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CYTOTOXIC ACTIVITY AND MECHANISM OF *GLYCOSMIS PARVA* LEAF EXTRACTS ON HUMAN B-LYMPHOMA CELLS

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A Thesis Submitted in Partial Fulfillment of the Requirements
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Thesis Title	CYTOTOXIC ACTIVITY AND MECHANISM OF *GLYCOSMIS PARVA* LEAF EXTRACTS ON HUMAN B-LYMPHOMA CELLS
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Field of Study	Medical Science (Pharmacology)
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อภิวันท์ รอเสนา: ฤทธิ์และกลไกการเป็นพิษของสิ่งสกัดจากใบส้มชื่นต่อบีเซลล์ลิมโฟมาของมนุษย์ (Cytotoxic activity and mechanism of *Glycosmis parva* leaf extracts on human B- lymphoma cells) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผู้ช่วยศาสตราจารย์ ดร. วัชรี ลิมปนสิทธิกุล, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รองศาสตราจารย์ ดร. นิจศิริ เรืองรังษี, ๗๓ หน้า

การศึกษานี้ประเมินความเป็นพิษของสิ่งสกัดด้วยตัวทำละลายชนิดต่างๆจากใบของส้มชื่นต่อเซลล์รามอส ซึ่งเป็นเซลล์ลิมโฟมาของมนุษย์ นำสิ่งสกัด เฮกเซน เอทิลอะซีเตท บูทานอล และ น้ำมาทดสอบความเป็นพิษด้วยวิธีรีซาซูนิน มีเพียงสิ่งสกัดเอทิลอะซีเตทเท่านั้นที่นำไปศึกษาต่อเนื่องจากมีความเป็นพิษต่อเซลล์มะเร็งรามอสสูง โดยมีค่าความเข้มข้นที่ทำให้เซลล์ตายร้อยละ ๕๐ เท่ากับ ๑๕.๖๘ ไมโครกรัม/มิลลิลิตรหลังจากได้รับสารนาน ๒๔ ชั่วโมง สิ่งสกัดนี้มีความเป็นพิษต่อเซลล์เม็ดเลือดขาวปกติของมนุษย์ต่ำกว่าความเป็นพิษที่มีต่อรามอสเซลล์มาก ทำการศึกษาลักษณะการตายจากการได้รับสิ่งสกัดโดยย้อมด้วย annexin V-FITC/propidium iodide และวัดด้วย fluorescence flow cytometer พบว่า สิ่งสกัดทำให้เซลล์รามอสตายแบบเอพอพโตซิสเป็นหลักหลังได้รับสารนาน ๘ ชั่วโมง ซึ่งสารทำให้เกิดการตายแบบเอพอพโตซิสโดยการกระตุ้นเอนไซม์ caspases เนื่องจาก ZVADFMK ซึ่งเป็นสารที่ยับยั้ง caspase หลายตัว ยับยั้งการออกฤทธิ์ของสิ่งสกัดได้เกือบสมบูรณ์ นอกจากนี้พบว่าสิ่งสกัดอาจไปกระตุ้นภายในเซลล์ให้เกิดการตายแบบเอพอพโตซิส เนื่องจากสิ่งสกัดมีผลต่อการแสดงออกของโปรตีนในตระกูล BCL-2 ที่ควบคุมการตายดังกล่าว สิ่งสกัดยับยั้งการแสดงออกในระดับ mRNA ของ BCL-XL ที่ยับยั้งการเกิดเอพอพโตซิส และเพิ่มการแสดงออกของ BAK ที่กระตุ้นการเกิดเอพอพโตซิส สิ่งสกัดออกฤทธิ์ทำลายเซลล์รามอสแบบเฉพาะต่อระยะในวัฏจักรเซลล์ โดยทำให้เซลล์สะสมที่ระยะ G1 และ S หลังจากเซลล์ได้รับสิ่งสกัดนาน ๑ ชั่วโมงและถูกนำไปเพาะเลี้ยงต่อในอาหารใหม่ที่ไม่มีสิ่งสกัดต่อเป็นเวลา ๔๘ ชั่วโมง การสะสมของเซลล์รามอสที่เกิดขึ้นสอดคล้องกับผลของสิ่งสกัดต่อการแสดงออกของโปรตีน cyclins และโปรตีนที่ยับยั้ง cyclin dependent kinases (Cdks) ที่ควบคุมการทำงานของ Cdks ซึ่งเป็นเอนไซม์ kinases สำคัญที่ควบคุมวัฏจักรเซลล์ สิ่งสกัดยับยั้งการแสดงออกในระดับ mRNA ของ cyclin D1 และ cyclin E ที่กระตุ้นการทำงานของ Cdks ในระยะเริ่มต้นของ G1 และในระยะ G1 เข้าสู่ S ตามลำดับ สิ่งสกัดเพิ่มการแสดงออกของ p21 ซึ่งจับและยับยั้งการทำงานของ Cdks ในระยะ G1 และ G2 ผลจากการศึกษานี้แสดงว่าสิ่งสกัดเอทิลอะซีเตทจากใบของส้มชื่นสามารถชักนำให้เซลล์มะเร็งตายแบบเอพอพโตซิสเป็นหลักและยับยั้งวัฏจักรเซลล์ในระยะ G1 และ S

สาขาวิชา.....วิทยาศาสตร์การแพทย์... ลายมือชื่อนิสิต .....

ปีการศึกษา ..๒๕๕๓..... ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

5174847030: MAJOR MEDICAL SCIENCE
KEYWORDS: *GLYCOSMIS PARVA* / CYTOTOXICITY / APOPTOSIS / CELL CYCLE ARREST

APIWAN ROSENA : CYTOTOXIC ACTIVITY AND MECHANISM OF *GLYCOSMIS PARVA* LEAF EXTRACTS ON HUMAN B-LYMPHOMA CELLS. ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., 73 pp.

This study evaluated cytotoxic activities of solvent extracts from the leaves of *Glycosmis parva* on human B-lymphoma Ramos cells. The cytotoxic activities of the hexane, ethyl acetate, butanol and water extracts on Ramos cells were determined by resazurin assay. Only the ethyl acetate extract was chosen to further study because it exhibited potent cytotoxic activity against Ramos cells with IC50 at 15.68 μg/ml after 24 h exposure. This extract has much lower cytotoxic effect on normal human peripheral blood mononuclear cells (PBMCs) than on Ramos cells. The patterns of cell death induced by this extract were determined by staining with annexin V-FITC/ propidium iodide staining and detecting with fluorescence flow cytometer. The extract induced Ramos cell death mainly by apoptosis after 8 h of treatment. This apoptotic effect was mainly dependent on caspase activation. A pan caspase inhibitor Z-VAD-FMK almost completely blocked the apoptotic effect of the extract. This study also demonstrated that the intrinsic pathway of apoptosis was involved in the apoptotic effect of the extract. The extract had effects on the expression of proteins in the BCL-2 family which regulate the intrinsic pathway of apoptosis. It significantly decreased the mRNA expression of anti-apoptotic BCL-XL and increased the expression of pro-apoptotic BAK. It acted as a cell cycle specific cytotoxic agent against Ramos cells. It caused Ramos cell accumulation at G1 and S phases after 1 h of treatment and further cultured the treated cells in a fresh media for 48 h without the extract. This effect was correlated to its effect on the expression of cyclins and a cyclin dependent kinase inhibitor which all regulate cyclin dependent kinases (Cdks), the key regulatory kinases in the cell cycle. The extract decreased the mRNA expression of cyclin D1 and cyclin E which activate Cdk functions at early G1 phase and late G1 to S phase progression, respectively. It significantly increased the mRNA expression of a CKI p21 which binds to and inhibits Cdk activities at G1 and G2 phases. These results demonstrate that ethyl acetate extract from leaves of *G. parva* can induce cancer cell death mainly by apoptosis and cause cell cycle arrest at G1 and S phases.

Field of study: ….Medical Science……Student's Signature …...……
Academic year: .. 2010……..Advisor's Signature ……...…
Co-advisor's Signature……...……

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LIST OF ABBREVAITIONS

AIF	Apoptosis-inducing factor
Apaf-1	Apoptosis protease-activating factor 1
APC	Anaphase promoting complex
Asp	Aspartate
ATCC	American Type Cell Culture
ATP	Adenosine triphosphate
BAD	BCL-2 antagonist of cell death
BAK	BCL-2-antagonist/kitter-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
Cdc	Cell-division-cycle gene
Cdk	Cyclin-dependent kinases
Cip/Kip	Cdk inhibitory protein/Kinase Inhibitor protein
CKI	Cdk inhibitors
CO_2	Carbon dioxide
COX-2	Cyclooxygenase 2
CR	Complement receptor
CRD	Cysteine-rich domain
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern

DD	Death domain
DED	Death effector domain
DEPC	Diethyl pyrocarbonate
DISC	Death inducing signaling complex
DNA	Deoxyribonucleic acid
DRs	Death receptors
eIF	Eukaryotic initiation factor
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
HCl	Hydrochloric acid
HMGB1	High mobility group protein B1
HSV -1	Herpes simplex virus type 1
HSV -2	Herpes simplex virus type 2
Htra2	High temperature requirement A2
IAPs	Inhibitors of apoptosis protein
ICAD	Inhibitor of caspase-activated DNase
INK4	Inhibitors of Cdk4
iNOS	Indicible nitric oxide synthase
mg	Milligram
ml	Milliliter
M	Molar (mole per liter)
MAPK	mitogen-activated protein kinase
MCL-1	Myeloid cell leukemia 1
MFG-E8	Milk fat globule-EGF factor-8 protein
MMP	Mitochondrial membrane permeabilization

MPF	Mitosis promoting factor
MPT	Mitochondrial permeability transition
ng	Nanogram
NaCl	Sodium chloride
NAIP	Neuronal apoptosis inhibitory protein
NF-κB	Nuclear factor κB
NK	Natural killer cell
NO	Nitric oxide
ox-LDL	Oxidized low-density lipoprotein
PBS	Phosphate buffer saline solution
PBMC	Peripheral blood mononuclear cell
PCNA	Proliferating cell nuclear antigen
PS	Phosphatidylserine
PUMA	P53-upregulated modulator of apoptosis
pH	The negative logarithm of hydrogen ion concentration
Rb	Retinoblastoma protein
rpm	Revolution per minute
SCF	Skp1, cullin, F-box protein
S.E.	Standard error
Smac	Second mitochondria-derived activator of caspases
SR-A	Scavenger receptor A
TGF-β	Transforming growth factor β
TM	Transmembrane domain
TNFR	Tumor necrosis factor receptor
TRAIL	TNF-related apoptosis-inducing ligand
XIAP	X-linked mammalian inhibitor of apoptosis protein
ºC	Degree Celsius
μg	Microgram
$\triangle\psi$m	Mitochondrial membrane potential

CHAPTER I

INTRODUCTION

Background and Rationale

The growing burden of cancer is still one of health problems in the world as well as Thailand. The incidence of cancer is predicted to increase by 50 percents in the next 20 years. Cancer cells arise from the imbalance between cell growth and cell death. Somatic cells normally proliferate under the regulation of the cell cycle progression. The cell cycle is tightly regulated by several positive and negative signal molecules that involve in cell cycle control. Program cell death or apoptosis is highly regulated for elimination of unwanted cells without eliciting an inflammatory response. Cancer cell can be treated in difference ways depending on types of cancer, location in the body and severity of the disease. Goal of cancer treatment is elimination of cancer cells as much as possible but harm normal cells at the least. Several treatment models are currently available. These include surgery, radiation, and anti-cancer drugs. Conventional anti-cancer drugs which are cytotoxic agents and hormones and hormone antagonists are still wildly used. Hormone and their antagonists have been used only for treatment cancer of sex organs. Cytotoxic agents are more widely used than hormones and their antagonists for treatment of several types of cancers. However, major problems of these drugs uses are side effects and drug resistance, leading to treatment failure. Most of them have some cytotoxic effects on rapid proliferating normal cells which lead to common side effects of these drugs such as bone marrow suppression and infection. Moreover, cancer cells can adapt to these cytotoxic drugs and then become resistant to them. Searching of new drugs or new strategies is always required for cancer therapy. Recently, target-based anticancer drugs have been emerged to play important role in cancer treatment. These drugs selectively act on cancer cells by targeting to molecules selectively express in cancer cells or highly express in cancer cells. Limitation of these drugs is high cost and long term usage.

Searching for anticancer drugs from natural sources is one of strategies for new anticancer drugs development. Many herbal medicines have been used as alternative medicine for cancer therapy in several parts of the world. *Glycosmis parva* Craib (Rutaceae) is a small shrub widely distributes in Thailand. It is used as a traditional medicine for treatments of abscess, scabies, and snakebite. Plants in the genus *Glycosmis* contain two main chemical compounds including acridone alkaloids and sulfur containing

propanamides in their branches and leaves. It has been reported that acridone alkaloids demonstrated antiviral activities while sulfur containing propanamides had antibacterial, antifungal, antiviral as well as anticancer activities. There are very few reports on pharmacological activities of active compounds from *G. parva*. The hexane and ethyl acetate extracts from leaves of *G. parva* inhibited expression of proinflammatory cytokines, cyclooxygenase 2 (COX-2), and nitric oxide synthase (iNOS) in lipopolysaccharide activated-macrophage J774A.1 cells. COX-2 is one of targets for target-based anticancer drug development due to overexpression of this enzyme in several types of cancers. There is no information about anticancer activities of the active compounds from *G. parva*. This study intended to investigate cytotoxic and apoptosis induction activities of *G. parva* leaves on human B-lymphoma cells.

Objectives

1. To screen the cytotoxicity of solvent extracts from the leaves of *G.parva* on human B-lymphoma cells.
2. To evaluated the selective cytotoxicity of these extracts on the tumor cells.
3. To study apoptotic induction activity and mechanism of apoptotic induction of the extracts on human B-lymphoma cells.
4. To investigate the effect of the extract on the cell cycle pattern and mechanism of cell cycle arrest of the extracts in human B-lymphoma cells.

Hypothesis

The extracts from leaves of *G. parva* have cytotoxicity on human B-lymphoma cells by apoptotic induction and or cell cycle arrest.

Expected benefits and applications

Results from this study may clarify the potential cytotoxic effect of *G. parva* on human B-lymphoma cells. The information from this study may benefit for further investigation of the compounds from this plant in animal research or in clinical trial and further investigation of the compound in another cancer cells in the future

Keywords

*Glycosmis parva***,** cytotoxicity, apoptosis, cell cycle arrest

CHAPTER II
LITERATURE REVIEWS

Tissue homeostasis requires a balance between cell proliferation and death (King. *et al*. 1998). An imbalance between these two processes can result in either unwanted tissue atrophy or tissue growth. Cancer is a problem of this imbalance, a decrease in cell death as well as an increase in cell proliferation. It is a disease of deregulated cell proliferation together with suppressed apoptosis (Fulda S. 2011). Apoptosis is the common and well-defined form of programmed cell death. It is a physiological cell suicide that is essential for the maintenance of homeostasis in embryonic, fetal and adult tissues (Agostini. *et al*. 2005). Most of the cytotoxic drugs in current use have been shown to induce cancer cell death by either apoptosis or necrosis or both types of cell death (Bezabeh. *et al*. 2001).

