM citral.

# $p < 0.01$ denotes statistically significant difference when compared between 150 µM and 75 µM of citral.

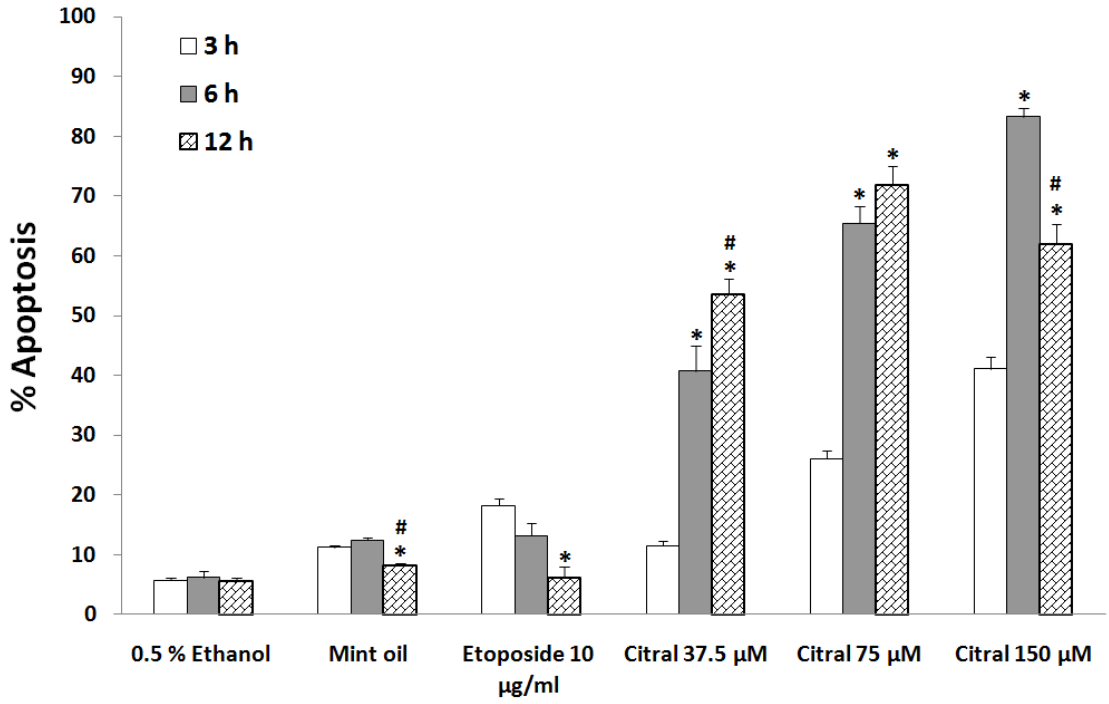


Figure 17: The time-dependent effect of citral on Ramos cells apoptosis. The cells were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. The percentage of apoptotic cells (annexin V^+/PI^-) were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

* $p < 0.01$ denotes statistically significant difference as compared to 3 h.

# $p < 0.01$ denotes statistically significant difference when compared between 12 h and 6 h.

Table 2: The effect of citral on Ramos cells death. The cells were treated with 37.5, 75 and 150 µM of citral for 3, 6 and 12 h. The patterns of cell death were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

Treatment	% Viable cells	% Death cells			% Total death
		Apoptosis	PI positive	Double positive	
3 h					
0.5% Ethanol	93.77 ± 0.42	5.73 ± 0.37	0.10 ± 0.00	0.33 ± 0.03	6.17 ± 0.38
Mint oil	87.13 ± 0.83a	11.20 ± 0.35	0.73 ± 0.19	0.90 ± 0.21	12.83 ± 0.45a
Etoposide 10 µg/ml	80.83 ± 1.03a	18.23 ± 1.25a	0.23 ± 0.09	0.73 ± 0.12	19.20 + 1.07a
Citral 37.5 µM	87.70 ± 0.82	11.50 ± 0.85	0.50 ± 0.12	0.33 ± 0.12	12.33 ± 0.80
Citral 75 µM	72.57 ± 1.09a*	26.07 ± 1.44a*	0.63 ± 0.24	0.73 ± 0.13	27.43 ± 1.10a*
Citral 150 µM	56.97 ± 1.90a*#	41.10 ± 1.93a*#	1.10 ± 0.11a	0.83 ± 0.12	43.03 ± 1.90a*#
6 h					
0.5% Ethanol	93.33 ± 1.13	6.22 ± 1.05	0.10 ± 0.05	0.32 ± 0.13	6.63 ± 1.15
Mint oil	86.65 ± 0.29	12.42 ± 0.41	0.35 ± 0.19	0.53 ± 0.14	13.30 ± 0.28
Etoposide 10 µg/ml	84.77 ± 2.47	13.17 ± 2.18	0.62 ± 0.28	1.43 ± 0.68	15.22 ± 2.47
Citral 37.5 µM	56.80 ± 4.68a	40.72 ± 4.30a	0.72 ± 0.22	1.77 ± 0.87	43.20 ± 4.69a
Citral 75 µM	29.17 ± 2.87a*	65.47 ± 2.91a*	3.85 ± 0.71a	1.52 ± 0.56	70.83 ± 2.87a*
Citral 150 µM	9.40 ± 2.27a*#	83.30 ± 1.49a*#	4.72 ± 0.99a*	2.55 ± 0.79	90.57 ± 2.28a*#
12 h					
0.5% Ethanol	94.13 ± 0.58	5.63 ± 0.60	0.03 ± 0.03	0.17 ± 0.03	5.83 ± 0.59
Mint oil	87.10 ± 1.74	8.23 ± 0.39	0.63 ± 0.44	4.00 ± 1.01	12.87 ± 1.77
Etoposide 10 µg/ml	73.97 ± 1.50a	6.10 ± 1.85	3.23 ± 1.77	16.70 ± 1.96a	26.03 ± 1.50a
Citral 37.5 µM	31.63 ± 4.37a	53.53 ± 2.62a	12.07 ± 2.12	2.77 ± 0.46	68.37 ± 4.37a
Citral 75 µM	6.97 ± 1.49a*	71.83 ± 3.16a*	17.03 ± 3.56a	4.20 ± 1.36	93.07 ± 1.47a*
Citral 150 µM	1.17 ± 0.03a*	61.97 ± 3.37a	17.57 ± 3.99a	19.33 ± 2.40a*#	98.87 ± 0.03a*
24 h					
0.5% Ethanol	95.62 ± 0.52	3.78 ± 0.64	0.07 ± 0.04	0.55 ± 0.08	4.40 ± 0.53
Etoposide 10 µg/ml	54.92 ± 0.69a	25.62 ± 1.38a	1.25 ± 0.33	18.20 ± 0.38a	45.07 ± 0.71a

a $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

* $p < 0.01$ denotes statistically significant difference between 75 and 150 µM citral compared with 37.5 µM citral.

# $p < 0.01$ denotes statistically significant difference when compared between 150 µM and 75 µM of citral.

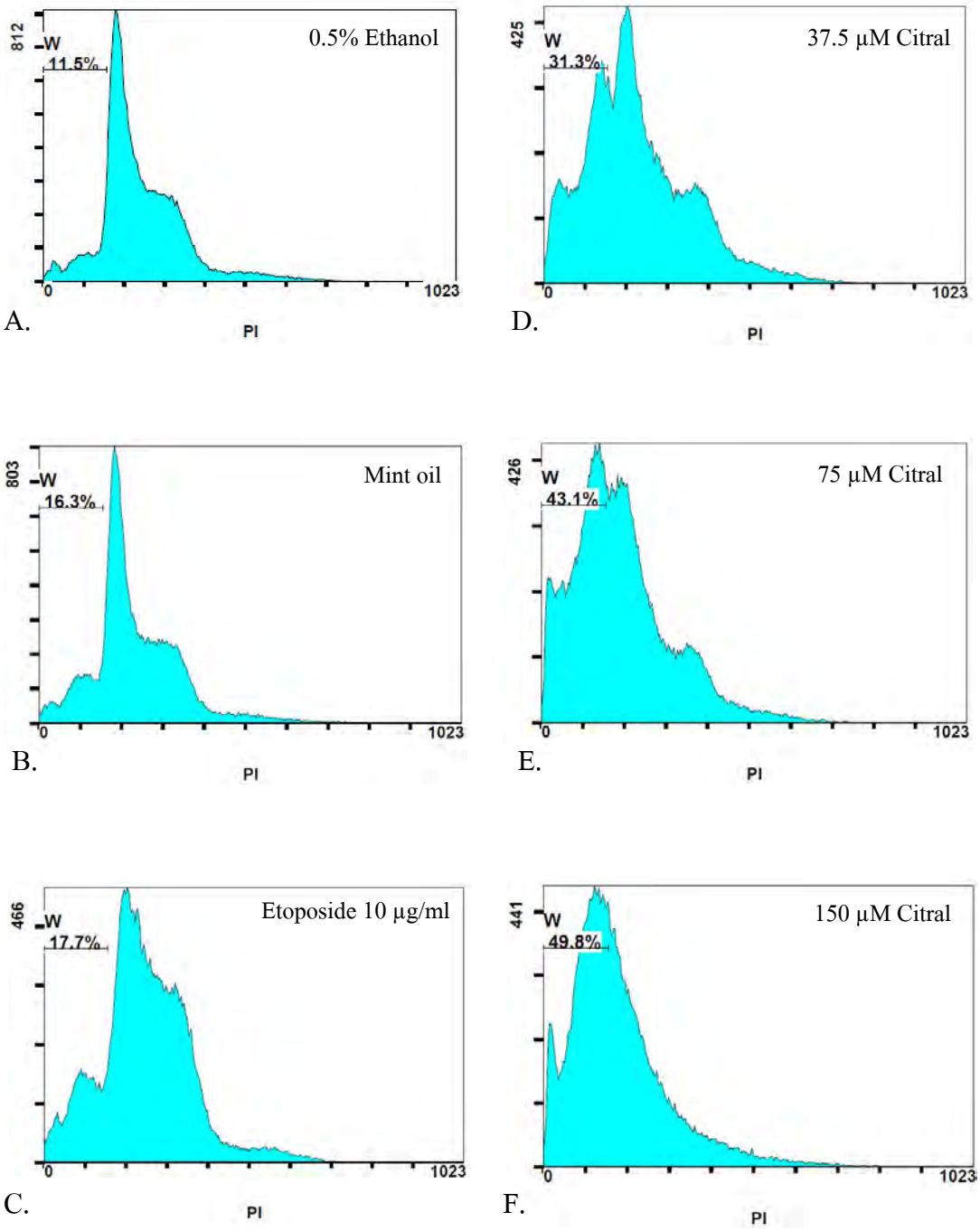


Figure 18: A representative histogram for determining sub-diploid cells population by PI staining assay using flow cytometer. Ramos cells were treated with 37.5, 75 and 150 µM citral for 12 h before being fixed, RNase treated, and stained with PI. The population of apoptotic cells was determined in a DNA histogram as a sub G1 hypodiploid population by flow cytometer. The percentage of hypodiploid cells was indicated in each sub-figure. (A) 0.5 % ethanol; (B) mint oil; (C) etoposide 10 µg/ml; (D, E, F) 37.5, 75 and 150 µM of citral.

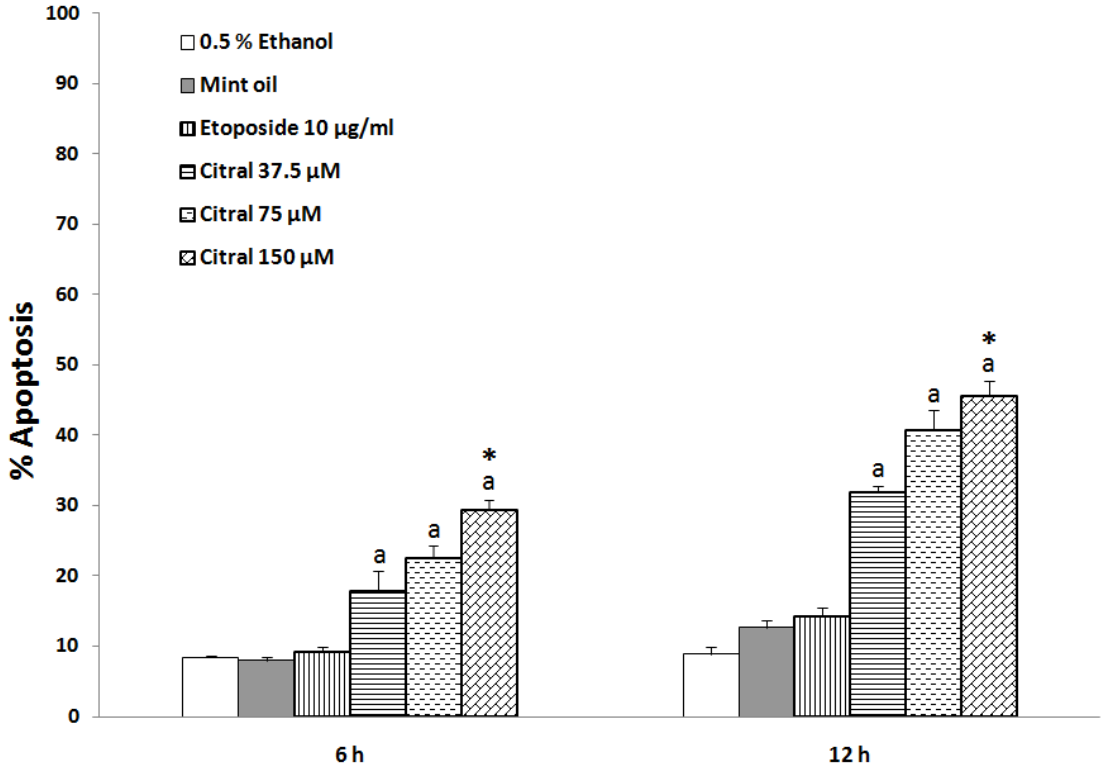


Figure 19: Citral induced sub-diploid population in Ramos cells. The cells were treated with 37.5, 75 and 150 µM citral for 6 and 12 h before being ethanol-fixed, RNase treated, and stained with PI. The percentage of sub-diploid cells were determined by fluorescent flow cytometer. The data is expressed as mean ± S.E. of four independent experiments (n=4).

a $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

* $p < 0.01$ denotes statistically significant difference between 75 and 150 µM citral compared with 37.5 µM citral.

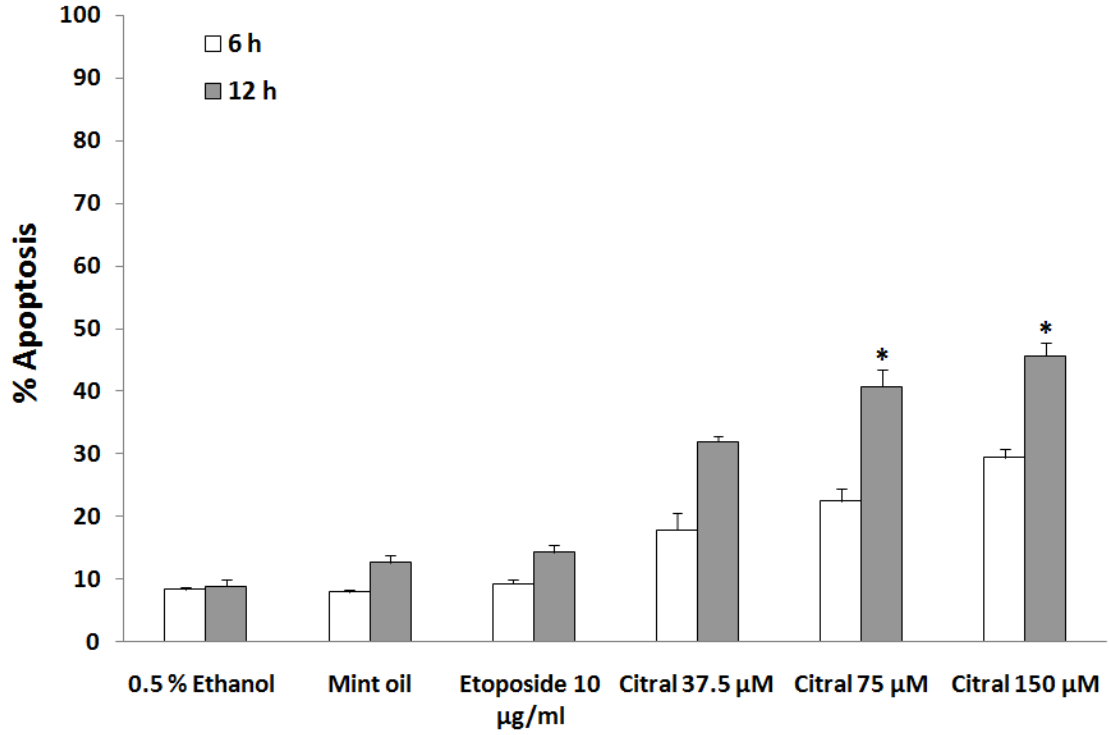


Figure 20: The comparison of citral induced sub-diploid population in Ramos cells between 6 h and 12 h of treatment. The cells were treated with 37.5, 75 and 150 µM citral for 6 and 12 h before being ethanol-fixed, RNase treated, and stained with PI. The percentage of sub-diploid cells were determined by fluorescent flow cytometer. The data is expressed as mean ± S.E. of four independent experiments (n=4).

* $p < 0.01$ denotes statistically significant difference as compared to 6 h.

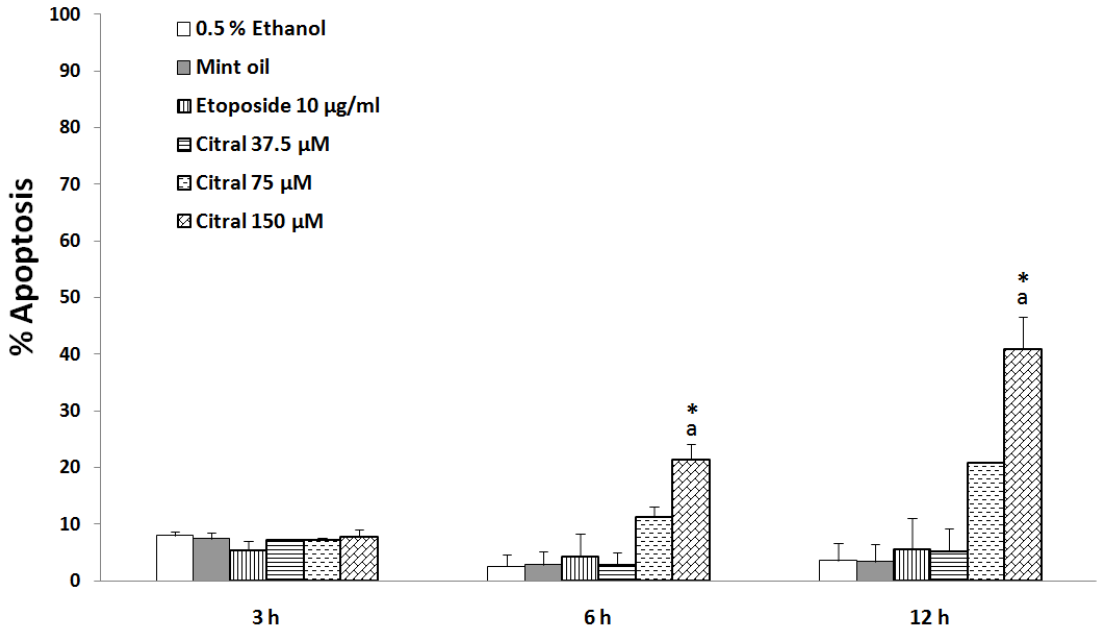


Figure 21: The effect of citral induced apoptosis in normal cells. Human PBMCs cells were treated with 37.5, 75 and 150 µM citral for 3, 6 and 12 h. The percentage of apoptotic cells (annexin V^+/PI^-) were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

a $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

* $p < 0.01$ denotes statistically significant difference between 75 and 150 µM citral compared with 37.5 µM citral.

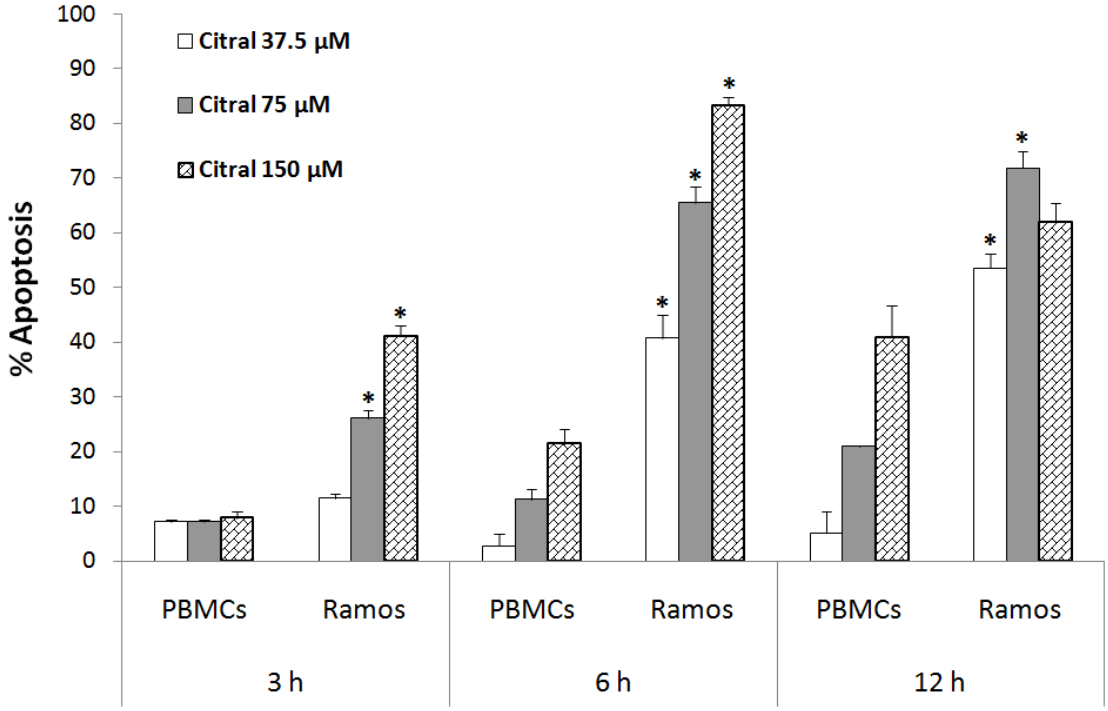


Figure 22: The comparison of citral induced apoptosis in between normal cells and Ramos cells. Human PBMCs and Ramos cells were treated with 37.5, 75 and 150 µM of citral for 3, 6 and 12 h. The percentage of apoptotic cells (annexin V^+/PI^-) were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

* $p < 0.01$ denotes statistically significant difference as compared to PBMCs.

2. Characterization of apoptosis induced by citral in Ramos cells

The apoptotic induction activity of citral was characterized whether it induced apoptosis via the mitochondrial pathway by determining the expression of some BCl-2 and p53 proteins or via the extrinsic pathway by using the Fas ligand (FasL) inhibitor, anti-FasL antibody. The caspase dependency of the citral activity was also evaluated by using a pan-caspase inhibitor, Z-VAD-FMK.

The mitochondria pathway of apoptosis is regulated by proteins in the Bcl-2 family. Some of these proteins (e.g., BCL-2, BCL-XL) prevent apoptosis while the others (e.g., BAX, BAK) promote apoptosis. The expressions of these proteins are partly regulated by p53 protein. In this study, the effect of 75 and 150 μM citral on the expressions of p53, pro-apoptotic (BAX and BAK), and anti-apoptotic (BCL-2 and BCL-XL) proteins in Ramos cells was determined by RT-PCR after 6 h of treatment. Citral significantly decreased the mRNA expression of BCL-2 (Fig. 23). The BCL-2 expressions were significantly decreased from 56.85 ± 5.48 % in the solvent (0.5% ethanol) treated cells to 6.77 ± 6.57% and 3.52 ± 3.08% in the 75 and 150 μM citral treated cells respectively. Citral did not have any effect on the mRNA expressions of p53, BCL-Xl, BAX and BAK. These results suggest that citral induces Ramos cells apoptosis using mitochondrial pathway by down regulating BCL-2 expression.

To evaluate whether the extrinsic pathway involved in the apoptotic effect of citral, the anti-human Fas ligand antibody, was used to inhibit Fas-Fas ligand interaction. Ramos cells were pretreated with the antibody before being treated with citral for 6 h. The percentage of apoptotic cells were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The result in Fig. 24 demonstrated that the antibody did not change the apoptotic induction activity of citral on Ramos cells. No difference was observed between the antibody-pretreated and the non-pretreated conditions at all concentrations (37.5, 75 and 150 μM) of citral. The Fas-Fas ligand interaction doesn't play role in the apoptotic induction of citral.

It is well known that the signal from the intrinsic pathway of apoptosis is mainly driven through caspase activation. However, the caspase independence of this pathway has been reported. To determine whether citral induced Ramos cells apoptosis depends on caspase activation, a pan-caspase inhibitor, Z-VAD-FMK, was used to pre-treat the cells before treating with citral. The cells were pretreated with the caspase inhibitor for 1 h prior to be treated with

citral for 6 h. The caspase inhibitor significantly inhibited citral-mediated apoptosis, from 40.71 ± 4.30% to 7.06 ± 1.21%; from 65.46 ± 2.91% to 19.40 ± 0.83% and from 83.30 ± 1.48% to 51.60 ± 6.75% at 37.5, 75 and 150 µM of citral respectively (Fig. 25). These results indicated that the apoptosis triggered by citral was mediated mainly by caspase activation.