Necrosis is an accidental and uncontrolled cell death by a consequence of extreme physicochemical stress, such as heat, osmotic shock, mechanical stress, freeze thawing, high concentration of hydrogen peroxide, and high concentration of cytotoxic agents. This type of cell death occurs quickly due to the direct effect of the stress on the cell. Necrotic cells become swell and then their plasma membrane collapse leading to rapid organelle lysis and release of intracellular mediators from dead cells. These intracellular mediators can trigger the innate immune system and induce inflammation of the cells nearby (Proskuryakov. *et al*. 2002).

Apoptosis is the other type of cell death which plays an important role in many physiologic and pathologic processes. It is important for the multicellular organisms to eliminate damaged, infected or unwanted cells that may interfere with normal function (Chen. *et al*. 2009). Cells with genetic damage caused by exposure to carcinogens can be deleted by undergoing apoptosis, thereby preventing their replication and the accumulation of clones of abnormal cells. There are increasing evidences to support the hypothesis that failure of apoptosis may be an important factor in the evolution of cancer and its poor response to chemotherapy and radiation. Inhibition of apoptosis causes an imbalance in normal tissue homeostasis promoting cell growth and it also allows the survival of genetically damaged cells, both contributing to tumor development and progression. Both induction and inhibition of apoptosis can be related to either physiological stimuli,

exposure to stress-inducing agents, or pathological conditions. Among pharmacological agents, many cancer chemotherapeutic drugs are known to activate apoptotic mechanisms of tumor cell death. Moreover, apoptosis has been proposed as a novel target for cancer chemoprevention, whose rationale is to remove cells undergoing neoplastic transformation, in situations where other defense mechanisms fail to block the carcinogenesis process upstream (Agostini. *et al*. 2005 and King. *et al*. 1998)

Programmed cell death by apoptosis

Apoptotic cells are characterized by morphological changes including cell shrinkage, chromatin condensation, DNA fragmentation and formation of apoptotic bodies. They are also identified by several biochemical changes including phosphatidylserine (PS) exposure on the outer leaflet of the plasma membrane and changes in mitochondrial membrane permeability leading to release of intermembrane space mitochondrial proteins (Agostini. *et al*. 2005). These morphological and biochemical changes are used as the markers to distinguish apoptosis from other forms of cell death. Many anticancer drugs kill tumor cells by inducing apoptosis (Bezabeh. *et al*. 2001). Generally apoptosis is mediated by the activation of the family of cysteine-containing aspartate-directed proteases (caspases). Caspases are sequentially activated into active caspases which can cleave their target molecules leading to cell to become apoptotic cell (Kim. *et al*. 2002).

Apoptotic cells are rapidly engulfed by professional phagocytes, macrophages and dendritic cells. The clearance of these apoptotic cells by phagocytes occurs so rapidly. This prevents the release of potentially toxic or immunogenic intracellular mediators from the dead cells. The phagocytosis of apoptotic cells renders the phagocytes immunosuppressive by producing anti-inflammatory cytokines, such as IL-10. Therefore, even tissues with high cell turnover rates are free from inflammation. Phagocytes engulf dead cell corpses but not living cells. It has been revealed that phagocytes express MFG-E8 which is specifically recognize phosphatidylserine (PS) on apoptotic cells. When MFG-E8 is engaged with PS on apoptotic cells it promotes the phagocytosis of apoptotic cells (Tanaka M. 2005).

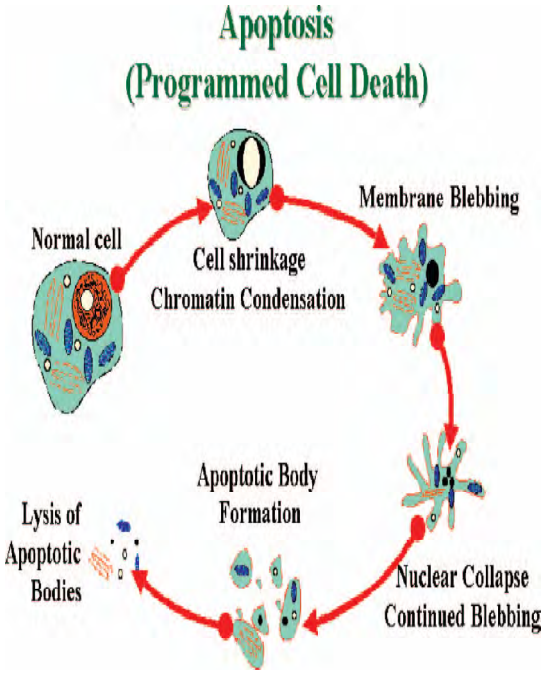


Figure 1: Characteristics of cell death by apoptosis. The progression of morphology changes are observed in apoptotic cell (Vedula. *et al*. 2008).

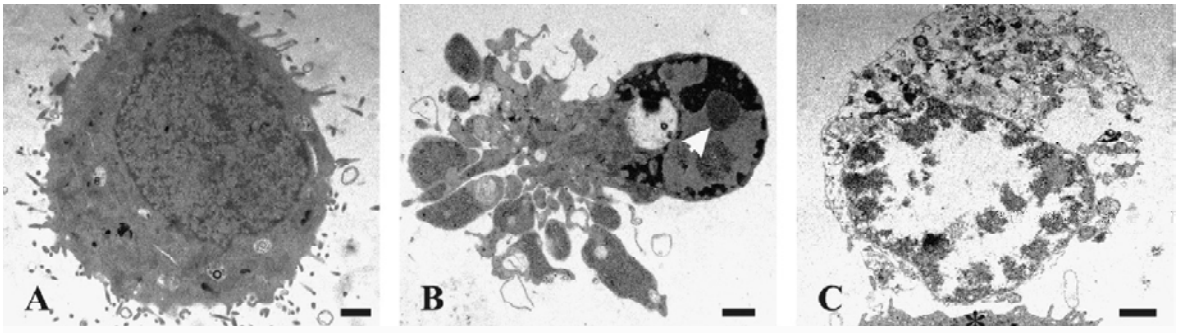


Figure 2: Photographs under electron microscope comparing a normal cell (A), an apoptotic cell (B) and a necrotic cell (C) (Krysko. *et al*. 2007).

Caspases

Caspases are essential effector molecules for inducing apoptosis in eukaryotic cells (Fan. *et al*. 2005). All of these enzymes consist of an N-terminal prodomain followed by a large subunit and a small subunit. They are synthesized as inactive proenzymes which are activated by cleavage at specific aspartate (Asp) residues to become active enzymes containing both large (p20) and small (p10) subunits which contain residues that are essential for catalysis and substrate recognition. All caspases contain an active-site

pentapeptide of general structure, Gln-Ala-Cys-Arg-Gly (QACRG), in the p20 subunit. The amino acids Cys-285 and His-237 involved in catalysis (Jekely G. 1998 and Cohe GM. 1997). There are 2 different types of these domains known as the death effector domain (DED) and the caspase-recruitment domain (CARD) (Weber CH. 2001). Based on their predominant functional roles and their substrate specificities, caspases are typically divided into 3 major groups. The caspases with large prodomains are referred to as inflammatory caspases (group I) and initiator of apoptosis caspases (group II), while caspases with a short prodomain of 20–30 amino acids are effector caspases (group III). The large prodomains of procaspases contain structural motifs that belong to death domain superfamily which involves in the transduction of the apoptotic signal. Caspases that involve in apoptosis process are initiation caspases (caspase-2,-8, -9 and -10) that activate execution or effector caspases (caspase-3, -6 and -7) (Grutter GM. 2000).

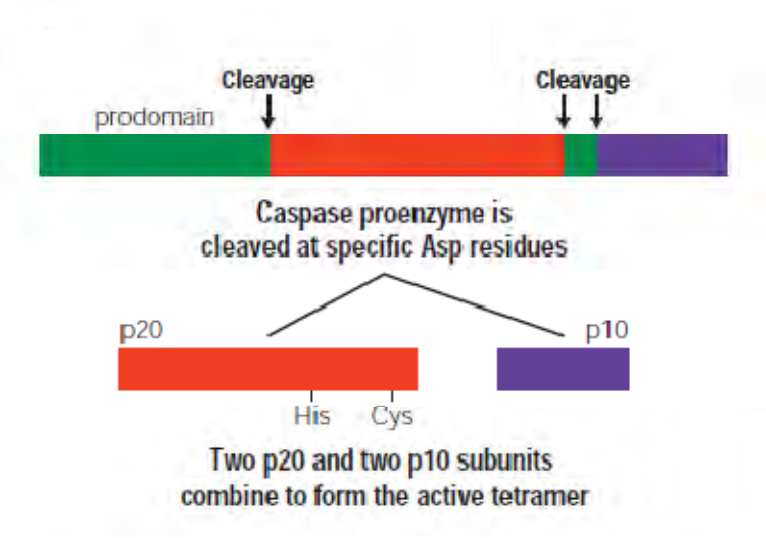


Figure 3: A schematic representation of structural features of mammalian caspase (Jekely G. 1998).

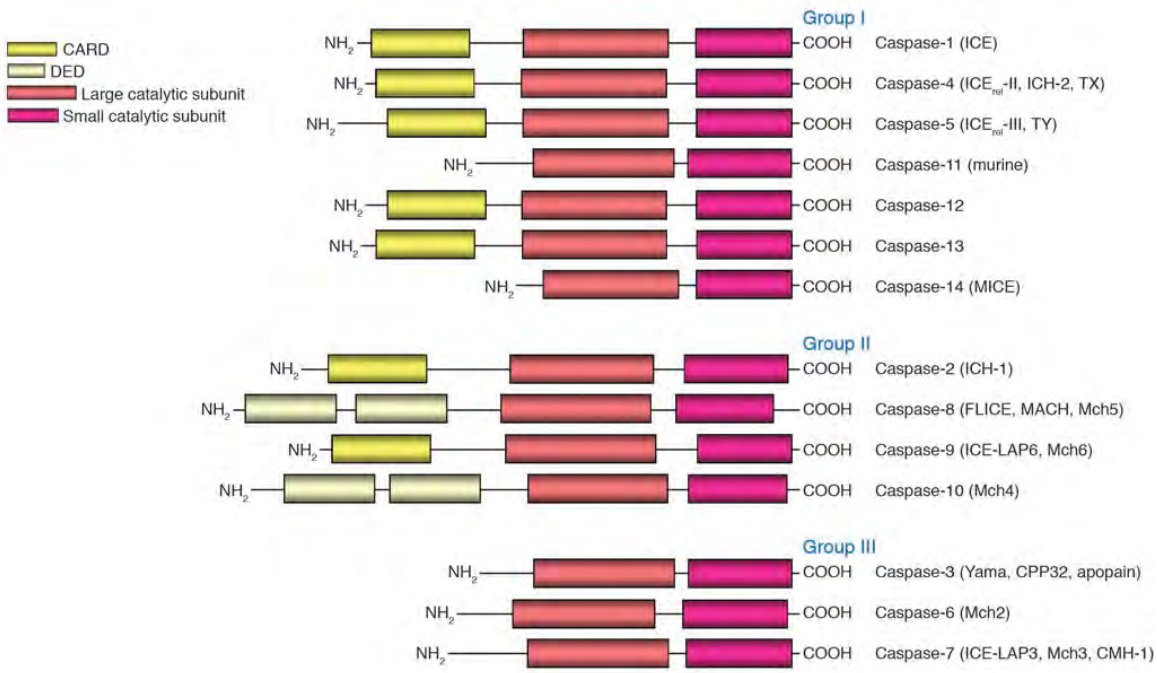


Figure 4: Three major groups of mammalian caspases (Lavrik. *et al*. 2005).

Caspase activation pathways

Caspases are activated by 3 major pathways including the extrinsic, the intrinsic and the granzyme B pathways. The extrinsic pathway is initiated through activation of death receptors on the cell surface, leading to caspase activation. The intrinsic pathway or mitochondria mediated apoptosis involves in the increase of mitochondrial permeability by stimuli and follows by the releases of apoptotic factors from the mitochondria to activate caspase. The granzyme B mediated pathway is the direct effect of granzyme B to activate caspases.

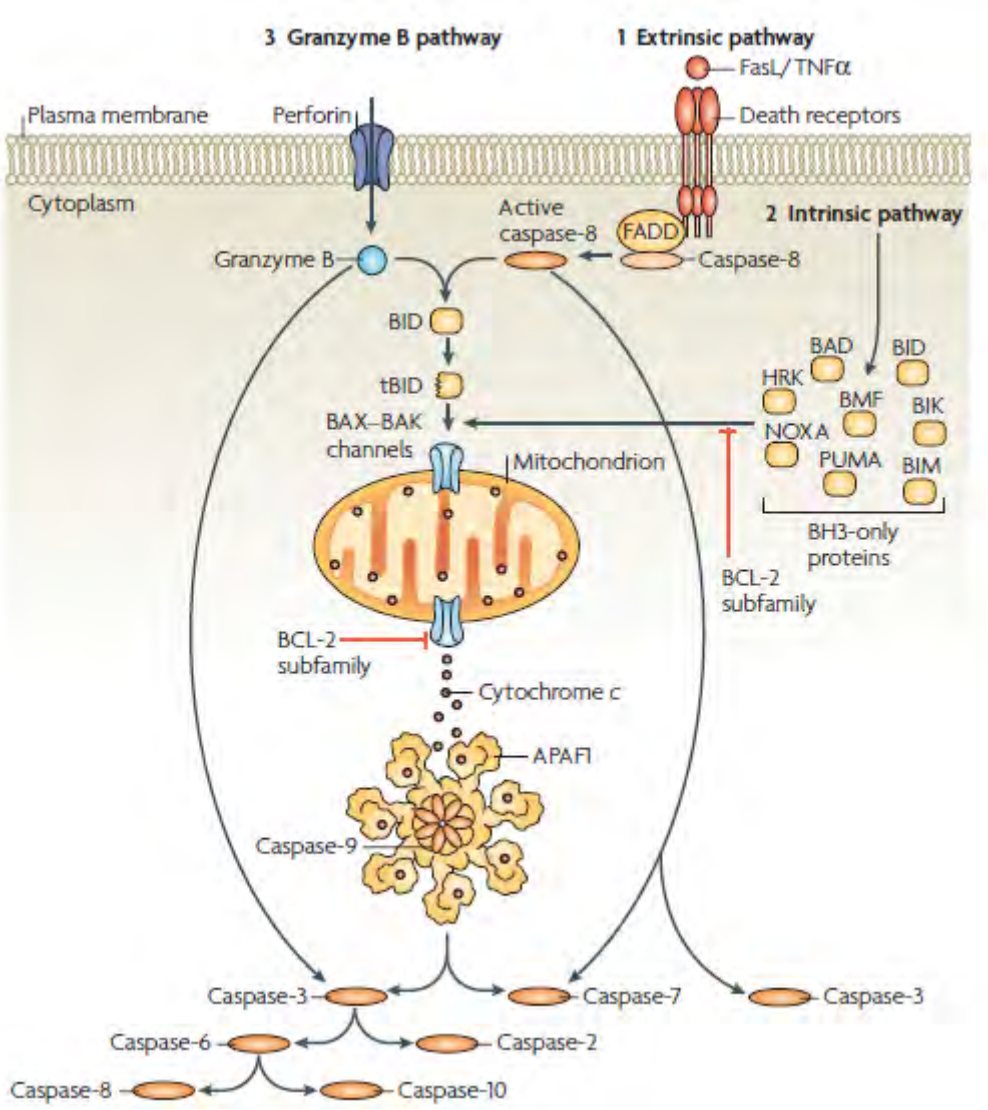


Figure 5: Caspase activation pathways (Taylor. *et al*. 2003).