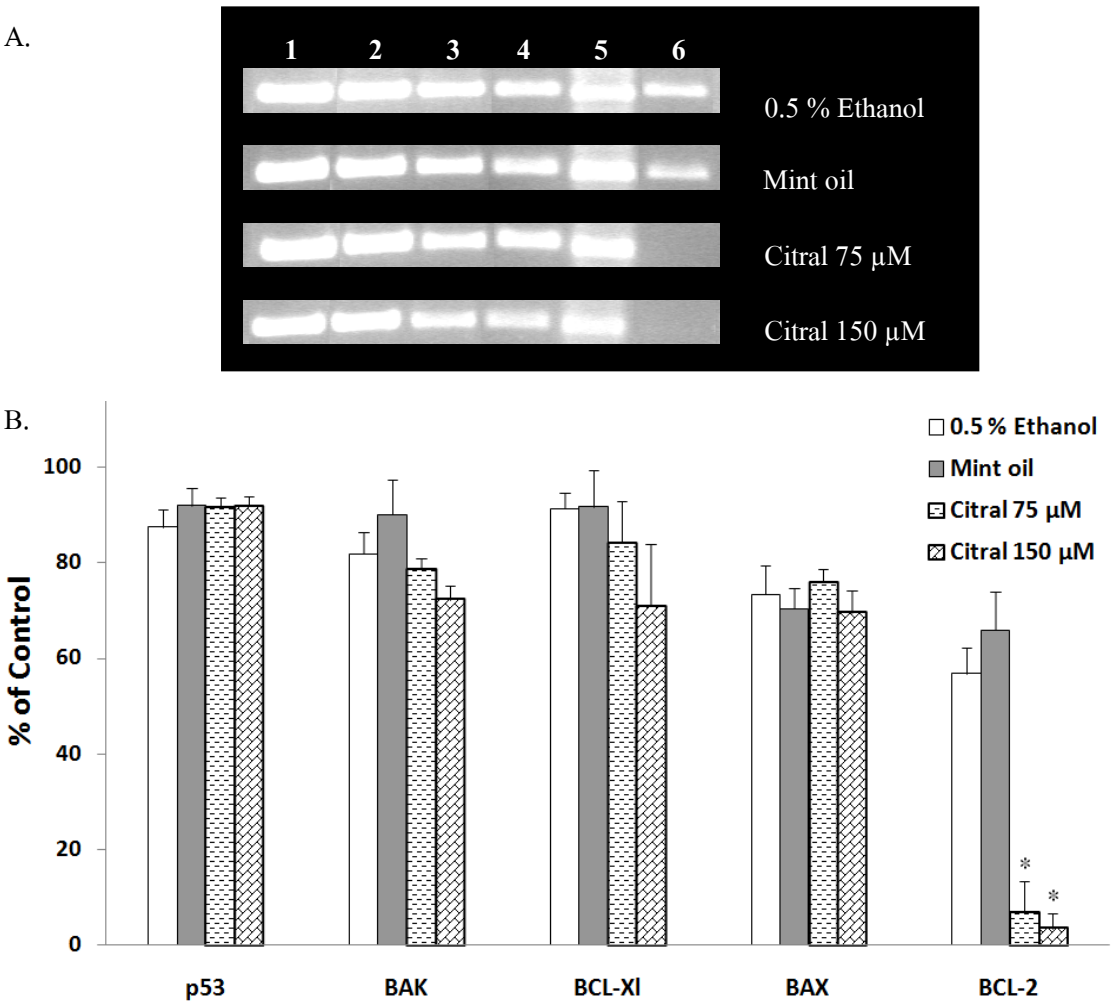


Figure 23: The effect of citral on mRNA expression of p53 and BCL-2 family proteins. Ramos cells were treated with 75 and 150 µM of citral for 6 h. The total RNA from the treated cells was reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. (A.) A representative of the PCR products of p53 and BCL-2 family proteins from citral-treated cells. Lanes: 1. GAPDH; 2. p53; 3. BAK; 4. BCL-XL; 5 BAX and 6. BCL-2. (B.) Densitometric analysis the PCR products relative to GAPDH represented as % of control. The data is expressed as mean ± S.E. of three independent experiments (n=3).

* $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

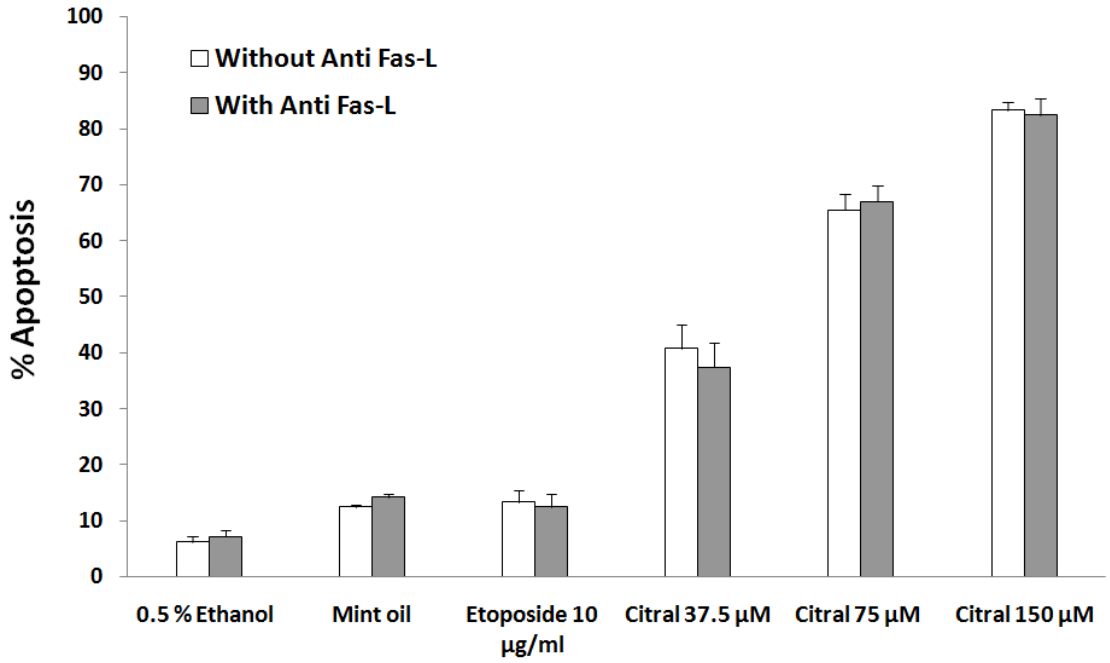


Figure 24: The involvement of Fas-FasL interaction on citral induced apoptosis. Ramos cells were pretreated with 200 ng/ml of anti-FasL antibody for 1 h before being treated with citral for 6 h. The percentage of apoptotic cells was determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

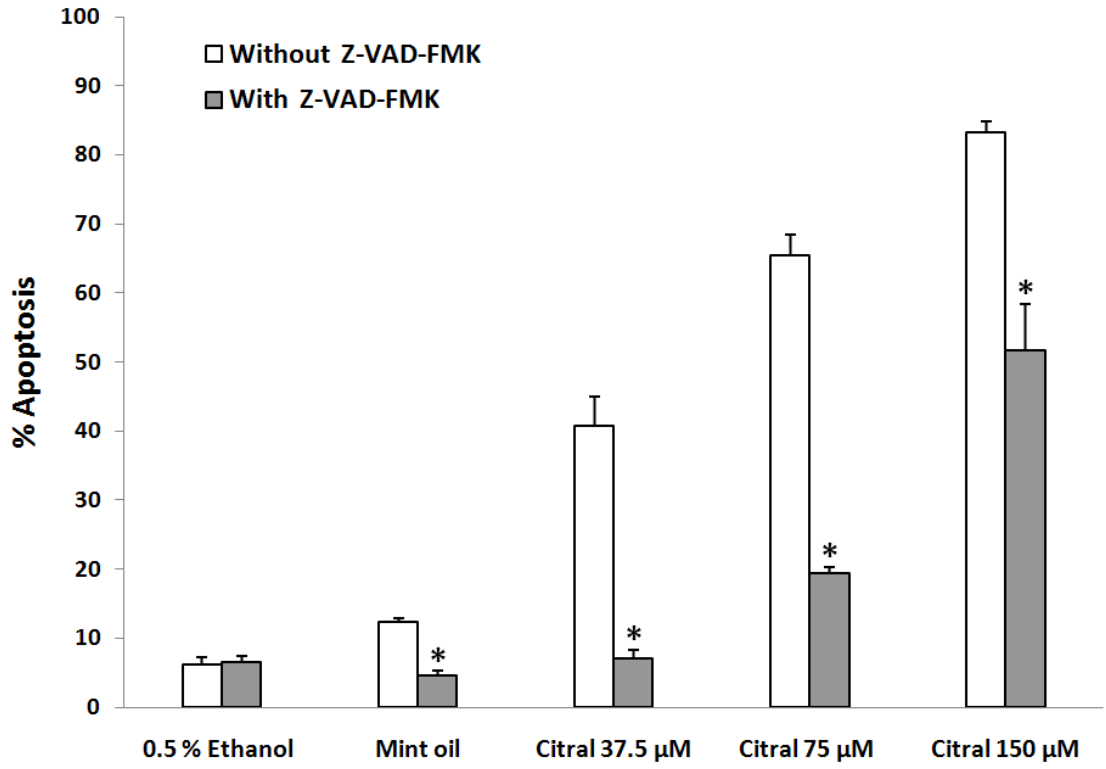


Figure 25: The involvement of caspase activation on citral induced Ramos cells apoptosis. The cells were pretreated with 50 µM of Z-VAD-FMK for 1 h prior to treated cells with citral for 6 h. The percentage of apoptotic cells were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

* $p < 0.01$ denotes statistically significant difference from without Z-VAD-FMK.

3. The effect of citral on the cell cycle

The effect of citral on the cell cycle was also investigated in this study. Ramos cells were treated with 37.5, 75 and 150 μM of citral for 3 h, washed, and then further incubated with fresh medium for 48 h. The cellular DNA content of the treated cells were analyzed by flow cytometer after being fixed, RNase treated, and stained with PI. Fig. 26 showed the DNA content profile of the cells treated with 0.5% ethanol and mint oil as the typical cellular distribution in G0/G1, S and G2/M phase. One μg/ml etoposide, which was used as the positive control, induced some cell death as shown in the sub-G1 DNA content. The viable cells were accumulated at the G2/M phase in Fig. 26 in the etoposide treated condition. This drug is known that it can arrest the cell cycle at the G2/M phase. In this experiment, citral also induced cell death by apoptosis in a dose-dependent manner. The 11.9%, 23.3% and 81.0% of sub-G1 DNA contents was detected when the cells were treated with 37.5, 75 and 150 μM, respectively. However, citral did not change the pattern of the viable cell distribution in the cell cycle. These results indicate that citral induces Ramos cells apoptosis but has no effect on the cell distribution in the cell cycle.

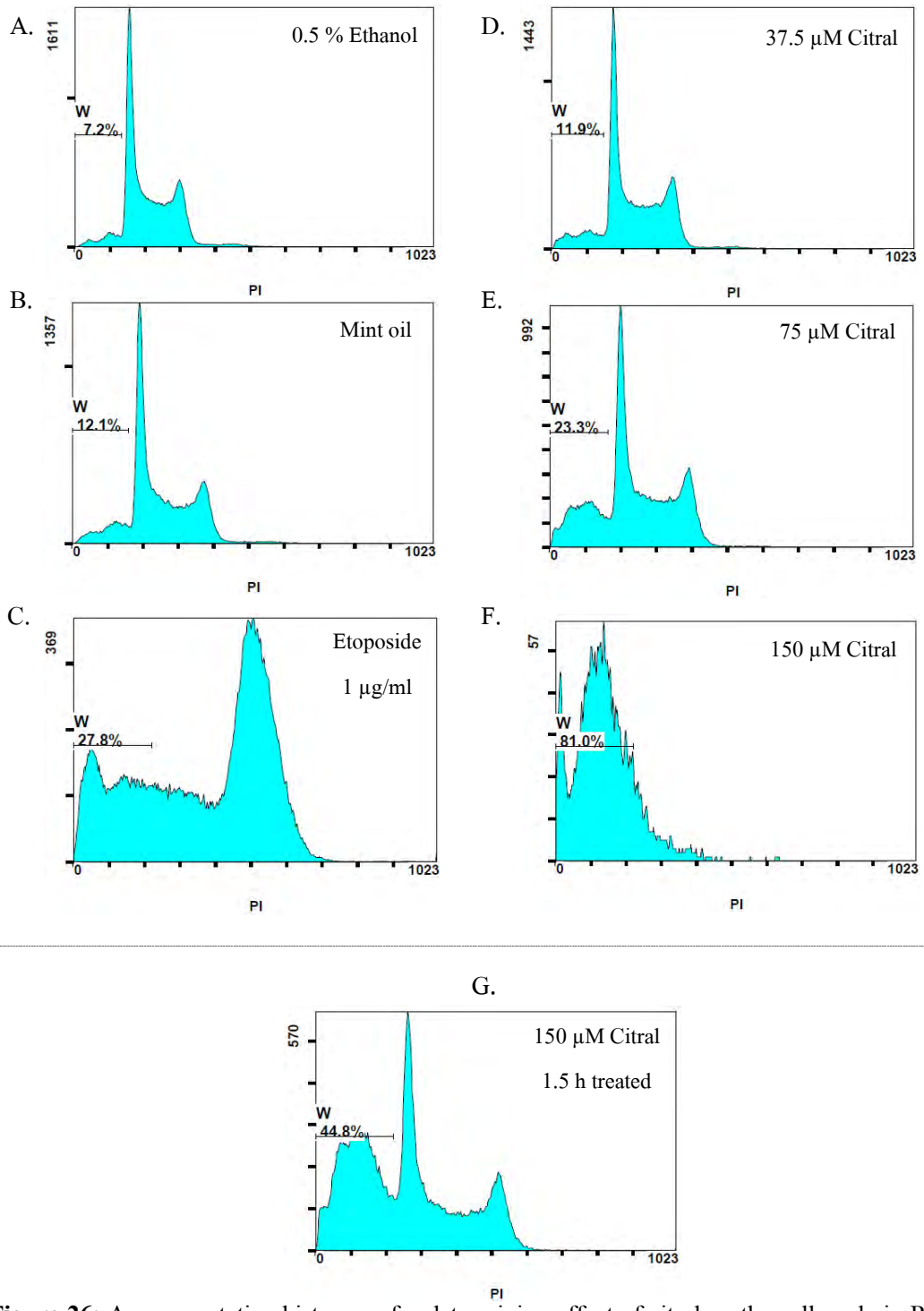


Figure 26: A representative histogram for determining effect of citral on the cell cycle in Ramos cells. The cells were treated with 37.5, 75 and 150 µM citral for 3 h, then washed and incubated in fresh medium for 48 h. The treated cells were fixed, RNase treated, and stained with PI. The cell cycle patterns were determined by fluorescent flow cytometer. (A) 0.5 % ethanol; (B) mint oil; (C) etoposide 1 µg/ml; (D, E, F) 37.5, 75 and 150 µM of citral; (G) 1.5 h treated with 150 µM of citral.

CHAPTER V

DISCUSSION AND CONCLUSION

This study aimed to evaluate the anti-cancer effects of citral on human B-lymphoma cells, Ramos cells. Apoptotic induction activity of citral was investigated by detecting the exposure of phosphatidylserine (PS) on the cell surface of apoptotic cells by using annexin V which specifically binds to this phospholipid. PS exposure on the extracellular surface is one of the characteristic of cells undergoing apoptosis. It translocates from the inner to the outer leaflet of the plasma membrane at the early stage of apoptosis **[31]**. Patterns of Ramos cells death induced by citral was determined by annexin V-FITC/PI staining monitored by fluorescence flow cytometer. Staining cells simultaneously with annexin V and PI allows the resolution of viable cells (double-negative), early apoptotic cells (annexin V-positive), necrotic cells (PI-positive), and necrotic and late apoptotic cells (double-positive). Because only cells with annexin V-positive are truly representative of apoptotic cells, the percentage of this cell population was quantified as apoptotic cells in this study. Citral induced Ramos cells death in the concentration-dependent (37.5, 75 and 150 μM) and the time-dependent (3, 6 and 12 h) manner. Citral, at 75 and 150 μM, induced cell death as early as 3 h after exposure. At the lowest concentration used in this study, 37.5 μM, it decreased cell survival almost 50% and 70% after 6 and 12 h of exposure, respectively. Citral acted faster with the higher potency on Ramos cells than its cytotoxic effect on human breast cancer cells, MCF-7 with IC_{50} 180 μM at 48 h **[4]**. The results in this study demonstrated that citral mainly induce Ramos cells death by apoptotic induction. The only apoptotic cells (annexin V positive) were detected at all concentrations used in the study after 3 and 6 h exposure. Some necrosis (PI positive) and necrosis/late apoptosis (double positive) were also detected when the cells were treated with 75 and 150 μM citral for 12 h. The positive control in this study, etoposide, didn't cause Ramos cells death after 3 to 12 h exposure. It induced these cell death after 24 h of treatment. The delayed effect of etoposide in these cells may due to its mechanism of action in cell cycle specific pattern. Etoposide is a topoisomerase II inhibitor that acts by stabilizing a DNA-topo II cleavable complex, resulting in DNA strand breaks. It induces cells in S and G2 phases of the cell cycle undergo cell cycle arrest or apoptosis. It effect observed after a considerable time of treatment in many cancer cell lines **[126]**.

The apoptotic effect of citral on Ramos cells was confirmed by analyzing hypodiploid or sub-diploid DNA of ethanol-fixed citral-treated cells with PI staining and flow cytometer. The effect of citral on these cancer cells in this assay was correlated with the study by detecting phosphatidylserine exposure. However the percentage of sub-diploid or apoptotic cells was not as high as the results from annexin V-FITC/PI staining assay. It is predicted that some apoptotic cells may progress to apoptotic bodies which are unable to be collected during fixing and washing steps before detecting by flow cytometer.

The harmful effect of citral on normal cells was also evaluated for selectivity and safety of this agent. Citral had no cytotoxic effect on human PBMCs from normal male subjects after 3 h of treatment. It induced apoptosis in PBMCs much less than in Ramos cells after 6 and 12 h of exposure. These results demonstrate that within a short time citral can induce apoptosis in these lymphoma cells with much higher potency than its apoptotic effect on normal cells.

The apoptotic pathway induced by citral was explored in this study. Citral, at 75 and 150 μM, dramatically decreased the mRNA expression of BCL-2. It didn't have effect on the mRNA expression of other BCL-2 family members, BCL-XL, BAX and BAK. A number of proteins in the BCL-2 family play roles in the mitochondrial apoptosis pathway. They either suppresses (anti-apoptotic) or promotes (pro-apoptotic) changes in mitochondrial membrane permeability **[63]**. BCL-2 and BCL-XL are anti-apoptotic proteins that preserve mitochondrial membrane integrity and prevent cell death. In contrast, BAK and BAX are pro-apoptotic BCL-2 members that are expressed abundantly and selectively during apoptosis to promote this type of cell death. The BCL-2 protein forms heterodimer with the BAX protein in different molar ratio. This ratio is critical for cells in several tissues to undergo or evade from apoptotic process **[127]**. A high BCL-2 to BAX ratio favors cell survival while a low ratio provokes apoptosis. Down-regulation of BCL-2 expression may lead to a decrease BCL-2 to BAX ratio and an increase of free BAX. These free BAX molecules can then translocate to mitochondrial membrane, form homodimer and trigger the caspase activation by alteration of mitochondrial membrane permeabilization. This results in the release of apoptosis promoting factors into the cytoplasm. Previous studies have shown that each cell type is protected from apoptosis by at least one member of anti-apoptotic BCL-2-like proteins. It was suggested that BCL-XL may play a more important role than BCL-2 in protecting B-cells from apoptosis. The overexpression of BCL-XL in immature mouse B-cells, WEH1-231, protects these cells from apoptosis induced by many stimuli **[128-129]**. In this study

citral had very little effect on the expression of BCL-XL mRNA. The results in this study suggest that citral may induce Ramos cells apoptosis through mitochondrial pathway by down-regulating BCL-2 expression. It cannot rule out that citral may have effect on other anti- and pro-apoptotic BCL-2 like proteins at the other levels, either translational or pos-translational levels. It is known that the activity of BCL-2 family proteins is strictly control at transcriptional, translational and post-translational levels. Their protein levels and the protein–protein interactions among these proteins also play critical roles in controlling their activity. Previous studies reported that the phosphorylation of BCL-XL might inhibit its anti-apoptotic function **[130]**. The effect of citral on proteins levels and phosphorylation of BCL-2 family members is needed to be elucidated.

The p53 is a tumor suppressor protein that is activated by DNA damage, oxidative stress, cellular calcium overload and other cellular stresses. It promotes apoptosis through the direct activation of pro-apoptotic BAX and the BH3-only proteins PUMA and Noxa gene expression. Citral didn't have any effect on the mRNA expressions of p53 as well as pro-apoptotic BCL-2 proteins, BAX and BAK. These results suggest that citral induces Ramos cells apoptosis independent on p53 activation.

The effect of citral on Ramos cells via the extrinsic pathway, Fas-Fas ligand interaction, was rule out in this study. Citral could still induce Ramos cells apoptosis in the presence of anti-Fas ligand antibody which inhibits Fas-Fas ligand interaction.

It is well known that caspases always play roles in the initiation of apoptosis in various cell types. Many apoptogenic factors from mitochondria such as cytochrome C and Smac/Diablo trigger caspase activation when they are released into cytosol. However, some apoptogenic factors can induce apoptosis in caspase-independent manner. These include EndoG, AIF and HtrA2/Omi **[131]**. HtrA2/Omi can trigger both caspase-dependent and caspase–independent apoptosis. The degree of caspase dependence is varying among apoptotic stimuli **[131-132]**. The caspase dependency of the citral activity was evaluated by using Z-VAD-FMK as a caspase inhibitor. Carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]- fluoromethylketone (Z-VAD-FMK) is a potent, cell permeable pan caspase inhibitor that irreversibly binds to the catalytic site of caspases and inhibit many caspase activities. Z-VAD-FMK attenuated the percentage of Ramos cells undergoing apoptosis in response to all concentrations citral used in the study. The activity of this inhibitor on the citral-treat cells was depending on the concentrations of citral. Apoptosis induced by 37.5 μM citral was abolished by Z-VAD-FMK. The inhibitor decreased apoptotic

induction activity of 75 μM citral more profound than its activity at 150 μM. These findings indicated that the apoptosis triggered by citral was mediated mainly by caspase activation. However, caspase-independent pathway of apoptosis may also be involved at high concentration of citral. This explanation needs to be investigated further.

Cell cycle arrest is one of the cytotoxic mechanisms of many anticancer agents. Citral was reported to exhibit cell cycle arrest in G2/M phase in human breast cancer cells MCF-7 **[4].** The effect of citral on the cell cycle of Ramos cells was also investigated in this study. The results showed that citral didn't change the pattern of the viable cell distribution in the cell cycle while 1 μg/ml etoposide induced cells accumulated at the G2/M phase. These results indicate that citral induces Ramos cells apoptosis without any change in the cell distribution in the cell cycle.

Base on the above results, citral appears to induce Ramos cells undergoing apoptosis in a concentration- and time-dependent manner. It might induce Ramos cells apoptosis through the mitochondrial pathway by down-regulating BCL-2 expression and mediated mainly by caspase activation. Its apoptosis effect was independent on any apparent changes in cell cycle distribution. The results from this study suggest that citral may be developed as an agent for the management of lymphoma cancer. Indeed, more in depth studies both *in vitro* as well as *in vivo* in appropriate relevant animal models are needed to strengthen this suggestion.

REFERENCES

[1] World Health Organization. Cancer: WHO cancer control programme; 2006 [Online]. Available from: http://www.who.int/cancer/en. [2006, November 18]

[2] Hsu S, Singh B, Schuster G. Induction of apoptosis in oral cancer cells: agents and mechanisms for potential therapy and prevention. Oral Oncology. 2004;40(5):461-73.

[3] Hengartner MO. The biochemistry of apoptosis. Nature. 2000 Oct 12;407(6805):770-6.

[4] Chaouki W, Leger DY, Liagre B, Beneytout JL, Hmamouchi M. Citral inhibits cell proliferation and induces apoptosis and cell cycle arrest in MCF-7 cells. Fundamental & Clinical Pharmacology. 2009 Jul 28. [Epub ahead of print]

[5] Foster I. Cancer: A cell cycle defect. Radiography. 2008;14(2):144-9.

[6] Oppenheim JJ, Yang D. Alarmins: chemotactic activators of immune responses. Current Opinion in Immunology. 2005 Aug;17(4):359-65.

[7] Ulloa L, Messmer D. High-mobility group box 1 (HMGB1) protein: Friend and foe. Cytokine & Growth Factor Reviews. 2006;17(3):189-201.

[8] Reed JC. Mechanisms of apoptosis. American Journal of Pathology. 2000 Nov;157(5):1415-30.

[9] Altmann K-H. Microtubule-stabilizing agents: a growing class of important anticancer drugs. Current Opinion in Chemical Biology. 2001;5(4):424-31.

[10] Danial NN, Korsmeyer SJ. Cell Death: Critical Control Points. Cell. 2004;116(2):205-19.

[11] Stennicke HR, Salvesen GS. Caspases - controlling intracellular signals by protease zymogen activation. Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology. 2000;1477(1-2):299-306.

[12] Arends MJ, Wyllie AH. Apoptosis: mechanisms and roles in pathology. International Review of Experimental Pathology. 1991;32:223-54.

[13] Chowdhury I, Tharakan B, Bhat GK. Caspases -- An update. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2008;151(1):10-27.

[14] Oliver L, Vallette FM. The role of caspases in cell death and differentiation. Drug Resistance Updates. 2005;8(3):163-70.

[15] Weber CH, Vincenz C. The death domain superfamily: a tale of two interfaces?. Trends in Biochemical Sciences. 2001 Aug;26(8):475-81.

[16] Fesik SW. Insights into Programmed Cell Death through Structural Biology. Cell. 2000;103(2):273-82.