The extrinsic pathway of apoptosis

Procaspase-8 and -10 are initiator caspases in the extrinsic pathway of apoptosis. This pathway is initiated by the activation of cell surface death receptors. These receptors are the members of TNF receptor (TNFR) superfamily including TNFR1, CD95 [Fas/APO-1], TRAIL-R1, TRAIL-R2, DR3, and DR6 which all contain similar extracellular cyteine-rich domains (CRDs) and intracellular death domains (DDs) of about 80 amino acids at the cytoplasmic tail. The CRDs are essential for receptor self-association and receptor–ligand interactions (Siegel. *et al*. 2000). The DD is important in transmitting the death signal from the cell surface to the intracellular signaling cascades. These death

receptors initiate apoptotic signaling cascades within seconds after binding to their specific death ligands and induce apoptosis within hours. Fas (apo-1 or CD95) is one of the most well known death receptor. It is a 45-52 kDa transmembrane glycoprotein expressed in various tissues and on activated T and B cell 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 express in many tumor cells such as melanomas, astrocytomas, lymphomas and various carcinomas (Balachandran. *et al*. 2000). Fas molecules undergo oligomerization and conformational change after binding to FasL. 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 procaspase-8 is auto-catalytically activated and becomes active caspase-8. This caspase then triggers execution caspases (caspase-3, -6, or -7) to induce apoptosis (Thomas. *et al*. 2004).

Caspase 8 and caspase-10 can activate the mitochondrion-mediated pathway by cleave Bid which is a pro-apoptotic BCL-2 protein to become active form tBid. tBid can trigger the activation of the mitochondrion pathway leading to apoptosis (Fan. *et al*. 2005).

Caspase-2 is also one of initiator caspases. Activated death receptors recruit procaspase-2 by adaptor molecules. Procaspase-2 is activated after the recruitment leading to activation of executioner caspases (caspase 3, -6, or -7) and finally induction of apoptosis (Read. *et al*. 2002).

The intrinsic pathway of apoptosis

The mitochondria or intrinsic pathway of apoptosis is initiated by intracellular apoptotic signals such as ultraviolet radiation, reactive oxygen and reactive nitrogen species, chemotherapeutic agents, heat shock, growth factor withdrawal and DNA damage (Bratton. *et al*. 2001). These stimuli cause the increase in the mitochondrial membrane permeability which leads to the release of pro-apoptotic factors in the intermembrane space of mitochondria into the cytosol. These factor are cytochrome c, second mitochondria-derived activator of caspase/direct IAP-binding protein with low isoelectric point (Smac/Diablo), a serine protease called high temperature requirement protein (HtrA2/Omi), apoptosis-inducing factor (AIF), endonuclease G (EndoG) and caspase-activated DNase (CAD) (Wang X. 2001). Cytochrome c, Smac/Diablo and HtrA2/Omi

activate the caspase dependent mitochondrial pathway where as AIF, Endo G and CAD released from the mitochondria at a late phase of apoptosis and play role in apoptosis in either a caspase independent or dependent manner. The release of these apoptotic factors is tightly regulated by several proteins in the BCL-2 family.

Cytochrome c interacts with apoptotic protease-activating factor-1 (Apaf-1), ATP/dATP, and procaspase-9 forming an apoptosome (Gupta S. 2005). Apaf-1 contains a CARD, which involves in the recruitment of procaspase-9 (Zou H. 1999). Procaspase-9 in apoptosome complex undergoes autoproteolytic cleavage to become active caspase-9. This initiator caspase then directly activates effector caspases, caspases-3, -6 and -7 (Fig. 8). These effector caspases result in the sequentially cell death by proteolytic clevage of a vast numbers of downstream targets. Smac/DIABLO and HtraA2/Omi can binds to the inhibitor of apoptosis proteins (IAPs) and disrupt IAPs association with active caspase-9. This allows caspase-9 to activate caspase-3, leading to apoptosis (Srinivasula. *et al*. 2001)

The mitochondrial membrane permeability in apoptotic process is tightly regulated by proteins in the B cell lymphoma-2 (BCL-2) family. These proteins are divided into anti-apoptotic BCL-2 and pro-apoptotic BCL-2 proteins. All of them contain BCL-2 homology domains (BH1–4 domains) (Fig. 9) (Chipuk JE. 2006). The anti-apoptotic BCL-2 proteins (BCL-2, BCL-W, BCL-XL, A1 and MCL-1) contain 4 BH domains (BH 1-4). They are transmembrane proteins integrated in the endoplasmic reticulum (ER), the nuclear envelope and the outer mitochondrial membranes. They preserve cell survival by playing negative regulatory role in apoptosis. They prevent cytochrome c release from mitochondria by directly binding to and inhibiting pro-apoptotic BCL-2 proteins (Chipuk. *et al*. 2008). The pro-apoptotic BCL-2 proteins (BAK and BAX) contain 3 BH domains (BH 1-3). They regulate and induce mitochondrial outer membrane permeability by forming a dimer or oligomers to generate proteolipid pores. Apoptotic factor such as cytochrome c are released from these pore leading to pro-caspase 9 activation (Martinou. *et al*. 2001)**.** The other pro-apoptotic BH3-only proteins [BAD, BID, BIK, BIM, BMF, Harakiri (HRK), Noxa and p53-upregulated modulator of apoptosis (PUMA)] contain only a conserved BH3 domain in their structure. They bind and inhibit anti-apoptotic BCL-2 proteins leading to apoptotic induction. They play role as initial sensors of various apoptotic signals that induce cell stress or damage. Activation of these proteins is a crucial for overcoming the effect anti-apoptotic BCL-2 proteins (Youle. *et al*. 2008) and inducing

the formation of BAK–BAX oligomers within mitochondrial outer membranes. These oligomers generate pores that permit the release of apoptotic factors.

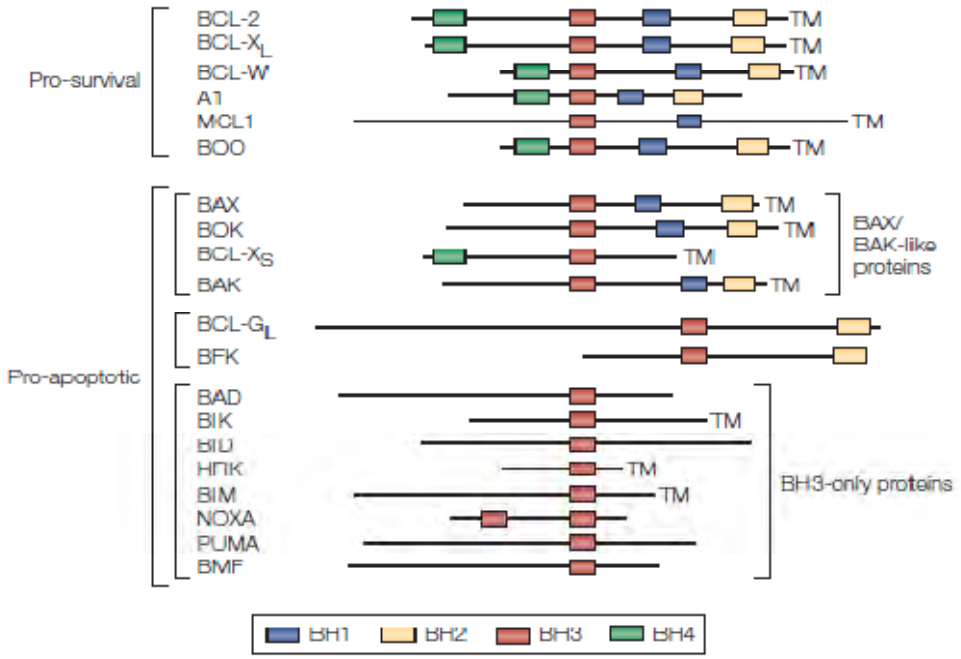


Figure 6: The BCL-2 family proteins (Strasser A. 2005)

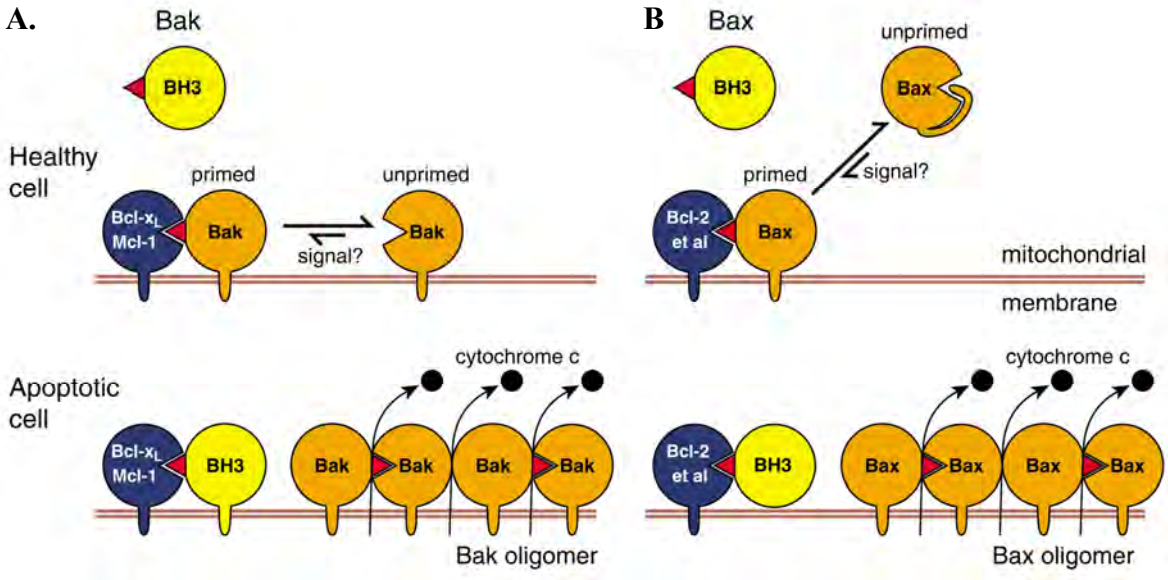


Figure 7: Model for the regulation of (A) BAK and (B) BAX (Adams. *et al.* 2007).

The granzyme-B dependent pathway

The granzyme B-dependent pathway of caspase activation is one of the killing mechanisms of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. This pathway plays a major physiological role in protecting the host against virus infection and tumor cells. Activated CTLs or NK cells can release two major granule-bound toxins, perforin and granzyme B from the cells to kill target cells. Perforin is a pore-forming protein that forms pores by polymerization on target cells. These pores facilitate the delivery of granzyme B inside the cells. Granzyme B is a serine protease that can cleave and activate procaspase-3 (Fig. 8) (Lieberman J. 2003)

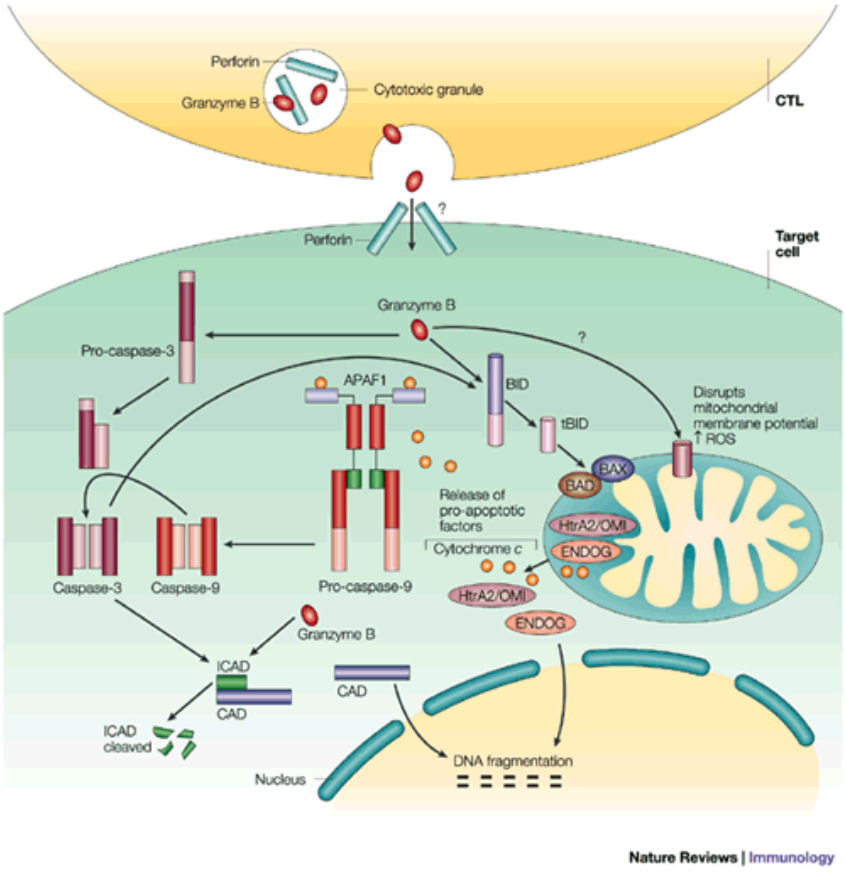


Figure 8: Granzyme B-mediated apoptosis pathway. (Lieberman J. 2003)

The cell cycle

The cell cycle is a sequence of events which leads to cell growth and proliferation. It is tightly control by many positive or negative regulatory mechanisms and proteins that either allow or arrest it progression. There are 4 major phases of the cell cycle including G1, S, G2 and M phases (Fig. 9). Cells in G1 phase commit to enter the cell-cycle and prepare to duplicate their DNA in S phase. After S phase, cells enter the G2 phase to prepare cellular constituents needed for mitosis in M phase. In the M phase, chromatids and daughter cells separate in to 2 identical cells. After M phase, the cells can enter G1 to the new round of the cell cycle or G0 which is the quiescent phase.