[17] Ghavami S, Hashemi M, Ande SR, Yeganeh B, Xiao W, Eshraghi M, et al. Apoptosis and cancer: mutations within caspase genes. Journal of Medical Genetics. 2009 Aug;46(8):497-510.

[18] Lavrik IN, Golks A, Krammer PH. Caspases: pharmacological manipulation of cell death. The Journal of Clinical Investigation. 2005 Oct;115(10):2665-72.

[19] Grutter MG. Caspases: key players in programmed cell death. Current Opinion in Structural Biology. 2000;10(6):649-55.

[20] Ashe PC, Berry MD. Apoptotic signaling cascades. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 2003 Apr;27(2):199-214.

[21] Woo E-J, Kim Y-G, Kim M-S, Han W-D, Shin S, Robinson H, et al. Structural Mechanism for Inactivation and Activation of CAD/DFF40 in the Apoptotic Pathway. Molecular Cell. 2004;14(4):531-9.

[22] Korn C, Scholz SR, Gimadutdinow O, Pingoud A, Meiss G. Involvement of conserved histidine, lysine and tyrosine residues in the mechanism of DNA cleavage by the caspase-3 activated DNase CAD. Nucleic Acids Research. 2002 Mar 15;30(6):1325-32.

[23] Rao L, Perez D, White E. Lamin proteolysis facilitates nuclear events during apoptosis. The Journal of Cell Biology. 1996 Dec;135(6 Pt 1):1441-55.

[24] Croft DR, Coleman ML, Li S, Robertson D, Sullivan T, Stewart CL, et al. Actin-myosin-based contraction is responsible for apoptotic nuclear disintegration. The Journal of Cell Biology. 2005 Jan 17;168(2):245-55.

[25] Ndozangue-Touriguine O, Hamelin J, Breard J. Cytoskeleton and apoptosis. Biochemical Pharmacology. 2008 Jul 1;76(1):11-8.

[26] Moss DK, Betin VM, Malesinski SD, Lane JD. A novel role for microtubules in apoptotic chromatin dynamics and cellular fragmentation. Journal of Cell Science. 2006 Jun 1;119(Pt 11):2362-74.

[27] Lane JD, Vergnolle MA, Woodman PG, Allan VJ. Apoptotic cleavage of cytoplasmic dynein intermediate chain and p150(Glued) stops dynein-dependent membrane motility. The Journal of Cell Biology. 2001 Jun 25;153(7):1415-26.

[28] Chiu R, Novikov L, Mukherjee S, Shields D. A caspase cleavage fragment of p115 induces fragmentation of the Golgi apparatus and apoptosis. The Journal of Cell Biology. 2002 Nov 25;159(4):637-48.

[29] Ura S, Masuyama N, Graves JD, Gotoh Y. Caspase cleavage of MST1 promotes nuclear translocation and chromatin condensation. Proceedings of the National Academy of Sciences USA. 2001 Aug 28;98(18):10148-53.

[30] Luthi AU, Martin SJ. The CASBAH: a searchable database of caspase substrates. Cell death and differentiation. 2007 Apr;14(4):641-50.

[31] Taylor RC, Cullen SP, Martin SJ. Apoptosis: controlled demolition at the cellular level. Nature Reviews Molecular Cell Biology. 2008 Mar;9(3):231-41.

[32] Borisenko GG, Matsura T, Liu S-X, Tyurin VA, Jianfei J, Serinkan FB, et al. Macrophage recognition of externalized phosphatidylserine and phagocytosis of apoptotic Jurkat cells-existence of a threshold. Archives of Biochemistry and Biophysics. 2003;413(1):41-52.

[33] Matsura T, Togawa A, Kai M, Nishida T, Nakada J, Ishibe Y, et al. The presence of oxidized phosphatidylserine on Fas-mediated apoptotic cell surface. Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids. 2005;1736(3):181-8.

[34] Wu Y, Tibrewal N, Birge RB. Phosphatidylserine recognition by phagocytes: a view to a kill. Trends in Cell Biology. 2006;16(4):189-97.

[35] Greenberg ME, Sun M, Zhang R, Febbraio M, Silverstein R, Hazen SL. Oxidized phosphatidylserine-CD36 interactions play an essential role in macrophage-dependent phagocytosis of apoptotic cells. The Journal of Experimental Medicine. 2006 Nov 27;203(12):2613-25.

[36] Trouw LA, Blom AM, Gasque P. Role of complement and complement regulators in the removal of apoptotic cells. Molecular Immunology. 2008;45(5):1199-207.

[37] Gardai SJ, McPhillips KA, Frasch SC, Janssen WJ, Starefeldt A, Murphy-Ullrich JE, et al. Cell-surface calreticulin initiates clearance of viable or apoptotic cells through trans-activation of LRP on the phagocyte. Cell. 2005 Oct 21;123(2):321-34.

[38] Oka K, Sawamura T, Kikuta K, Itokawa S, Kume N, Kita T, et al. Lectin-like oxidized low-density lipoprotein receptor 1 mediates phagocytosis of aged/apoptotic cells in endothelial cells. Proceedings of the National Academy of Sciences USA. 1998 Aug 4;95(16):9535-40.

[39] Boatright KM, Renatus M, Scott FL, Sperandio S, Shin H, Pedersen IM, et al. A Unified Model for Apical Caspase Activation. Molecular Cell. 2003;11(2):529-41.

[40] Zimmermann KC, Bonzon C, Green DR. The machinery of programmed cell death. Pharmacology & Therapeutics. 2001;92(1):57-70.

[41] Wajant H. The Fas signaling pathway: more than a paradigm. Science. 2002 May 31;296(5573):1635-6.

[42] Chen G, Goeddel DV. TNF-R1 signaling: a beautiful pathway. Science. 2002 May 31;296(5573):1634-5.

[43] Pan G, Bauer JH, Haridas V, Wang S, Liu D, Yu G, et al. Identification and functional characterization of DR6, a novel death domain-containing TNF receptor. FEBS Letters. 1998 Jul 24;431(3):351-6.

[44] Siegel RM, Frederiksen JK, Zacharias DA, Chan FK, Johnson M, Lynch D, et al. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. Science. 2000 Jun 30;288(5475):2354-7.

[45] Wang ZB, Liu YQ, Cui YF. Pathways to caspase activation. Cell Biology International. 2005 Jul;29(7):489-96.

[46] Poulaki V, Mitsiades CS, Mitsiades N. The role of Fas and FasL as mediators of anticancer chemotherapy. Drug Resistance Updates. 2001 Aug;4(4):233-42.

[47] Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble form of human fas ligand in activated lymphocytes. The EMBO Journal. 1995 Mar 15;14(6):1129-35.

[48] Rupinder SK, Gurpreet AK, Manjeet S. Cell suicide and caspases. Vascular Pharmacology. 2007;46(6):383-93.

[49] Gupta S. Molecular mechanisms of apoptosis in the cells of the immune system in human aging. Immunological Reviews. 2005 Jun;205:114-29.

[50] Scaffidi C, Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. Journal of Biological Chemistry. 1999 Aug 6;274(32):22532-8.

[51] Golks A, Brenner D, Fritsch C, Krammer PH, Lavrik IN. c-FLIPR, a new regulator of death receptor-induced apoptosis. Journal of Biological Chemistry. 2005 Apr 15;280(15):14507-13.

[52] Madesh M, Antonsson B, Srinivasula SM, Alnemri ES, Hajnoczky G. Rapid kinetics of tBid-induced cytochrome c and Smac/DIABLO release and mitochondrial depolarization. Journal of Biological Chemistry. 2002 Feb 15;277(7):5651-9.

[53] Bratton SB, Cohen GM. Apoptotic death sensor: an organelle's alter ego?. Trends in Pharmacological Sciences. 2001 Jun;22(6):306-15.

[54] Riedl SJ, Shi Y. Molecular mechanisms of caspase regulation during apoptosis. Nature Reviews Molecular Cell Biology. 2004 Nov;5(11):897-907.

[55] Wang X. The expanding role of mitochondria in apoptosis. Genes & Development. 2001 Nov 15;15(22):2922-33.

[56] Zou H, Li Y, Liu X, Wang X. An APAF-1.cytochrome c multimeric complex is a functional apoptosome that activates procaspase-9. Journal of Biological Chemistry. 1999 Apr 23;274(17):11549-56.

[57] Srinivasula SM, Hegde R, Saleh A, Datta P, Shiozaki E, Chai J, et al. A conserved XIAP-interaction motif in caspase-9 and Smac/DIABLO regulates caspase activity and apoptosis. Nature. 2001 Mar 1;410(6824):112-6.

[58] Youle RJ, Strasser A. The BCL-2 protein family: opposing activities that mediate cell death. Nature Reviews Molecular Cell Biology. 2008 Jan;9(1):47-59.

[59] Lorenzo HK, Susin SA, Penninger J, Kroemer G. Apoptosis inducing factor (AIF): a phylogenetically old, caspase-independent effector of cell death. Cell death and differentiation. 1999 Jun;6(6):516-24.

[60] Li LY, Luo X, Wang X. Endonuclease G is an apoptotic DNase when released from mitochondria. Nature. 2001 Jul 5;412(6842):95-9.

[61] Chipuk JE, Bouchier-Hayes L, Green DR. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell death and differentiation. 2006 Aug;13(8):1396-402.

[62] Skommer J, Wlodkowic D, Deptala A. Larger than life: Mitochondria and the Bcl-2 family. Leukemia Research. 2007;31(3):277-86.

[63] Chipuk JE, Green DR. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?. Trends in Cell Biology. 2008;18(4):157-64.

[64] Martinou JC, Green DR. Breaking the mitochondrial barrier. Nature Reviews Molecular Cell Biology. 2001 Jan;2(1):63-7.

[65] Zinkel S, Gross A, Yang E. BCL2 family in DNA damage and cell cycle control. Cell death and differentiation. 2006 Aug;13(8):1351-9.

[66] Wu Y, Xing D, Liu L, Gao B. Regulation of Bax activation and apoptotic response to UV irradiation by p53 transcription-dependent and -independent pathways. Cancer Letters. 2008;271(2):231-9.

[67] Nakano K, Vousden KH. PUMA, a novel proapoptotic gene, is induced by p53. Molecular Cell. 2001 Mar;7(3):683-94.

[68] Fortin A, Cregan SP, MacLaurin JG, Kushwaha N, Hickman ES, Thompson CS, et al. APAF1 is a key transcriptional target for p53 in the regulation of neuronal cell death. The Journal of Cell Biology. 2001 Oct 15;155(2):207-16.

[69] Shresta S, Pham CTN, Thomas DA, Graubert TA, Ley TJ. How do cytotoxic lymphocytes kill their targets? Current Opinion in Immunology. 1998;10(5):581-7.

[70] Trapani JA, Sutton VR. Granzyme B: pro-apoptotic, antiviral and antitumor functions. Current Opinion in Immunology. 2003 Oct;15(5):533-43.

[71] Pinkoski MJ, Waterhouse NJ, Heibein JA, Wolf BB, Kuwana T, Goldstein JC, et al. Granzyme B-mediated apoptosis proceeds predominantly through a Bcl-2-inhibitable mitochondrial pathway. Journal of Biological Chemistry. 2001 Apr 13;276(15):12060-7.

[72] Sutton VR, Wowk ME, Cancilla M, Trapani JA. Caspase activation by granzyme B is indirect, and caspase autoprocessing requires the release of proapoptotic mitochondrial factors. Immunity. 2003 Mar;18(3):319-29.

[73] Barry M, Heibein JA, Pinkoski MJ, Lee SF, Moyer RW, Green DR, et al. Granzyme B short-circuits the need for caspase 8 activity during granule-mediated cytotoxic T-lymphocyte killing by directly cleaving Bid. Molecular and Cellular Biology. 2000 Jun;20(11):3781-94.

[74] Sutton VR, Davis JE, Cancilla M, Johnstone RW, Ruefli AA, Sedelies K, et al. Initiation of apoptosis by granzyme B requires direct cleavage of bid, but not direct granzyme B-mediated caspase activation. The Journal of Experimental Medicine. 2000 Nov 20;192(10):1403-14.

[75] Heibein JA, Goping IS, Barry M, Pinkoski MJ, Shore GC, Green DR, et al. Granzyme B-mediated cytochrome c release is regulated by the Bcl-2 family members bid and Bax. The Journal of Experimental Medicine. 2000 Nov 20;192(10):1391-402.

[76] Deveraux QL, Reed JC. IAP family proteins-suppressors of apoptosis. Genes & Development. 1999 Feb 1;13(3):239-52.

[77] Shi Y. Mechanisms of caspase activation and inhibition during apoptosis. Molecular Cell. 2002 Mar;9(3):459-70.

[78] LeBlanc AC. Natural cellular inhibitors of caspases. Progress in Neuro Psychopharmacology and Biological Psychiatry. 2003;27(2):215-29.

[79] Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC. The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. The EMBO Journal. 1997 Dec 1;16(23):6914-25.

[80] Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, et al. IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. The EMBO Journal. 1998 Apr 15;17(8):2215-23.