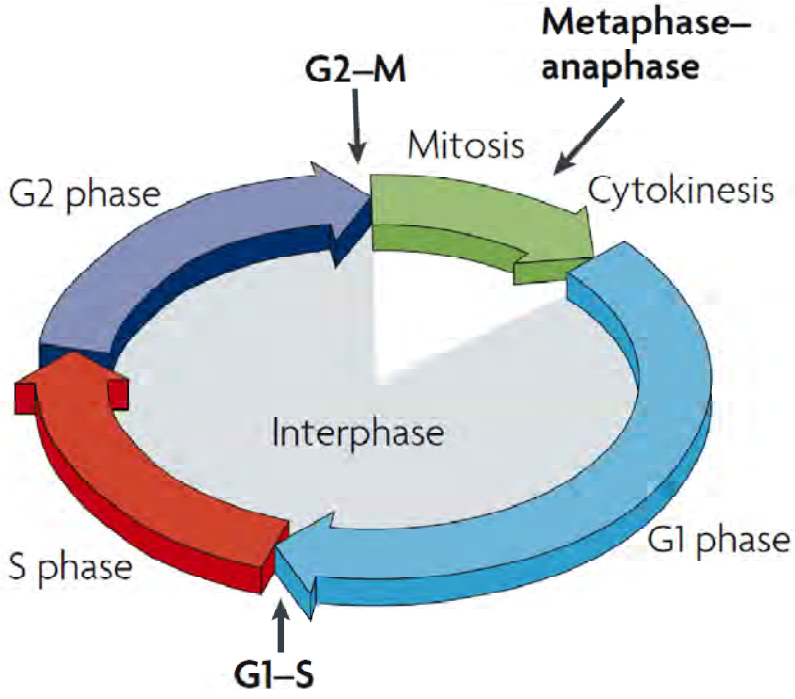


Figure 9: The stages of the cell cycle and the cell cycle check points (Hochegger H. 2008)

Cells entry into each phase of the cell-cycle is strictly regulated at the cell-cycle checkpoints. The regulation at these checkpoints ensures that cell progression in each phase takes place completely and only once in the correct sequence **(**King. *et al*. 1998). There are 3 regulatory 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. 9). Both G1/S and G2/M checkpoints are the DNA damage checkpoints. The G1/S checkpoint works in response to DNA damage to

prevent cell entry into S phase until the damage is fixed. The G2/M checkpoint functions during G2 phase to prevent entry into M phase before the DNA synthesis is fully 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 (Vermeulen. *et al* 2003)**.**

Key regulatory proteins of the cell cycle checkpoints are the cyclin-dependent kinases (Cdks) which are serine/threonine protein kinases. There are 4 Cdks function at different phase of the cell cycle, Cdk1 at G2 and M phases, Cdk2 at G1 and S phase, and Cdk4 and Cdk6 at G1 phase. Each Cdk are active at specific point in the cell cycle. It becomes an active kinase after binding to its specific regulatory subunit called cyclin to be a Cdk-cyclin complexe. Each Cdk associates with its specific cyclin. Different Cdk-cyclins are required at different phases of the cell cycle (Fig. 11, 12). During the cell cycle, the Cdks constitutively express but each cyclin is cyclically synthesized and destroyed at specific time in the cycle. Thus the each Cdk activity is regulated in a time dependent manner (Malumbres M. 2009).

Cyclin D is the first cyclin that is induced when a resting cell commits to enter the cell cycle. This cyclin binds to and activates Cdk 4 and Cdk 6 to become Cdk4-cylin D and Cdk6-cyclin D complexes. These activated Cdks phosphorylate retinoblastoma protein (Rb) which is a product of tumor suppressor gene. Rb is a key regulator at the restriction point just before entering the G1 phase. It binds to and inhibits E2F transcription factors which regulate the expression of many proteins involved in cell cycle progression and DNA synthesis such as Cdk1, cyclin E and A, DNA polymerase, thymidine kinase, and dihydrofolate reductase (Vermeulen. *et al* 2003). Phosphorylation of Rb by Cdk4/6 causes phosphorylated Rb which can dissociate from E2F (Schafer AK. 1998). This leads to active E2F that can turns on the expression of several proteins as mentioned before. The expression of cyclin E is induced for the progression of cells from G1 to S phase. Cyclin E binds to and activates Cdk2 to be Cdk2-cyclin E complex which is required for the transition of cells from G1 to S phase (Ohtsubo. *et al*. 1995).

E2F also turns on the expression of cyclin A which binds to and activates Cdk2 to be Cdk2-cyclin A complex in G1/S phase. Cyclin A also activates Cdk1 in late S phase. Cdk1-cyclin A complex is required for entry into S phase, completion of S phase, and entry into M phase (Johnson. *et al*. 1999). The M phase is regulated by Cdk1-cyclin A and

Cdk1-cyclin B complexes (Arellano. *et al*. 1997). This activated Cdk phosphorylates several 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. It also activates the anaphase promoting complex (APC) which causes the ubiquitin-mediated proteolysis of anaphase inhibitor securin, resulting in the degradation of the cross-linking proteins connecting sister chromatids (Fasanaro. *et al*. 2010). 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, resulting in inactivation of Cdk1 (Bloom. *et al*. 2007). The cells then enter telophase with 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.

Cdks are also regulated by phosphorylation/dephosphorylation on conserved threonine and tyrosine residues of these kinases and by Cdk inhibitors (CKIs). Full activation of Cdks requires phosphorylation of Thr161 in Cdk1, Thr160 in Cdk2, and Thr172 in Cdk4. These phosphorylations induce conformational changes of Cdks and enhance the binding of cyclins (Kaldis. *et al*. 1996). Phosphorylation of Cdk1 at Tyr15 and/ or Thr14 results in inactivating this Cdk. Dephosphorylation at these residues by the phosphatase enzyme Cdc25 is essential for activation of Cdk1 (Lew. *et al*. 1996).

Cdk inhibitors (CKIs) are the other regulators of Cdk activity. They inhibit Cdk activities by binding to either Cdks or to Cdk-cyclin complexes. There are 2 CKI families according to their substrate specificity, the inhibitors of Cdk4 (INK4) family and the Cdk inhibitory Protein/Kinase Inhibitor protein (Cip/Kip) family (Fig. 13) (Maddika. *et al*. 2007). The CKIs in the INK4 family are p15 $^{(INK4b)}$, p16 $^{(INK4a)}$, p18 $^{(INK4c)}$, and p19 $^{(INK4d)}$. These CKIs specifically inactivate G1-Cdk (Cdk4 and Cdk6) by bining to and forming stable complexes with these Cdks before their association with cyclin D (Ortega. *et al*. 2002). The members of the Cip/Kip family are p21$^{(Waf1, Cip1)}$, p27 $^{(Cip2)}$, and p57 $^{(Kip2)}$. These CKIs bind to and inactivate Cdk-cyclin complexes. They inhibit the activity the G1 Cdk-cyclin complexes, and to a lesser extent, Cdk1-cyclin B complexes (Besson. *et al*. 2003). P21 also 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 (Molinari M. 2000). The expression and

activation of p15 and p27 is regulated by transforming growth factor β (TGF-β), resulting in growth arrest (Reynisdottir. *et al*. 1995). CKIs mediate cell cycle arrest in response to several anti-proliferative signals. Dysfunction of CKIs has been identified in many human cancers (Malumbres. *et al*. 2001), suggesting a role of CKIs in preventing abnormal cell proliferation and tumor formation.

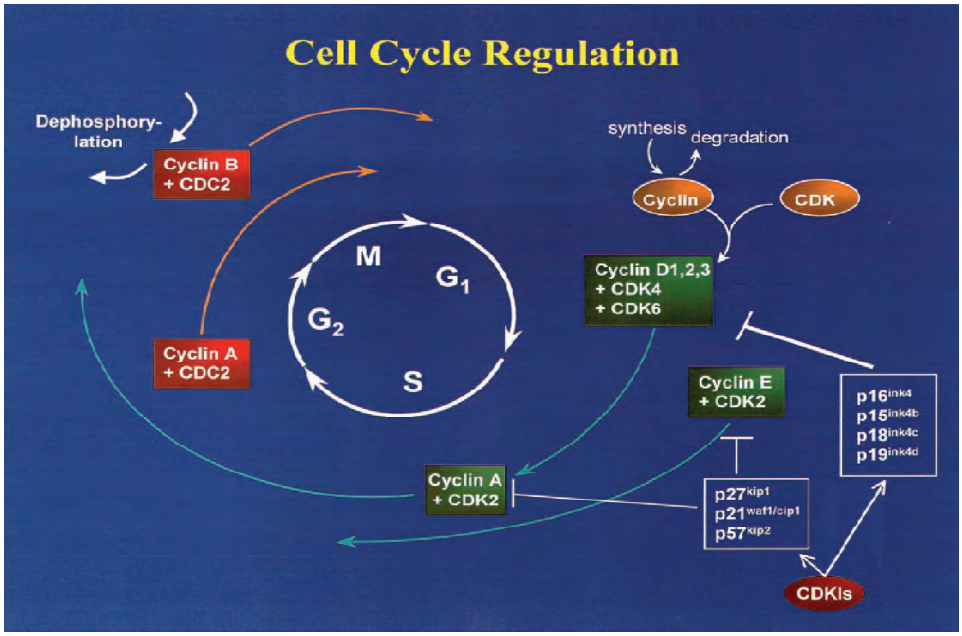


Figure 10: Activities of mammalian cyclin-Cdk complexes and Cdk-inhibitors though the cell cycle (Manish. *et al*. 2001).

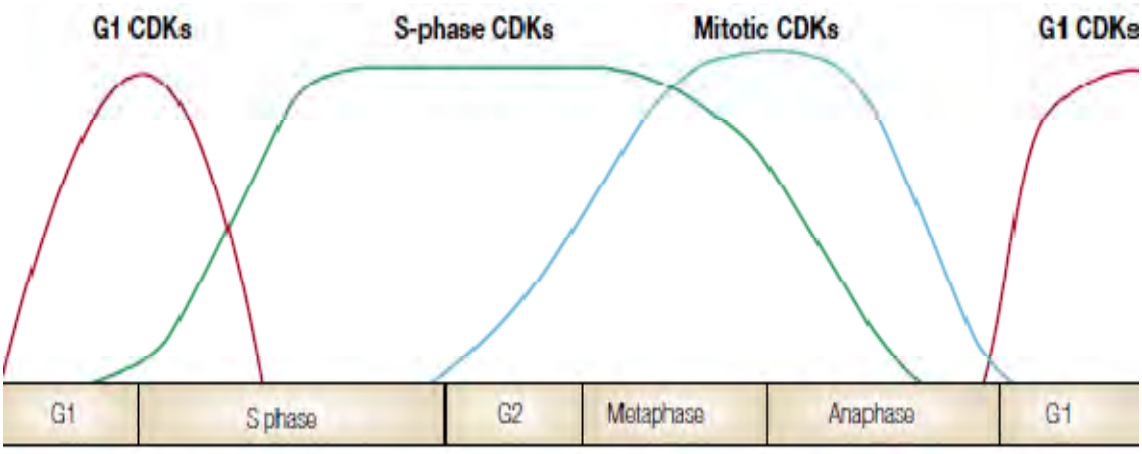


Figure 11: Concentration changes in cyclins during the cell cycle (Bardin. *et al*. 2001)

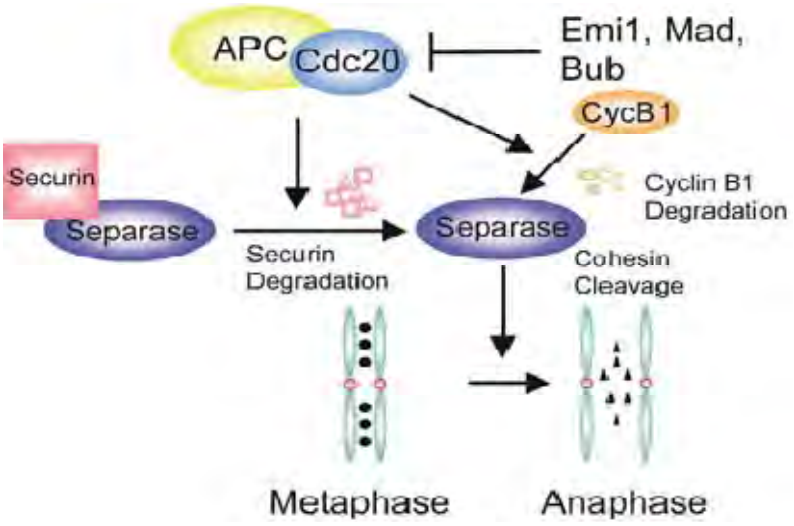


Figure 12: Regulation of sister chromatid separation by the APC. Proteolysis of securin mediated by APCCdc20 at metaphase (Wasc. *et al*. 2005).

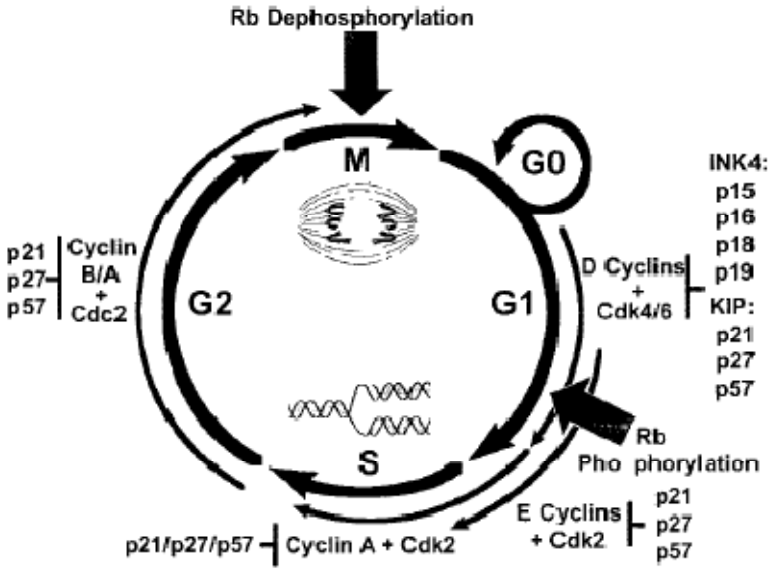


Figure 13: The cell cycle inhibitor proteins, cyclin dependent kinase inhibitors (CKIs) (Slingerland. *et al*. 2000).

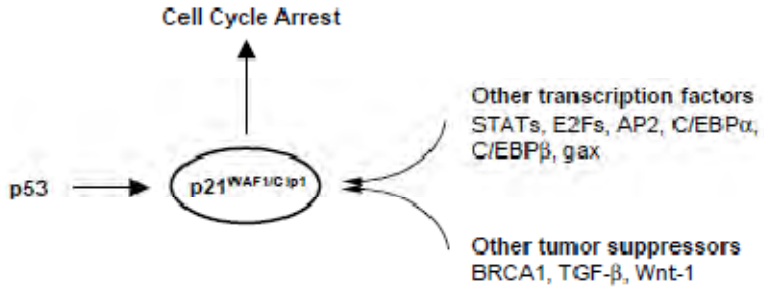


Figure 14: The expression of $p21^{WAF1/Cip1}$ can be transcriptionally activated by p53-dependent and p53-independent mechanisms (Liu. *et al*. 2003)

Chemotherapeutic drugs

Chemotherapeutic or cytotoxic drugs were discovered through chemically synthesis and from natural sources. Most of them kill rapidly proliferating cancer cells by interacting with DNA, interfering production of DNA precursors or DNA synthesis, inhibiting the function of mitotic spindle leading to damage of DNA. They are classified in to 2 groups according to their effects on the cell cycle as cell cycle specific and cell cycle nonspecific drugs.

1. **Cell cycle nonspecific cytotoxic drugs**

Alkylating agents

Alkylating agents are cell cycle nonspecific anticancer drugs. They can kill cancer cells in any phase of the cell cycle. They act by alkylating one or both strands of DNA helix leading to DNA cross-linking and DNA damage. This DNA damage can induce apoptosis. The common clinically used alkylating agents are cyclophosphamide, ifosfamide, alkylating-related drugs as cisplatin and oxaliplatin. They are widely used to treat a variety of tumors in combination with other anticancer drugs.

Antitumor antibiotics

Antitumor antibiotics are products from microbial organisms. Most of them act as cell cycle nonspecific anticancer drugs except bleomycin which causes cell accumulation

in G2 phase. Doxorubicin is the most widely used antitumor antibiotics. It kills cancer cells by intercalating DNA, inhibiting topoisomerase II. This leads to DNA damage and apoptosis. It binds to cellular membrane to alter fluidity and ion transport. It also generates free radicals which cause cardiotoxicity. Doxorubicin is one of the most important anticancer in combined regimen for treatment of several types of tumors.

2. Cell cycle specific cytotoxic drugs

Antimetabolites

Antimetabolites are cell cycle specific anticancer drugs that inhibit the progression of S phase in the cell cycle. They have the most effective effect in tumors with high growth fraction. They are structural analogues to endogenous intracellular metabolites involve in DNA and RNA synthesis. Therefore, they interfere with normal synthesis of nucleic acids. Antimetabolites are divided in to 2 groups, folic acid antagonists and nucleoside analogs. Folic acid antagonists inhibit the production of tetrahydrofolate which is a co-factor in synthesis of DNA precursors (thymidylate), purine nucleotides, and some amino acids. This leads to inhibition of DNA, RNA, and proteins syntheses. Methotrexate is a folic acid antagonist commonly used for treatment of several types of tumors. Nucleoside analogs are sub–divided in to purine and pyrimidine analogs. They are converted to nucleotides (nucleoside triphosphate analogs) by intracellular enzymes. They inhibit the synthesis of DNA precursors thus inhibit DNA synthesis. They also act as DNA chain terminators by incorporating in to newly synthesized DNA. This leads to DNA damage and apoptosis. 6-Mercaptupurine is a well known purine analog and 5-fluorouracil is a pyrimidine analog. Both of them are commonly used to treat cancer.

Plant alkaloids

Products from natural sources make an enormous contribution to anticancer drug discovery. Several currently used cytotoxic drugs are derived from plant alkaloids. These drugs are in the followings;

- Vinca alkaloids (vinblastine and vincristine) are isolated from the periwinkle *Catharanthus roseus*. They bind to tubulin dimers thereby inhibit microtubule assembly and inhibit mitotic spindle function during mitosis phase of the cell cycle. They are M phase specific drugs.

- Taxanes (paclitaxel and docetaxel) are cell cycle specific anticancer drugs. They arrest the cell cycle at M phase by binding to tubulin and stabilizing mitotic spindle during M phase. Paclitaxel is isolated from the bark of the yew tree *Taxus brevifolia*.

- Etoposide is an epipodophyllotoxin derivative and derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *Podophyllum emodi*. It is a topoisomerase II inhibitor. It forms ternary complex with topoisomerase II and DNA, leading to an accumulation of DNA breaks and cell death. Cells in the S and G2 phases of the cell cycle are most sensitive to etoposide

- Camptothecin derivatives (irinotecan and topotecan) are topoisomerase I inhibitors. Both topoisomerase I and II mediate DNA strand breakage and resealing to permit replication, recombination, repair and transcription. The camptothecins bind to and stabilize the normally transient DNA-topoisomerase I cleavable complex, leading to an irreversible double-strand DNA break and cell death. They are S phase specific drugs. (Chabner. *et al*. 2011and Chu. *et al.* 2007)

Glycosmis parva

Glycosmis parva Criab is a plant in the genus *Glycosmis* in the Rutaceae family. It is widely distributes in Thailand with the local names Som-chuen and Prayon-Kluean. It is an evergreen shrub; leaves alternate, 1-5 foliate; flower usually small, auxiliary panicles, calyx 4 or 5 partial imbricate; petals 4-5 imbricate, stamen 8-10 free, filaments dilated below. Ovary 2-5 celled, the style very short, not jointed ovule 1 in each cell; fruits globose, fleshy, berry; seed 1-3 oblong, testa membranous. Some plants in the genus *Glycosmis* have been used as Thai traditional medicines for treatments of abscess, scabies, and snakebite (Chansriniyom. *et al*. 2009 and Hofer. *et al*. 1998)

Acridone alkaloids and sulfur containing propanamides are two main compound groups found rich in branches and leaves of plants in the genus *Glycosmis*. It has been identified that the ethyl acetate extract from branches of *G. parva* contain mainly acridone alkaloids (Chansriniyom. *et al*. 2009). These alkaloids are glycosparvarine, [*N*-methylatalaphylline, *N*-methylcyclo-atalaphylline-A, glycofolinine, and citramine. These leaves also contain 3 limonoids which are limonin, limonexic acid and isolimonexic acid. The ethyl acetate extract from the leaves of this plant are rich in sulfur-containing propanamides which are *S*-deoxydihydroglyparvin, *S*-deoxytetrahydroglyparvin, glyparvin-A, dihydroglyparvin. They also contain an acridone alkaloid named arborinine. (Chansriniyom. *et al*. 2009).

Acridone alkaloids have main molecular structure of two benzene rings fused together with nitrogen atom at 10^{th} position and a keto group at 9^{th} position. They exhibit a wide range of biological activities including antiviral, antimalarial and anticancer activities

(Giridhar. *et al*. 2010). Glycosparvarine and glycofolinine which are found in the branches of *G. parva* had moderate antiviral activity against herpes simplex viruses (HSV-1 and HSV-2) (Chansriniyom. *et al*. 2009). Arborinine which is identified in the leaves of *G. parva* had cytotoxic activity against some cancer cell lines including HeLa, MCF7 and A431 (Rethy. *et al*. 2007). The ethyl acetate extract from the branches of this plants inhibited lipopolysaccharide-stimulated macrophage J774A.1 cells. It inhibited nitric oxide production and the expression of inducible nitric oxide synthase (iNOS), cyclo-oxygenase 2 (COX 2), and pro-inflammatory cytokines (interleukin-1, interleukin-6 and tumor necrosis factor-α) (Chumseng S. 2009).

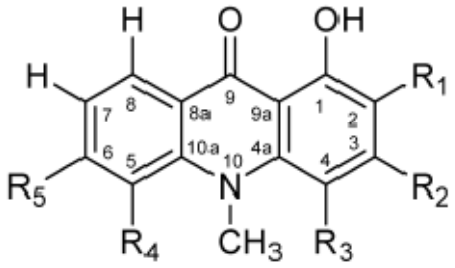


Figure 15: Acridone alkaloid structure (Chansriniyom. *et al*. 2009)

There are very few reports about pharmacological activities of sulfur-containing propanamides. They demonstrated antifungal activity (Greger. *et al*. 1993). *S*-deoxydihydroglyparvin from the leaves of *G. parva* exhibited anti-HSV activity (Chansriniyom. *et al*. 2009). The ethyl acetate extract from the leaves of *G. parva* which are rich in sulfur containing propanamides also inhibited LPS-stimulated macrophage J774A.1 cells similar to the extract from the branches of this plant as mentioned above (Chumseng S. 2009).

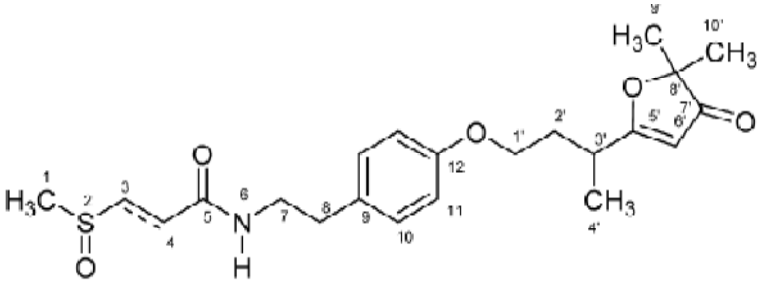


Figure 16: Sulfur-containing propanamide structure (Chansriniyom. *et al*. 2009)

CHAPTER III
MATERIAL AND METHOD

1. Material

1.1 Extracts of *Glycosmis parva*

The hexane, ethyl acetate, butanol and water extracts from the leaves of *Glycosmis parva* were prepared and identified by Associate Professor Dr. Nijsiri Ruangrungsi and Dr. Chaisak Chansriniyom, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand. All solvents were removed by evaporation and the extracts in solid form were stored at -20^{o}C. The TLC fingerprints of these extracts are in the Appendix A-1. The abbreviations of the solvent extracts were in the followings;

Solvent extracts	Abbreviations
Hexane extract	GPH
Ethyl acetate extract	GPE
Butanol extract	GPB
Water extract	GPW

1.2 Cells

- Human peripheral blood mononuclear cells (Human PBMCs)

Human PBMCs were prepared from heparinized blood from healthy male blood donors, age 25-30 years old, at the National Blood Bank, Thai Red Cross Society, with the informed consent. This study was approved by the Human Research Ethics Committee from the Faculty of Medicine, Chulalongkorn University with IRB No. 098/53. The cells were isolated from the whole blood by ficoll gradient centrifugation. They were maintained in complete RPMI 1640 medium contain 10% (v/v) fetal bovine serum (FBS), 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 obtained from the American Type Cell Culture (ATCC) (Rockville, MD). The cells were maintained in complete RPMI 1640 medium at 37 ^{o}C in humidified atmosphere of 5% CO_2.

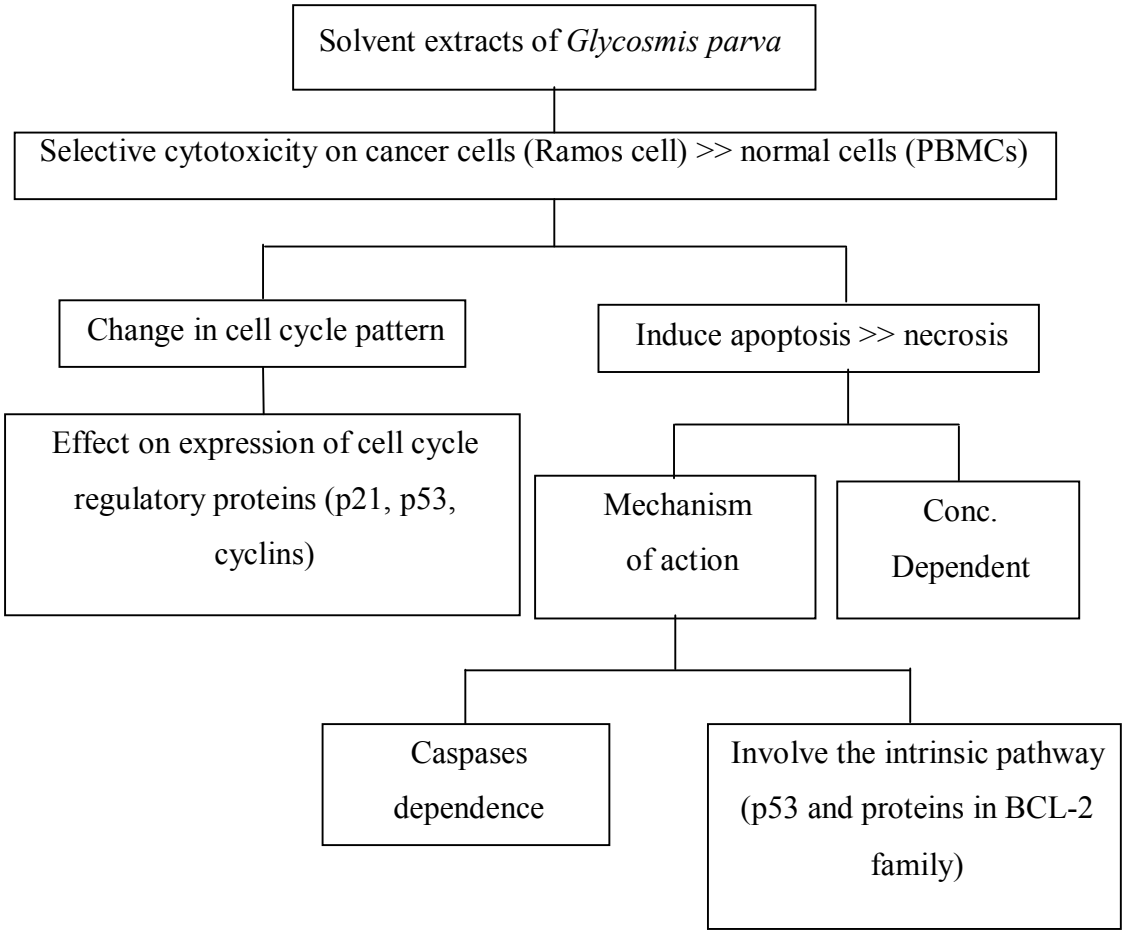
1.3 Equipments and Instruments

The following instruments were used in this study; analytical balance (GMPH, Satorius, Germany and UMT2, Mettler Toledo, Switzerland), autoclave (Hirayama, Japan), autopipette (Gilson, USA), biohazard laminar flow hood (Science, Germany and Labconco, USA), centrifuge (Hettich, USA and Eppendrof, Germany), ELISA microplate reader (Labsystems Multiskan, USA), fluorescence flow cytometer (Coulter, USA), gel electrophoresis system (Thermo, USA), gel documentation (Quantum ST4, Germany) , incubator (Thermo, USA), light microscope (Nikon, Japan), 24 and 96 well plates (Corning, USA), PCR thermal cycler (Eppendorf, Germany), PH meter (Mettler Tuledo, Switzerland), refrigerator 4 ^{o}C and -20 ^{o}C (Sanyo, Japan), Tissue culture flasks (Corning, USA) and vortex mixer (Scientific Industries, USA)

1.4 Reagents

The following reagents and reagents kits were used in this study; annexin V-FITC apoptosis detection kit (Santa Cruz Biotechnology, USA), caspase inhibitor Z-VAD-FMK (Promega- USA), fetal bovine serum (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, Malasia), absolute ethanol (Merck, Germany), TRIzol Reagent (Invitrogen, USA) and dietyl pyrocarbonate (DEPC) (Molekula, UK)

Conceptual framework



2. Methods

2.1 Preparation of the stock solutions of *G. parva*

All extracts were dissolved in 100% DMSO at 50 mg/ml as the stock solutions. These solutions were stored at -20^{o}C until use. They were diluted with sterile double-distilled water to 2% DMSO solutions before treating cells at 1:10 ratio. These made the final required concentrations of the extracts in medium containing constant 0.2% DMSO.