[81] Shiozaki EN, Shi Y. Caspases, IAPs and Smac/DIABLO: mechanisms from structural biology. Trends in Biochemical Sciences. 2004;29(9):486-94.

[82] Ekert PG, Silke J, Hawkins CJ, Verhagen AM, Vaux DL. DIABLO promotes apoptosis by removing MIHA/XIAP from processed caspase 9. The Journal of Cell Biology. 2001 Feb 5;152(3):483-90.

[83] Suzuki Y, Imai Y, Nakayama H, Takahashi K, Takio K, Takahashi R. A serine protease, HtrA2, is released from the mitochondria and interacts with XIAP, inducing cell death. Molecular Cell. 2001 Sep;8(3):613-21.

[84] Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. 2000 Jan 7;100(1):57-70.

[85] Sandal T. Molecular aspects of the mammalian cell cycle and cancer. Oncologist. 2002;7(1):73-81.

[86] Hochegger H, Takeda S, Hunt T. Cyclin-dependent kinases and cell-cycle transitions: does one fit all?. Nature Reviews Molecular Cell Biology. 2008 Nov;9(11):910-6.

[87] King KL, Cidlowski JA. Cell cycle regulation and apoptosis. Annual Review of Physiology. 1998;60:601-17.

[88] Vermeulen K, Van Bockstaele DR, Berneman ZN. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Proliferation. 2003 Jun;36(3):131-49.

[89] Malumbres M, Barbacid M. Cell cycle, CDKs and cancer: a changing paradigm. Nature Reviews Cancer. 2009 Mar;9(3):153-66.

[90] Kaldis P. The cdk-activating kinase (CAK): from yeast to mammals. Cellular and Molecular Life Sciences. 1999 Feb;55(2):284-96.

[91] Lew DJ, Kornbluth S. Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Current Opinion in Cell Biology. 1996 Dec;8(6):795-804.

[92] van den Heuvel S. Cell-cycle regulation. WormBook. 2005:1-16.

[93] Hartwell LH, Kastan MB. Cell cycle control and cancer. Science. 1994 Dec 16;266(5192):1821-8.

[94] Vermeulen K, Berneman ZN, Van Bockstaele DR. Cell cycle and apoptosis. Cell Proliferation. 2003 Jun;36(3):165-75.

[95] Schafer KA. The cell cycle: a review. Veterinary Pathology. 1998 Nov;35(6):461-78.

[96] Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Molecular and Cellular Biology. 1995 May;15(5):2612-24.

[97] Johnson DG, Walker CL. Cyclins and cell cycle checkpoints. Annual Review of Pharmacology and Toxicology. 1999;39:295-312.

[98] Arellano M, Moreno S. Regulation of CDK/cyclin complexes during the cell cycle. The International Journal of Biochemistry & Cell Biology. 1997 Apr;29(4):559-73.

[99] Fasanaro P, Capogrossi MC, Martelli F. Regulation of the endothelial cell cycle by the ubiquitin-proteasome system. Cardiovascular Research. 2009 Aug 7. [Epub ahead of print]

[100] Bloom J, Cross FR. Multiple levels of cyclin specificity in cell-cycle control. Nature Reviews Molecular Cell Biology. 2007 Feb;8(2):149-60.

[101] Maddika S, Ande SR, Panigrahi S, Paranjothy T, Weglarczyk K, Zuse A, et al. Cell survival, cell death and cell cycle pathways are interconnected: Implications for cancer therapy. Drug Resistance Updates. 2007 Apr;10(1-2):13-29.

[102] Ortega S, Malumbres M, Barbacid M. Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochimica et Biophysica Acta. 2002 Mar 14;1602(1):73-87.

[103] Besson A, Dowdy SF, Roberts JM. CDK inhibitors: cell cycle regulators and beyond. Developmental Cell. 2008 Feb;14(2):159-69.

[104] Molinari M. Cell cycle checkpoints and their inactivation in human cancer. Cell Proliferation. 2000 Oct;33(5):261-74.

[105] Reynisdottir I, Polyak K, Iavarone A, Massague J. Kip/Cip and Ink4 Cdk inhibitors cooperate to induce cell cycle arrest in response to TGF-beta. Genes & Development. 1995 Aug 1;9(15):1831-45.

[106] Malumbres M, Barbacid M. To cycle or not to cycle: a critical decision in cancer. Nature Reviews Cancer. 2001 Dec;1(3):222-31.

[107] Herrup K, Yang Y. Cell cycle regulation in the postmitotic neuron: oxymoron or new biology?. Nature Reviews Neuroscience. 2007 May;8(5):368-78.

[108] Manosroi J, Dhumtanom P, Manosroi A. Anti-proliferative activity of essential oil extracted from Thai medicinal plants on KB and P388 cell lines. Cancer Letters. 2006;235(1):114-20.

[109] Kumar A, Malik F, Bhushan S, Sethi VK, Shahi AK, kaur J, et al. An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells. Chemico-Biological Interactions. 2008;171(3):332-47.

[110] Verma M, Singh SK, Bhushan S, Pal HC, Kitchlu S, Koul MK, et al. Induction of mitochondrial-dependent apoptosis by an essential oil from *Tanacetum gracile*. Planta Medica. 2008 Apr;74(5):515-20.

[111] Murakami A, Nakamura Y, Koshimizu K, Ohigashi H. Glyceroglycolipids from *Citrus hystrix*, a traditional herb in Thailand, potentially inhibit the tumorpromoting activity of 12-O-tetradecanoylphorbol 13-acetate in mouse skin. Journal of Agricultural and Food Chemistry. 1995;43:2779-83.

[112] Pihlasalo J, Klika KD, Murzin DY, Nieminen V. Conformational equilibria of citral. Journal of Molecular Structure: THEOCHEM. 2007;814(1-3):33-41.

[113] Silva Cde B, Guterres SS, Weisheimer V, Schapoval EE. Antifungal activity of the lemongrass oil and citral against *Candida spp*. Brazilian Journal of Infectious Diseases. 2008 Feb;12(1):63-6.

[114] Nakamura Y, Miyamoto M, Murakami A, Ohigashi H, Osawa T, Uchida K. A phase II detoxification enzyme inducer from lemongrass: identification of citral and involvement of electrophilic reaction in the enzyme induction. Biochemical and Biophysical Research Communications. 2003;302(3):593-600.

[115] Yang Z, Xi J, Li J, Qu W. Biphasic effect of citral, a flavoring and scenting agent, on spatial learning and memory in rats. Pharmacology Biochemistry and Behavior. 2009;93(4):391-6.

[116] Phillips JC, Kingsnorth J, Gangolli SD, Gaunt IF. Studies on the absorption, distribution and excretion of citral in the rat and mouse. Food and Cosmetics Toxicology. 1976 Dec;14(6):537-40.

[117] Jackson GM, Hall DE, Walker R. Comparison of the short-term hepatic effects of orally administered citral in Long Evans hooded and Wistar albino rats. Food and Chemical Toxicology. 1987 Jul;25(7):505-13.

[118] Roffey SJ, Walker R, Gibson GG. Hepatic peroxisomal and microsomal enzyme induction by citral and linalool in rats. Food and Chemical Toxicology. 1990 Jun;28(6):403-8.

[119] Vinitketkumnuen U, Puatanachokchai R, Kongtawelert P, Lertprasertsuke N, Matsushima T. Antimutagenicity of lemon grass (*Cymbopogon citratus* Stapf) to various known mutagens in salmonella mutation assay. Mutation Research. 1994 Nov;341(1):71-5.

[120] Hayes AJ, Markovic B. Toxicity of Australian essential oil *Backhousia citriodora* (Lemon myrtle). Part 1. Antimicrobial activity and in vitro cytotoxicity. Food and Chemical Toxicology. 2002 Apr;40(4):535-43.

[121] Sandri IG, Zacaria J, Fracaro F, Delamare APL, Echeverrigaray S. Antimicrobial activity of the essential oils of Brazilian species of the genus *Cunila* against foodborne pathogens and spoiling bacteria. Food Chemistry. 2007;103(3):823-8.

[122] Tzortzakis NG, Economakis CD. Antifungal activity of lemongrass (*Cympopogon citratus* L.) essential oil against key postharvest pathogens. Innovative Food Science & Emerging Technologies. 2007;8(2):253-8.

[123] Minami M, Kita M, Nakaya T, Yamamoto T, Kuriyama H, Imanishi J. The inhibitory effect of essential oils on herpes simplex virus type-1 replication *in vitro*. Microbiology and Immunology. 2003;47(9):681-4.

[124] Hierro I, Valero A, Navarro MC. *In vivo* larvicidal activity of monoterpenic derivatives from aromatic plants against L3 larvae of *Anisakis simplex* s.l. Phytomedicine. 2006 Jul;13(7):527-31.

[125] Dudai N, Weinstein Y, Krup M, Rabinski T, Ofir R. Citral is a new inducer of caspase-3 in tumor cell lines. Planta Medica. 2005 May;71(5):484-8.

[126] Rello-Varona S, Gamez A, Moreno V, Stockert JC, Cristobal J, Pacheco M, et al. Metaphase arrest and cell death induced by etoposide on HeLa cells. The International Journal of Biochemistry & Cell Biology. 2006;38(12):2183-95.

[127] Oltval ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programed cell death. Cell. 1993;74(4):609-19.

[128] Fang W, Rivard JJ, Ganser JA, LeBien TW, Nath KA, Mueller DL, et al. Bcl-xL rescues WEHI 231 B lymphocytes from oxidant-mediated death following diverse apoptotic stimuli. The Journal of Immunology. 1995;155:66-75.

[129] Gottschalk AR, Boise LH, Thompson CB, Quintans J. Identification of immunosuppressant-induced apoptosis in a murine B-cell line and its prevention by bcl-x but not bcl-2. Proceedings of the National Academy of Sciences USA. 1994;91:7350-4.

[130] Basu A, Haldar S. Identification of a novel Bcl-xL phosphorylation site regulating the sensitivity of taxol- or 2-methoxyestradiol-induced apoptosis. FEBS Letters. 2003 Mar 13;538(1-3):41-7.

[131] Lockshin RA, Zakeri Z. Caspase-independent cell deaths. Current Opinion in Cell Biology. 2002 Dec;14(6):727-33.

[132] Donovan M, Cotter TG. Control of mitochondrial integrity by Bcl-2 family members and caspase-independent cell death. Biochimica et Biophysica Acta. 2004 Mar 1;1644 (2-3):133-47.

APPENDICES

APPENDIX A

Buffers and Reagents

1. RPMI 1640 stock solution 1 liter

RPMI powder	10.4	g
$NaHCO_3$	1.5	g
Glucose	4.5	g
Sodium pyruvate	0.11	g
HEPES (1M)	10	ml
Penicillin/Streptomycin	10	ml
ddH_2O	900	ml

Adjust pH to 7.2 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by filtering through a 0.45 membrane filter

2. Complete RPMI 1640 medium 200 ml

RPMI stock	180	ml
Fetal Bovine Serum	20	ml

3. 10x Phosphate Buffered Saline (PBS) 1 liter

NaCl	80.65	g
KCl	2	g
KH_2PO_4	2	g
Na_2HPO_4	11.5	g
ddH_2O	900	ml

Adjust pH to 7.4 with 1M HCl

Add ddH_2O to 1 liter and Sterilized by autoclaving

4. 10x Assay Buffered 100 ml

HEPES (1M)	10	ml
$CaCl_2$ (0.1M)	28	ml
NaCl (5M)	25	ml
ddH_2O	37	ml

5. Tris-HCl 1M pH 8.0 100 ml

Tris-base	12.114	g
ddH_2O	80	ml

Adjust pH to 8.0 with conc. HCl

Add ddH_2O to 100 ml and Sterilized by autoclaving

6. EDTA 0.5M pH 8.0 100 ml

EDTA	18.612	g
ddH_2O	80	ml

Adjust pH to 8.0 with NaOH

Add ddH_2O to 100 ml and Sterilized by autoclaving

7. 1x TE Buffered 100 ml

Tris-HCl 1M pH 8.0	1	ml
EDTA 0.5M pH 8.0	0.2	ml
ddH_2O	98.8	ml

Sterilized by autoclaving

8. 5x TBE Buffered 1 liter

Tris-base	54	g
Boric acid	27.5	g
EDTA 0.5M pH 8.0	20	ml

Sterilized by autoclaving

APPENDIX B

Results

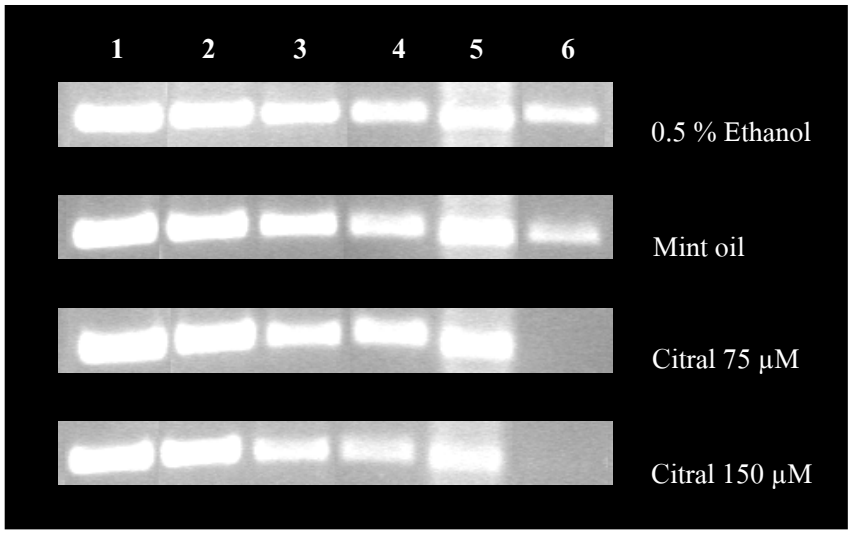


Figure 27: The effect of citral on mRNA expression of p53 and BCL-2 family proteins (n1). Ramos cells were treated with 75 and 150 µM of citral for 6 h. The total RNA from the treated cells was reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. Lanes: 1. GAPDH; 2. p53; 3. BAK; 4. BCL-XL; 5. BAX and 6. BCL-2.