2.2 Preparation of human peripheral blood mononuclear cells (PBMCs)

Human PBMCs were isolated from heparinized blood by ficall (Histoplaque-1077) density gradient centrifugation as in following procedure:

1. Equally mix each blood sample with RPMI 1640 medium containing 1µl/ml heparin.
2. Slowly layer 9 ml of diluted blood sample on 5 ml ficoll solution in a 15 ml sterile polypropylene tube.
3. Centrifuge the tube at 3,200 rpm for 30 min at room temperature.
4. Carefully remove the top layer of solution without disturbing interface.
5. Collect cells at the interface in to a new 15 ml sterile polypropylene tube.
6. Wash the cells twice with 12.5 ml RPMI medium (plus 2 µl/ml heparin) by centrifugation at 1,200 rpm for 10 min at room temperature.
7. Discard the supernatant and resuspend the pellet with 5 ml completed RPMI medium.
8. Determine viable cells by staining with 0.4% trypan blue dye solution at the ratio 1:1 and count the number of the cells on hemocytometer. The cells were adjusted to required density with the completed RPMI 1640 medium.

   $1x10^{6}$ cells/ml PBMCs at more than 95% viability were used in this study.

2.3 Determination of cytotoxic activities of the *G. parva* extracts

2.3.1 Selection of the extracts exhibiting cytotoxic activities against Ramos cells

All solvent extracts from the leaves of *G. parva* were preliminary screened for their cytotoxicity against Ramos cells by using resazurin assay. The assay was performed in triplication with 3 independent experiments (n=3).

1. Treat $1x10^6$ cells/ml Ramos cells with the solvent extracts at the concentration 25, 50 and 100 µg/ml for 24 hour at 37 ^oC. 0.2% DMSO and 20 µg/ml etoposide were used as the negative and positive controls, respectively.
2. Add resazurin to final concentration 5 µg/ml, further incubate for 6 hour at 37 ^oC.
3. Determine the production of resorufin product in viable cells by measuring OD at 570 and 600 nm with a microplate reader.
4. Calculate the percentage of cytoxicity of the extracts by the following equation;

$$\% \text{ Cytotoxicity} = \frac{[\Delta OD_{0.2\ \% DMSO} - \Delta OD_{sample}] \times 100}{\Delta OD_{0.2\ \% DMSO}}$$

Where Δ OD = Absorbance $_{570\ nm}$ - Absorbance $_{600\ nm}$

The extracts which had cytotoxicity against Ramos cells higher than 50% at 50 µg/ml were further investigated.

2.3.2 Determination of cytotoxic activities at IC50's of the extracts

The extracts which had cytotoxicity against Ramos cells higher than 50% at 50 µg/ml were assess for their cytotoxic activities at IC50's. The assay was performed in triplication of 3 independent experiments (n=3) in the following procedure.

1. Treat $1x10^6$ cells/ml Ramos cells or PBMCs with 5 concentrations of the extract for 24 h at 37 ^oC.
2. Add resazurin to final concentration 5 µg/ml further incubate for 6 hour at 37 ^oC.
3. Determine the production of resorufin product in viable cells by measuring OD at 570 and 600 nm with a microplate reader.
4. Calculate the percentage of toxicity of extracts.

5. Calculate the concentration of each extract which caused 50% cytotoxicity (IC50) from the percentage of toxicity after 24 h exposure.
6. Compare the cytotoxicity of the solvent extracts between on Ramos cells and on PBMCs.
7. Select the solvent extracts which had cytotoxicity on tumor cells higher than PBMCs for next studies.

2.4 Determination of apoptotic induction

Apoptotic induction activities of the solvent extracts which had cytotoxic activity against Ramos cell higher than PBMCs were evaluated at 2-3 optimum concentrations base on their IC50 values. Apoptotic cells were detected by using annexin V-FITC which specifically binds to exposed phosphatidylserine (PS) on the outer membrane of early apoptotic cells. The assay was performed in duplication of 3 independent experiments (n=3) as follow:

1. Treat $1x10^6$ cells/ml Ramos cells in 24 well plate with 2-3 concentrations of the solvent extract at 37 ^oC for 8 and 16 h. 0.2% DMSO was used as the solvent control, Twenty µg/ml etoposide and 37.5 µM citral were used as the positive control.
2. Collect the treated cells in each well to microcentrifuge 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 collect the cells by centrifugation at 12,000 rpm, 25 ^oC for 1 min.
4. Resuspend the cell pellet in 100 µl of the assay buffer and transferred in to flow cytometer tube.
5. Add 1 µl of 0.05 µg/ml PI and 0.5 µl of annexin V-FITC in each tube.
6. Incubate these tubes in the dark at room temperature for 15 min.
7. Subsequently add 400 µl of the assay buffer in to each tube and immediately analyze $1x10^4$ cells/ tube by fluorescence flow cytometer.
8. Assess type of the cells as in following; the annexin V-$FITC^-$/PI^- cells as viable cells, the annexin V-$FITC^+$ cells as apoptotic cells, the PI^+ cells as necrosis cells and the V-$FITC^+$/PI^+ as late apoptotic or secondary necrotic cells.

In this study, the solvent extracts which induced cells death mainly by apoptosis were further assessed in the next study.

2.5 Determination of apoptotic induction mechanism

The solvent extracts which induced cell death mainly by apoptosis were further investigated for their mechanisms of apoptotic induction. The dependency on caspase activation was evaluated by using a pan-caspase inhibitor, Z-VAD- FMK. The effect of the extract on the intrinsic pathway of apoptosis was evaluated by determining the mRNA expression of pro-apoptotic and anti apoptotic proteins in BCL-2 family.

2.5.1 Determination of the effect of the extract on caspase activation

1. In 24 well plate, pre- treat $1x10^6$ cells/ml Ramos cell with 50 μM Z-VAD-FMK for 1 h at 37 ^oC.
2. Treat the cells with 2-3 concentrations of the solvent extracts for 8 h at 37 ^oC
3. Detect types cell death by performing as in the 2^{nd} to 8^{th} steps of 2.4.

2.5.2 Determination of the effect of the extract on the expression of proteins in BCL-2 family

The mRNA expression of proteins involve in apoptosis including *p53* gene, pro-apoptotic BCL-2(BAK and BAX) and anti apoptotic BCL-2 (BCL-XL and BCL-2) genes was determined as in the following procedures;

1. Treat $1x10^6$ cells/ml Ramos cells with 2-3 concentrations of the solvent extracts for 8 h at 37 ^oC.
2. Transfer the treated cells into 15 ml centrifuge tubes and collect the cells by centrifugation at 1,200 rpm 25 ^oC for 10 min.
3. Lyses and homogenize the cells in 1 ml of TRIzol® reagent, transfer the lysate to 1.5 ml eppendorf tubes and incubate for 5 min at room temperature.
4. Add 0.2 ml chloroform, vigorously shake the tubes for 15 sec, and incubate at room temperature for 2-3 min.
5. Centrifuged at 12,000 rpm for 15 min at 4 ^oC, and transfer the aqueous phase to fresh eppendorf tubes.

6. Add 0.5 ml isopropyl alcohol, incubate at -20 ^{o}C for 1 h and centrifuge 12,000 rpm for 10 min at 4 ^{o}C.
7. Removed the supernatant and air dry the RNA pellet for 5-10 min.
8. Dissolve the pellet in DEPC- treated water and then incubate at 55-60 ^{o}C for 10 min.
9. Determine the concentration and protein contamination of total RNA by spectrophotometer at 260 and 280 nm. The total RNA samples should have optical density ratio; OD_{260}:OD_{280} > 1.8.
10. Stored the total RNA samples at -70^{o}C for complementary DNA (cDNA) production.
11. For cDNA production; mix 1 µl total RNA of each sample and 1 µl oligo dT15 primer in 0.2 ml PCR tube. Heat the tubes at 70^{o}C for 5 min and immediately chill on ice for 5 min. Prepare transcription mixture solution containing 25 mM $MgCl_2$, Mixed dNTP, ribonuclease inhibitor, and reverse transcriptase. Add 15 µl of mixture solution in to each tube. Generate cDNA in thermocycle machine by using the following conditions; 25^{o}C for 5 min, then 42^{o}C for 1 h and 30 min, and finally 70^{o}C for 15 min. Store the cDNA samples at -20^{o}C for using as the template to determine gene expression.
12. Determine mRNA expression of proteins in the BCL-2 family and p53 as follow; Carry out PCR in 50 µl reaction mixture containing PCR buffer, 0.5-1 µl cDNA, 1.5 mM $MgCl_2$, 0.2 mM mixed dNTP, 0.4 µM specific primers for interested gene (p53, BCL-2, BCL-XL, BAX and BAK) and 1 unit platinum® Taq DNA polymerase. Perform PCR by the following conditions; 94^{o}C for 2 min followed by 35 cycle of 30 sec denaturation at 94 ^{o}C, 30 sec annealing at appropriate melting temperature (Tm) of the primers, 1 min extension at 72^{o}C, and finally 10 min extension at 72^{o}C. Analyze the PCR products by electrophoresis in 1.5 % agarose gel at 100 V, stain the gel with ethidium bromide in 1xTBE buffer and determine their densities by gel documentation. Express the densities of the PCR products as % of internal control gene (GAPDH).

Table 1: Primers for RT-PCR and their annealing temperatures (Tm).

Gene	Primer sequences	Tm ^{o}C	PCR product (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'-TGG AGC TGC AGA GGA TGA TTG-3' Reverse: 5'-GAA GTT GCC GTC AGA AAA CAT G-3'	60	96
BCL-XL	Forward: 5'-CCA 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

2.6 Determination of the effect of the extract on the cell cycle

1. Treat 1x10^{6} cells/ml Ramos cells with 2-3 concentrations of the solvent extract in 24 well plate at 37^{o}C for 1 h.
2. Collect the treated cell and wash them twice by centrifugation at 1,200 rpm for 10 min at 25^{o}C.
3. Resuspend the cells in 1 ml fresh RPMI 1640 medium containing 10% FBS.
4. Incubate the cells for 48 h at 37^{o}C.
5. Transfer the cells in each well to a flow cytometer tube, collect the cells by centrifugation at 1,500 rpm 4^{o}C for 5 min, and wash them twice with 500 µl cold PBS.
6. Resuspend the cells in 150 µl cold PBS.
7. Fix the cells by slowly adding 350µl absolute ethanol with continuously shaking.

8. Incubate the cell for 15 min at -20^{o}C, and then collect them by centrifugation at 1,500 rpm at 4^{o}C for 5 min.
9. Wash the cells twice with 500 µl cold PBS and collect them by centrifugation at 1,500 rpm at 4^{o}C for 5 min.
10. Resuspend the cells in 500 µl assay buffer, add 4 mg/ml RNase 5 µl, and incubate for 30 min at room temperature.
11. Add 5 µl of 0.05 µg/ml PI and incubate in the dark for 15 min at room temperature.
12. Analyze the cell cycle pattern of 1x10^{6} cells/sample by fluorescence flow cytometer.

2.7 Determination of the effect of the extracts on the mRNA expression of regulatory proteins in the cell cycle

The extracts which changed Ramos cell cycle pattern were future investigated for their effect on the mRNA expression of regulatory proteins in the cell cycle, including p21, p53 and various cyclins (cyclin A, cyclin B1, cyclin D1 and cyclin E) as in the following procedure;

1. Treat Ramos cells as in the 2nd-11th steps of 2.6.
2. Collect the treated cells for isolating total RNA, producing cDNA, and generating PCR products of p21, p53, cyclin A, cyclin B1, cyclin D1 and cyclin E genes by using gene-specific primers (Table 2) as in the 2.5.2.

Table 2: Primers for RT-PCR and their annealing temperature

Gene	Primer sequences	Tm ^{o}C	PCR product (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
P21	Forward: 5'-GCG ATG GAA CTT CGA CTT TGT-3' Reverse: 5'-GGG CTT CCT CTT GGA GAA GAT-3'	54	352
Cyclin A	Forward: 5'-GTC ACC ACA TAC TAT GGA CAT G-3' Reverse: 5'-AAG TTT TCC TCT CAG CAC TGA C-3'	53	300
Cyclin B1	Forward: 5'-CGG GAA GTC ACT GGA AAC AT-3' Reverse: 5'-AAA CAT GGC AGT GAC ACC AA-3'	55	177
Cyclin D1	Forward: 5'-CTG GCC ATG AAC TAC CTG GA-3' Reverse: 5'-GTC ACA CTT GAT CAC TCT GG-3'	54	482
Cyclin E	Forward: 5'-AAT AGA GAG GAA GTC TGG-3' Reverse: 5'-AGA TAT GAC ACC TGC ATG-3'	55	440

2.8 Statistical analysis

Data were presented as mean with standard error of mean (mean $\pm$ S.E.). Statistical comparisons were made by one-way ANOVA followed by Turkey's post hoc test. All statistical analysis was performed according to the statistic program SPSS version 17. Any p-value<0.05 was considered statistically significance.

CHAPTER IV

RESULTS

1. Cytotoxic activity of *G. parva* leaves extracts on human B-lymphoma cells

Hexane, ethyl acetate, butanol and water extracts from the leaves of *G. parva* were screened for their cytotoxic effects against Ramos cells by rezasurin assay. The cells were treated with 25, 50 and 100 µg/ml of these extracts for 24 h. The ethyl acetate extract demonstrated cytotoxic effect against Ramos cells at all concentrations used in the screening (Fig. 17). The hexane extract had some cytotoxicity to these cancer cells but its effect is less than 50% at all concentrations. The other solvent extracts, butanol and water extracts, had no cytotoxicity against the cells. The ethyl acetate extract was chosen to further investigate for its IC50 of cytotoxicity, its apoptotic effect and its effect on the cell cycle pattern of Ramos cells.

The half maximal inhibitor concentration (IC50) of cytotoxicity of the ethyl acetate extract against Ramos cells was determined. The cells were treated with 3.125-50 µg/ml of the extract for 24 h. The extract induced Ramos cell death in a concentration dependent manner with its IC50 value at 15.68 µg/ml (Fig. 18). This IC50 was used for choosing 3 concentrations of the extract to use in the next experiments.

The cytotoxic effect of the extract against Ramos cells of GPE was compared to its harmful effect on normal human PBMCs. Both cell types were treated with 3.125- 50 µg/ml of the extract for 24 h. The extract had cytotoxic effect against Ramos cells higher than against normal cells (Fig. 18).

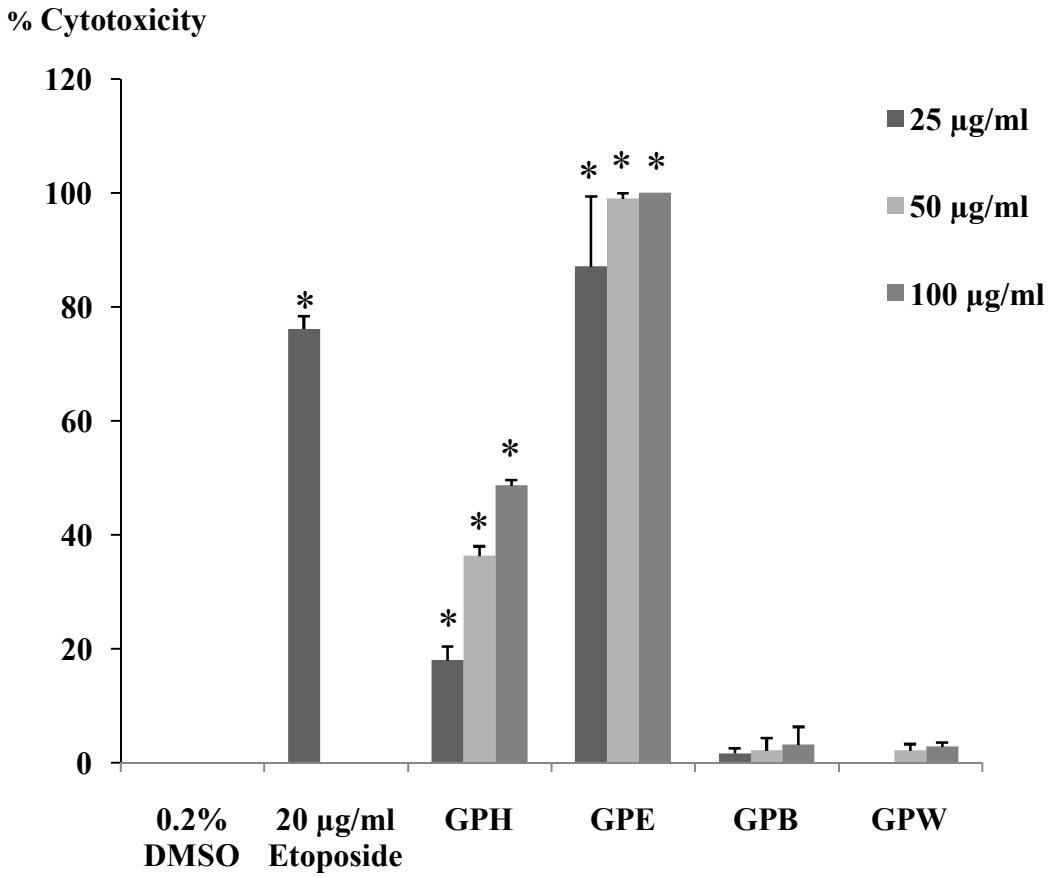


Figure 17: The cytotoxic effects of hexane, ethyl acetate, butanol and water extracts (GPH, GPE, GPB and GPW) from the leaves of *G.parva* on Ramos cells by rezasurin assay. The cells were treated with 25, 50 and 100 µg/ml of the extracts for 24 h. The data are expressed as mean $\pm$ S.E. of three independent experiments (n=3).

* $p < 0.05$ denotes statistically significant difference from the solvent control (0.2% DMSO).

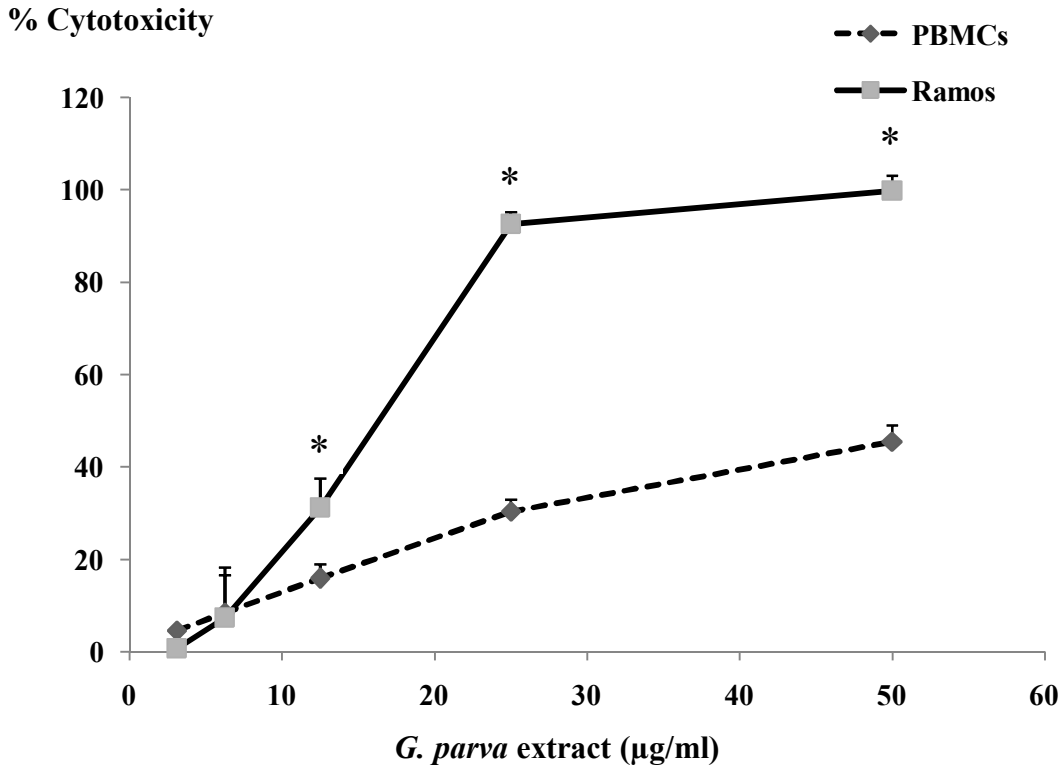


Figure 18: The comparison of the cytotoxic effect of the ethyl acetate extract from the leaves of *G. parva* between on Ramos cells and on human PBMCs by rezasurin assay. Both cell types were treated with 3.125-50 µg/ml of the extract for 24 h. The data are expressed as mean ± S.E. of the independent experiments (n=3)

* $p<0.05$ denotes statistically significance of difference when compared to PMBCs at the same concentration of extract.

2. Apoptotic effect of the extract on Ramos cells

The apoptotic effect of the ethyl acetate extract from the leaves of *G. parva* on Ramos cells was evaluated. The cells were treated with 7.5, 15 and 30 µg/ml of the extract for 8 and 16 h at 37^{o}C. The patterns of cell death were determined by staining with annexin V-FITC/PI and detecting with fluorescence flow cytometer. These patterns were identified as follow; annexin V-FITC^{-}/PI^{-} cells as viable cells, PI^{+} as necrosis cells, annexin V-FITC^{+} as apoptotic cells, and annexin V-FITC^{+}/PI^{+} as necrosis plus late apoptotic cells (Fig. 19). Etoposide which can induce cancer cell death by apoptosis and citral which has been reported to induce cell death mainly by apoptosis were used as positive control in this experiment.

The ethyl acetate induced total cell death in a concentration- and time-dependent manner after 8 and 16 h exposures (Table 1). It induced cell death mainly by apoptosis after 8 h exposure (Fig. 20 A.). This effect was similar to the effect of citral. At 30 µg/ml, it caused 55.80$\pm$1.09% total cell death and 45.73$\pm$1.22% apoptosis. This apoptosis is 81.95% of total cell death. However, the cell death induced by the extract at 16 h had changes in the pattern from mainly apoptosis to increase in late apoptosis and necrosis (Fig. 20 B.). The molecular effect of the extract on apoptosis at 8 h exposure was further evaluated.

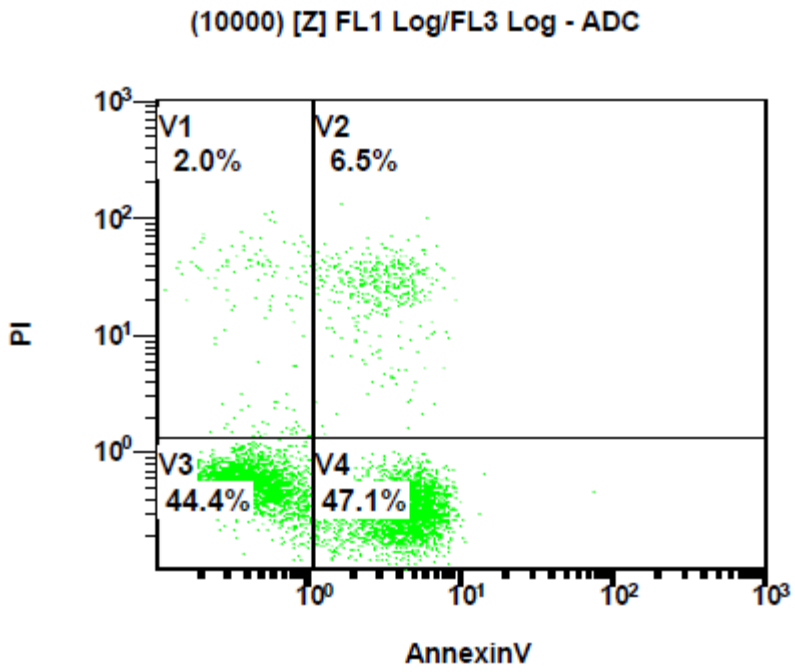


Figure 19: A representative patterns of Ramos cell death induced by the ethyl acetate extract from leaves of *G. parva*. The cells were treated with 30 µg/ml of the extract for 8 h. The types of cell death were determined by staining the treated cell with annexin V-PITC/PI and detecting with fluorescence flow cytometer. The patterns of cells death were assessed as in the followings; V1 or PI^+ cells as necrosis cells, V2 or annexin $V\text{-}FITC^+/PI^+$ cells as necrotic plus late apoptosis cells, V3 or $V\text{-}FITC^-/PI^-$ cells as viable cells, and V4 or annexin $V\text{-}FITC^+$ cells as apoptotic cells.

Table 3: The apoptotic effect of the ethyl acetate extract from leaves of *G. parva* on Ramos cells. The cells were treated with 7.5, 15 and 30 µg/ml of the extract for 8 and 16 h. The patterns of cells death were determined by staining with annexin V-FITC/PI and detecting with fluorescence flow cytometer. The data are expressed as mean ± S.E of three independents experiments (n=3).

Treatment	% Viable	Death cell			% total death
		Apoptosis	PI positive	Double positive	
8 hr					
0.2% DMSO	92.05 ± 0.15	1.65 ± 0.64	0.33 ± 0.03	5.52 ± 0.47	8.08 ± 0.25
Etoposide 10 µg/ml	65.43 ± 6.40*	15.68 ± 3.15	12.87 ± 6.05	5.98 ± 3.02	34.57 ± 6.40*
Citral 37.5	52.62 ± 8.77*	36.27 ± 8.24*	7.03 ± 4.92	11.18 ± 2.41*	47.38 ± 11.07*
G6 7.5 µg/ml	85.17 ± 2.71	8.17 ± 2.6	1.43 ± 0.13	5.18 ± 1.23	14.83 ± 2.71
G6 15 µg/ml	77.92 ± 3.47	15.57 ± 3.61	1.00 ± 0.13	5.53 ± 1.19*	21.98 ± 3.42
G6 30 µg/ml	44.17 ± 1.11*	45.73 ± 1.22*	3.80 ± 2.35	4.20 ± 1.21	55.82 ± 1.09*
16 hr					
0.2% DMSO	93.51 ± 1.60	1.67 ± 0.40	0.58 ± 0.07	4.18 ± 1.26	6.49 ± 1.60
Etoposide 10 µg/ml	66.18 ± 5.48*	8.12 ± 0.39	10.80 ± 4.75	15.30 ± 1.32*	33.82 ± 5.48*
Citral 37.5	28.02 ± 8.62*	36.80 ± 5.79*	30.83 ± 2.60*	5.65 ± 1.27	71.98 ± 8.62*
G6 7.5 µg/ml	80.12 ± 2.99	7.80 ± 1.84	1.46 ± 0.41	10.68 ± 1.52	19.88 ± 2.90
G6 15 µg/ml	55.73 ± 3.80*	21.97 ± 1.51*	2.66 ± 1.11	19.60 ± 1.65*	44.27 ± 3.80*
G6 30 µg/ml	13.62 ± 4.41*	34.62 ± 8.14*	43.28 ± 4.31*	5.65 ± 2.51	86.38 ± 4.41*

* $p < 0.05$ denotes statistically significant difference from the solvent control (0.2 % DMSO).