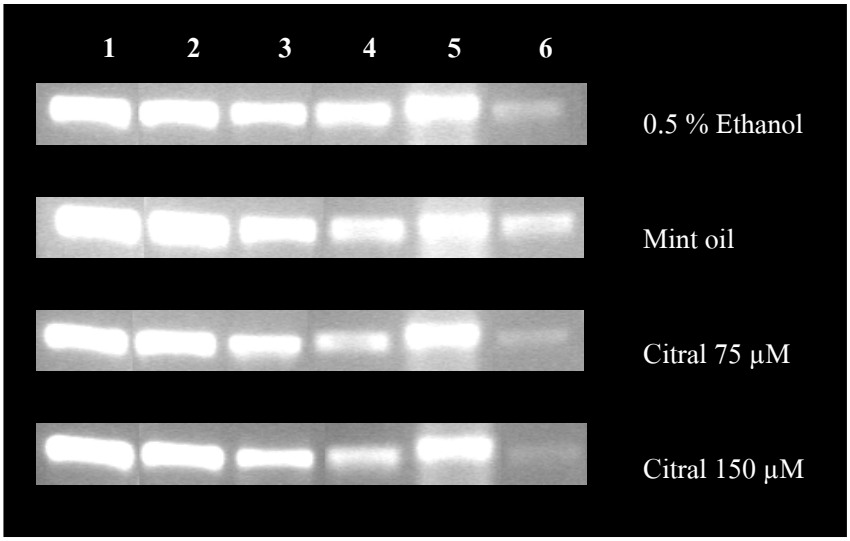


Figure 28: The effect of citral on mRNA expression of p53 and BCL-2 family proteins (n2). Ramos cells were treated with 75 and 150 µM of citral for 6 h. The total RNA from the treated cells was reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. Lanes: 1. GAPDH; 2. p53; 3. BAK; 4. BCL-XL; 5. BAX and 6. BCL-2.

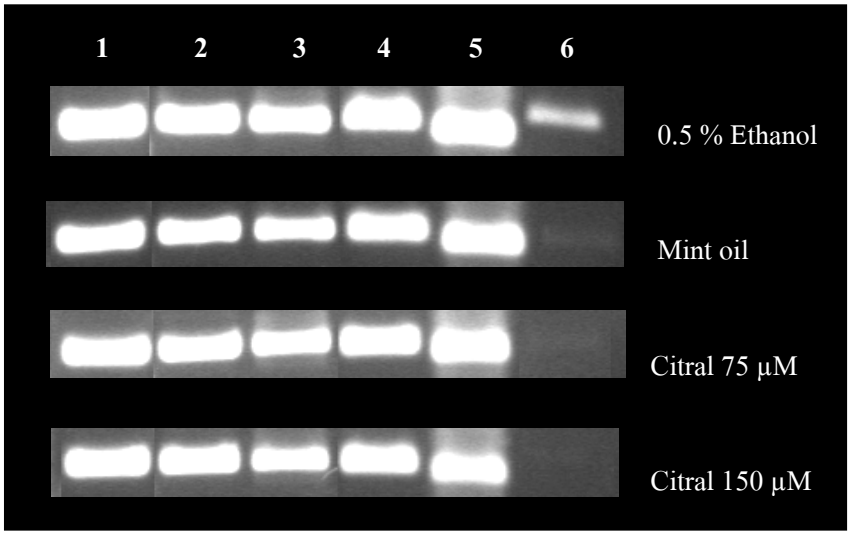


Figure 29: The effect of citral on mRNA expression of p53 and BCL-2 family proteins (n3). Ramos cells were treated with 75 and 150 µM of citral for 6 h. The total RNA from the treated cells was reverse transcribed and amplified with specific primers by RT-PCR. The PCR products were run on 1.5% agarose gel and analyzed by gel documentation. Lanes: 1. GAPDH; 2. p53; 3. BAK; 4. BCL-XL; 5. BAX and 6. BCL-2.

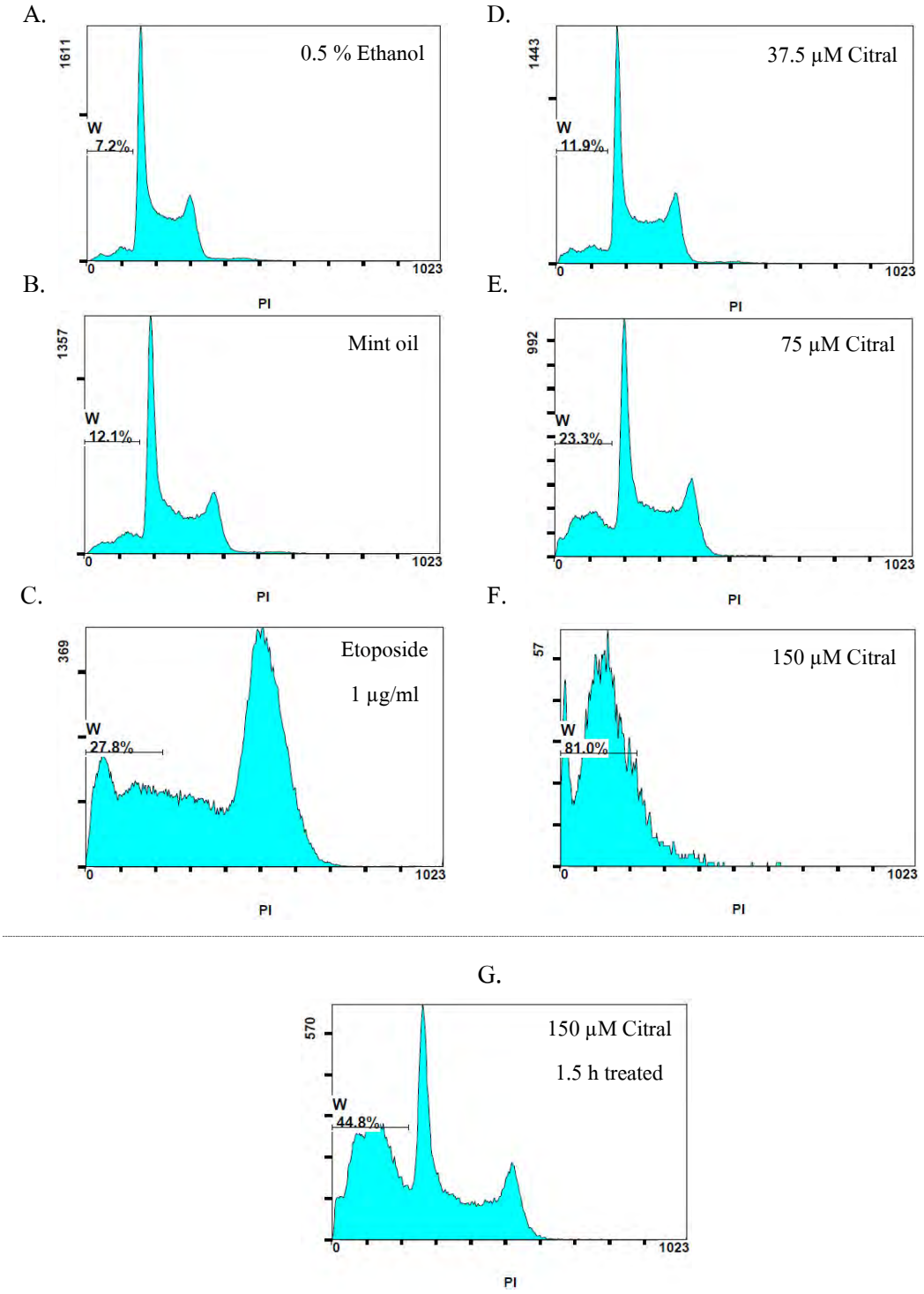


Figure 30: The effect of citral on the cell cycle in Ramos cells (n1). The cells were treated with 37.5, 75 and 150 µM citral for 3 h, then washed and incubated in fresh medium for 48 h. The treated cells were fixed, RNase treated, and stained with PI. The cell cycle patterns were determined by fluorescent flow cytometer. (A) 0.5 % ethanol; (B) mint oil; (C) etoposide 1 µg/ml; (D, E, F) 37.5, 75 and 150 µM of citral; (G) 1.5 h treated with 150 µM of citral.

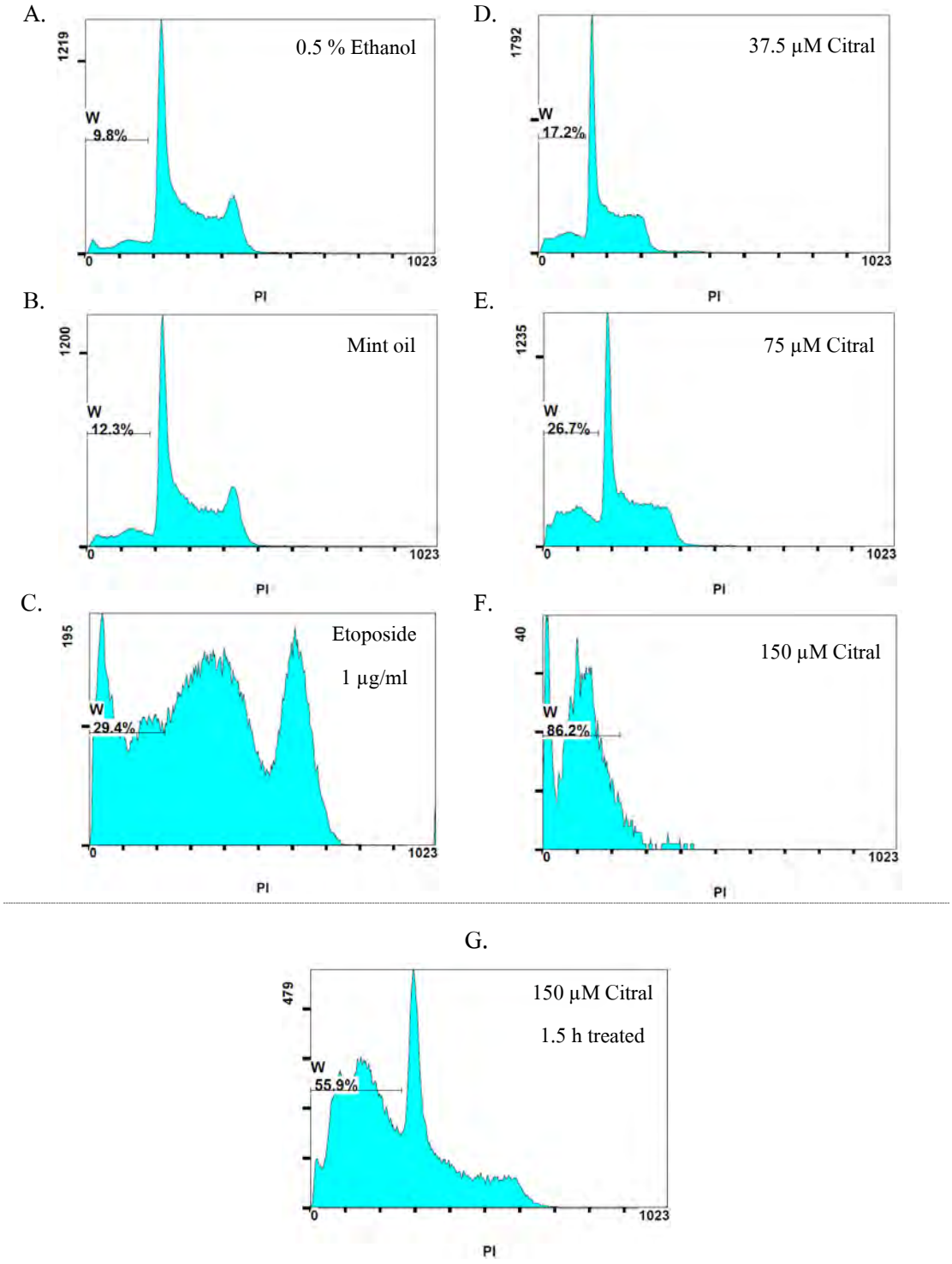


Figure 31: The effect of citral on the cell cycle in Ramos cells (n2). The cells were treated with 37.5, 75 and 150 µM citral for 3 h, then washed and incubated in fresh medium for 48 h. The treated cells were fixed, RNase treated, and stained with PI. The cell cycle patterns were determined by fluorescent flow cytometer. (A) 0.5 % ethanol; (B) mint oil; (C) etoposide 1 µg/ml; (D, E, F) 37.5, 75 and 150 µM of citral; (G) 1.5 h treated with 150 µM of citral.

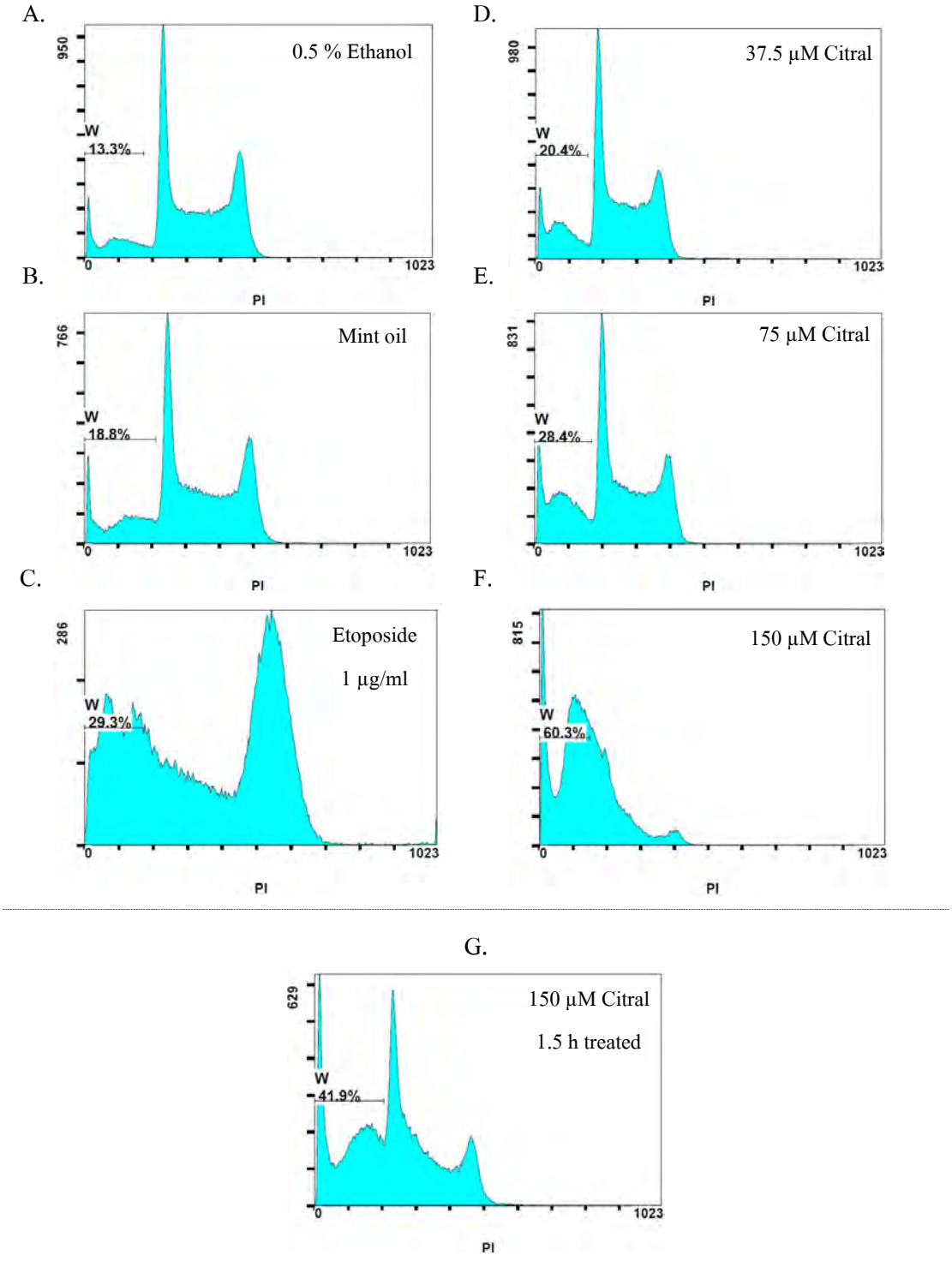


Figure 32: The effect of citral on the cell cycle in Ramos cells (n3). The cells were treated with 37.5, 75 and 150 µM citral for 3 h, then washed and incubated in fresh medium for 48 h. The treated cells were fixed, RNase treated, and stained with PI. The cell cycle patterns were determined by fluorescent flow cytometer. (A) 0.5 % ethanol; (B) mint oil; (C) etoposide 1 µg/ml; (D, E, F) 37.5, 75 and 150 µM of citral; (G) 1.5 h treated with 150 µM of citral.

Table 3: The effect of citral on normal cells death. Human PBMCs cells were treated with 37.5, 75 and 150 µM of citral for 3, 6 and 12 h. The patterns of cell death were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

Treatment	% Viable cells	% Death cells			% Total death
		Apoptosis	PI positive	Double positive	
3 h					
0.5% Ethanol	89.82 ± 0.63	8.00 ± 0.70	0.27 ± 0.12	1.92± 0.08	10.20 ± 0.65
Mint oil	90.50 ± 0.70	7.37 ± 1.08	0.35 ± 0.30	1.80 ± 0.10	9.52 ± 0.68
Etoposide 10 µg/ml	92.82 ± 1.63	5.30 ± 1.65	0.55 ± 0.40	1.32 ± 0.43	7.17 + 1.68
Citral 37.5 µM	90.62 ± 0.28	7.20 ± 0.25	0.27 ± 0.07	1.87 ± 0.08	9.35 ± 0.25
Citral 75 µM	90.82 ± 0.08	7.20 ± 0.30	0.32 ± 0.17	1.62 ± 0.08	9.15 ± 0.05
Citral 150 µM	89.92 ± 0.88	7.85 ± 1.25	0.70 ± 0.50	1.52 ± 0.13	10.07 ± 0.88
6 h					
0.5% Ethanol	91.11 ± 0.13	2.53 ± 2.08	5.66 ± 2.66	0.70 ± 0.68	8.90 ± 1.08
Mint oil	89.25 ± 0.65	2.81 ± 2.29	7.05 ± 3.46	0.90± 0.85	10.76 ± 0.67
Etoposide 10 µg/ml	89.75 ± 2.51	4.31 ± 3.94	5.06 ± 2.49	0.88 ± 0.85	10.26 ± 2.49
Citral 37.5 µM	90.91 ± 0.19	2.76 ± 2.12	5.68 ± 2.74	0.61 ± 0.59	9.06 ± 0.20
Citral 75 µM	82.83 ± 3.06	11.28 ± 1.80	5.06 ± 2.50	0.81 ± 0.69	17.16 ± 3.04
Citral 150 µM	67.46 ± 4.61a*#	21.40 ± 2.63a*	8.98 ± 4.22	2.13 ± 1.08	32.51 ± 4.59a*#
12 h					
0.5% Ethanol	93.66 ± 2.59	3.55 ± 3.08	2.23 ± 1.11	0.55 ± 0.53	6.33 ± 2.59
Mint oil	93.03 ± 2.49	3.33 ± 3.03	2.88 ± 1.46	0.70 ± 0.70	6.91 ± 2.49
Etoposide 10 µg/ml	85.11 ± 5.10	5.60 ± 5.38	7.18 ± 3.47	2.11 ± 2.09	14.90 ± 5.10
Citral 37.5 µM	91.01 ± 3.58	5.16 ± 3.94	2.91 ± 1.38	0.90 ± 0.85	8.98 ± 3.58
Citral 75 µM	74.80± 0.28a	20.91 ± 0.12	3.25 ± 0.65	1.03 ± 0.81	25.20 ± 0.28a
Citral 150 µM	47.18 ± 0.27a*#	40.86 ± 5.77a*#	8.95 ± 2.67	3.00 ± 1.34	52.81 ± 6.27a*#

a $p < 0.01$ denotes statistically significant difference from 0.5% ethanol.

* $p < 0.01$ denotes statistically significant difference between 75 and 150 µM citral compared with 37.5 µM citral.

# $p < 0.01$ denotes statistically significant difference when compared between 150 µM and 75 µM of citral

Table 4: The involvement of caspase activation on citral induced Ramos cells apoptosis. The cells were pretreated with 50 µM of Z-VAD-FMK for 1 h prior to treated cells with citral for 6 h. The patterns of cell death were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

Treatment	% Viable cells	% Death cells			% Total death
		Apoptosis	PI positive	Double positive	
Without Z-VAD-FMK					
0.5% Ethanol	93.33 ± 1.13	6.21 ± 1.05	0.10 ± 0.05	0.31 ± 0.13	6.63 ± 1.15
Mint oil	86.65 ± 0.29	12.41 ± 0.41	0.35 ± 0.19	0.53 ± 0.14	13.30 ± 0.28
Citral 37.5 µM	56.80 ± 4.68	40.71 ± 4.31	0.71 ± 0.22	1.76 ± 0.87	43.20 ± 4.69
Citral 75 µM	29.16 ± 2.87	65.46 ± 2.91	3.85 ± 0.71	1.51 ± 0.56	70.83 ± 2.87
Citral 150 µM	9.40 ± 2.27	83.30 ± 1.49	4.72 ± 0.99	2.55 ± 0.79	90.56 ± 2.28
With Z-VAD-FMK					
0.5% Ethanol	93.06 ± 0.82	6.53 ± 0.84	0.00 ± 0.00	0.40 ± 0.15	6.93 ± 0.82
Mint oil	94.86 ± 0.85*	4.66 ± 0.69*	0.00 ± 0.00	0.46 ± 0.19	5.13 ± 0.85*
Citral 37.5 µM	92.23 ± 1.32*	7.06 ± 1.22*	0.06 ± 0.03	0.63 ± 0.09	7.76 ± 1.32*
Citral 75 µM	79.00 ± 0.85*	19.40 ± 0.84*	0.30 ± 0.20*	1.33 ± 0.50	21.03 ± 0.87*
Citral 150 µM	45.60 ± 6.53*	51.60 ± 6.75*	0.10 ± 0.00*	2.70 ± 0.26	54.40 ± 6.53*

* $p < 0.01$ denotes statistically significant difference from without Z-VAD-FMK.

Table 5: The involvement of Fas-FasL interaction on citral induced apoptosis. Ramos cells were pretreated with 200 ng/ml of anti-FasL antibody for 1 h before being treated with citral for 6 h. The patterns of cell death were determined by annexin V-FITC/PI staining assay using fluorescent flow cytometer. The data is expressed as mean ± S.E. of three independent experiments (n=3).

Treatment	% Viable cells	% Death cells			% Total death
		Apoptosis	PI positive	Double positive	
Without Anti-FasL					
0.5% Ethanol	93.33 ± 1.13	6.21 ± 1.05	0.10 ± 0.05	0.31 ± 0.13	6.63 ± 1.15
Mint oil	86.65 ± 0.29	12.41 ± 0.41	0.35 ± 0.19	0.53 ± 0.14	13.30 ± 0.28
Etoposide 10 µg/ml	84.76 ± 2.47	13.16 ± 2.18	0.61 ± 0.28	1.43 ± 0.68	15.21 ± 2.47
Citral 37.5 µM	56.80 ± 4.68	40.71 ± 4.31	0.71 ± 0.22	1.76 ± 0.87	43.20 ± 4.69
Citral 75 µM	29.16 ± 2.87	65.46 ± 2.91	3.85 ± 0.71	1.31 ± 0.41	70.78 ± 2.82
Citral 150 µM	9.40 ± 2.27	83.30 ± 1.49	4.71 ± 0.99	2.55 ± 0.79	90.56 ± 2.28
With Anti-FasL					
0.5% Ethanol	92.46 ± 1.16	7.16 ± 1.16	0. 01 ± .017	0.33 ± 0.07	7.51 ± 1.15
Mint oil	85.11 ± 0.76	14.20 ± 0.57	0.13 ± 0.03	0.56 ± 0.20	14.9 ± 0.75
Etoposide 10 µg/ml	85.83 ± 2.89	12.46 ± 2.40	0.35 ± 0.23	1.36 ± 0.77	14.18 ± 2.89
Citral 37.5 µM	60.70 ± 4.66	37.36 ± 4.41	0.66 ± 0.15	1.25 ± 0.51	39.28 ± 4.68
Citral 75 µM	28.28 ± 2.90	67.00 ± 2.79	2.56 ± 0.29	2.20 ± 0.59	71.76 ± 2.90
Citral 150 µM	11.36 ± 3.37	82.46 ± 2.96	4.11 ± 0.99	2.01 ± 0.71	88.60 ± 3.38

BIOGRAPHY

Name	Mr. Chatikorn Bunkrai
Date of birth	November 23, 1983
Place of birth	Nakhon Si Thammarat, Thailand
Natianality	Thai
Education	2005: Bachelor of Science (Biotechnology) King Mongkut's Institute of Technology Ladkrabang