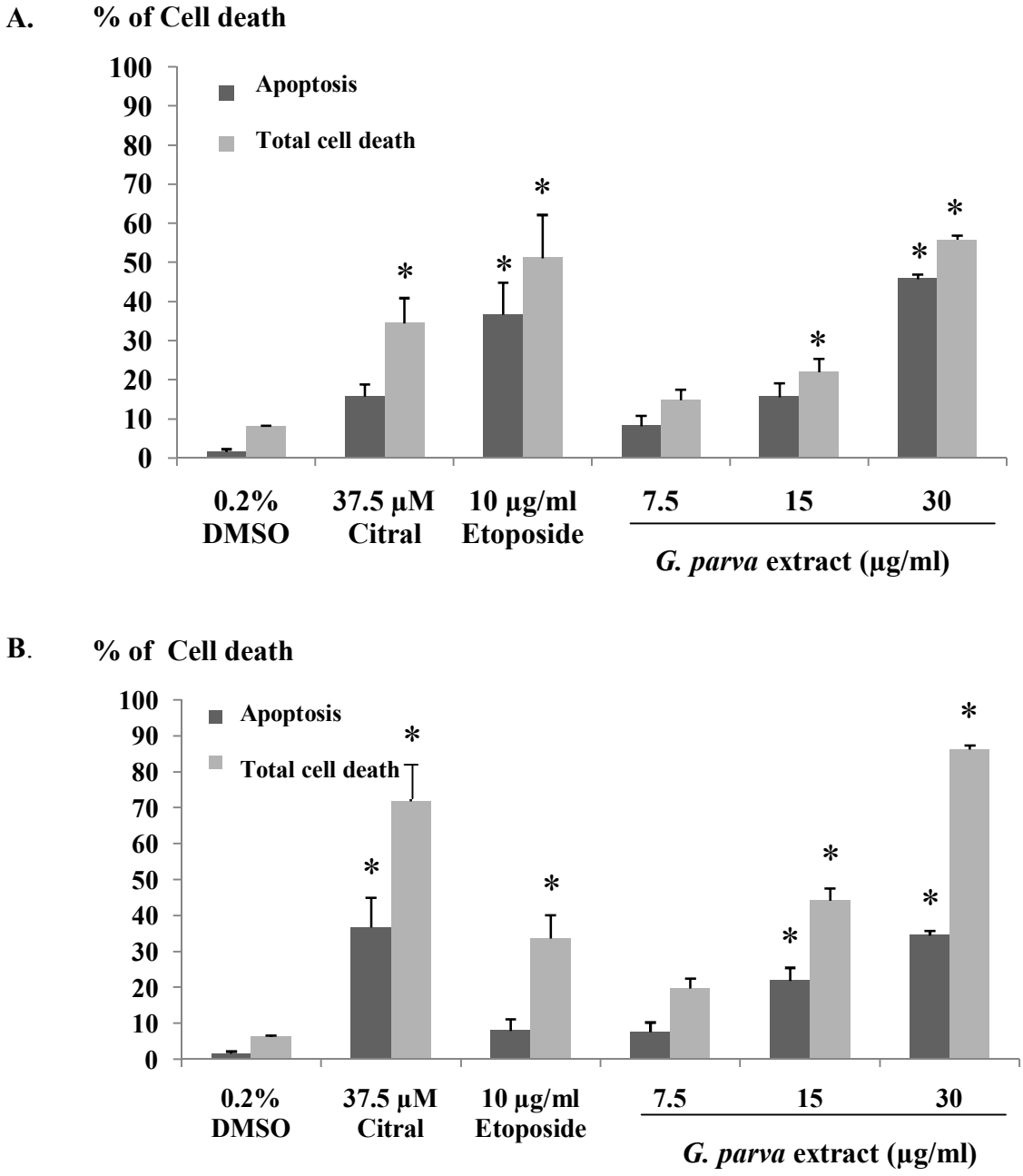


Figure 20: The apoptotic effect of the ethyl acetate extract from the leaves of *G. parva* on Ramos cells. The cells were treated with 7.5, 15 and 30 µg/ml of the extract for 8 (A) and 16 h (B). The patterns of cells death were determined by staining with annexin V-FITC/PI and detecting with fluorescence flow cytometer. The data are expressed as mean ± S.E of three independents experiments (n=3).

*p< 0.05 denotes statistically significant difference from the solvent control (0.2 % DMSO).

3. The apoptotic effect of the extract at the molecular level.

3.1 Molecular effect on the intrinsic pathway of apoptosis

Mitochondria play important role in the intrinsic pathway of apoptosis. Several proteins in the BCL-2 family are regulatory proteins in this pathway. They are divided into anti apoptotic BCL-2 proteins (e.g., BCL-2, BCL-XL) which inhibit apoptosis and pro-apoptotic BCL-2 proteins (e.g., BAX, BAK) which promote apoptosis. The expressions of some of these proteins are regulated by p53 transcription factor. Effects of the ethyl acetate extract on the expression of these proteins in the BCL-2 family and p53 were evaluated. Ramos cells were treated with 7.5, 15 and 30 µg/ml of the extract for 8 h. The mRNA expression of p53, pro apoptotic BCL-2 proteins (e.g., BAX, BAK) and anti apoptotic BCL-2 proteins (e.g., BCL-2, BCL-XL) in the treated cells was determined by RT-PCR.

The apoptotic effect of the extract correlates to its effect on the mRNA expression of proteins in the BCL-2 family. The extract at 30 µg/ml significantly decreased the mRNA expression of anti-apoptotic protein BCL-XL and increased the expression of pro-apoptotic protein BAK (Fig. 21). It did not change the expression of the other proteins in this family as well as p53.

3.2 Caspase dependence of the apoptotic effect of the extract.

The cysteine proteases of the caspase family play key roles in apoptosis. These enzymes are activated in the cascade way during apoptosis process both by the intrinsic and the extrinsic pathways. However, apoptosis can also be induced by caspase-independent mechanisms. The roles of caspases on the apoptotic effect of the ethyl acetate extract were investigated. Ramos cell were pretreated with a pan caspase inhibitor Z-VAD-FMK for 1 h and then treated with 15 and 30 µg/ml of the ethyl acetate extract for 8 h. The apoptotic effect of the extract was markedly decreased by Z-VAD-FMK. The inhibitor decreased the percentage of apoptotic cells from 7.35$\pm$ 0.85 % and 41.30 $\pm$ 0.30 % to 2.85$\pm$ 0.05 % and 4.05 $\pm$ 0.15 % when Ramos cells were treated with 15 and 30 µg/ml of the extract, respectively (Fig. 22). These results indicate that the apoptosis triggered by the ethyl acetate extract was medicated mainly by caspase activation.

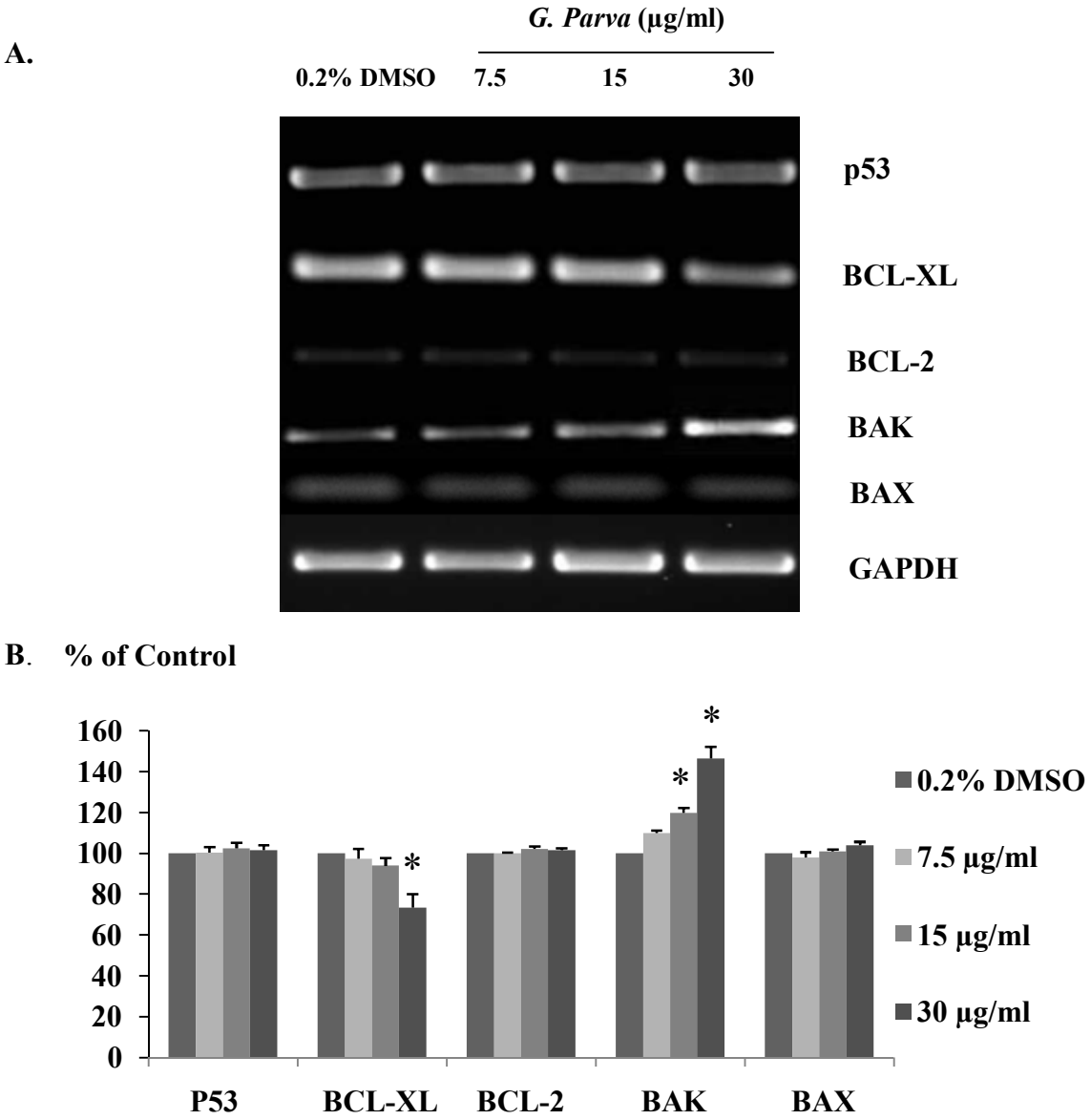


Figure 21: The effect of the ethyl acetate extract from the leaves of *G. parva* on the mRNA expression of p53 and proteins in the BCL-2 family. Ramos cells were treated with 7.5, 15 and 30 µg/ml of the extract for 8 h. The mRNA expression of p53 and proteins in the BCL-2 family was determined by RT-PCR total RNA from the treated cells were reverse transcribed and amplified with the specific primers by RT-PCR. The PCR products were identified by 1.5% agarose gel electrophoresis and analyzed by gel documentation. (A) A representative of PCR products of p53 and proteins in the BCL-2 family. (B) Densitometric analysis the PCR products relative to GAPDH represented as % of control. The data expressed as mean $\pm$ S.E. of three independent experiments (n=3). * $p < 0.05$ denotes statistically significant difference from the solvent control (0.2% DMSO).

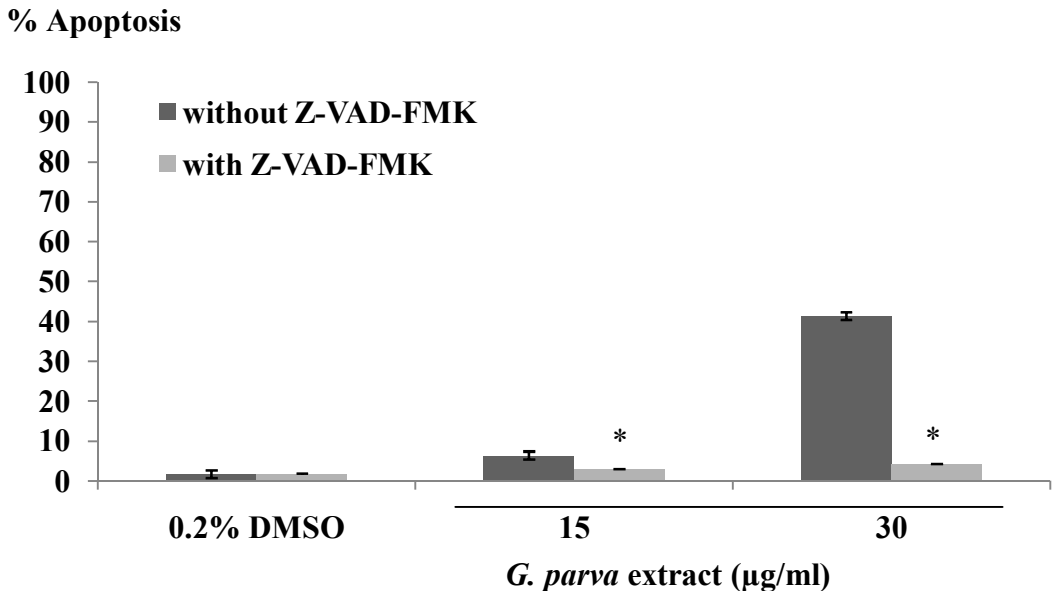


Figure 22: Caspase dependence of the apoptotic effect of the ethyl acetate extract from leaves of *G. parva* on Ramos cells. The cells were pretreated with 50 µM of Z-VAD-FMK for 1 h and then treated with 15 and 30 µg/ml of the extract for 8 h exposure. The percentage of apoptotic cells were determined by annexin V-FITC/PI staining assay using fluorescence flow cytometer. The data were expressed as mean $\pm$ S.E. of two independence experiments (n=2)

*P < 0.05 denotes statistically significant difference from without Z-VAD-FMK conditions.

4. Effect of the extract on the cell cycle and its regulatory proteins of Ramos cells.

This study evaluated whether the extract acts as a cell cycle specific cytotoxic agent on Ramos cells or not. The cells were treated with 7.5, 15 and 30 µg/ml of the extract for 1h and then incubated further in fresh RPMI 1640 medium without the extract for 48 h. The cell cycle patterns of the treated cells were determined by ethanol fixing, staining with PI, and DNA content detecting by fluorescence flow cytometer. The normal patterns of Ramos cells in the cell cycle were presented in the Fig. 23 (A). Etoposide which arrests the cell cycle at late S to G2 phase and causes cell accumulation at G2/M phase was used as the positive control (Fig. 23 B). The extract at the concentrations of 15 and 30 µg/ml changed the pattern of the cell cycle of Ramos cells. It cause cells accumulated at late G1-S phases (Fig. 23 D and E). It is postulated that this extract arrests the cell cycle of Ramos cells at G1 and S phase. This postulation was proven by determining the mRNA expression of regulatory proteins in the cell cycle. These proteins are cyclins (cyclin A, cyclin B1, cyclin D1 and Cyclin E), cyclin dependent kinase inhibitor (p21), as well as p53 in the extract-treated Ramos cells. The extract at 15 and 30 µg/ml significantly increased the mRNA expression of p21 which as Cdk inhibitor at G1/S transition. At 30 µg/ml, it significantly decreased the expression of cyclin D1, and cyclin E. These cyclins bind to their specific cyclin-dependent kinases (Cdks), leading to Cdk activation and cell cycle progress. Cyclin D1 binds to and activates Cdk4 and Cdk6 during early G1 phase. Cyclin E activates Cdk2 activity during G1 to S phase. The extract had no effect to the mRNA expression of transcription factor p53 which suppressor and the expression of cyclin A which activates Cdk2 activity during S phase and the G2 to M phase transition of the cell cycle.

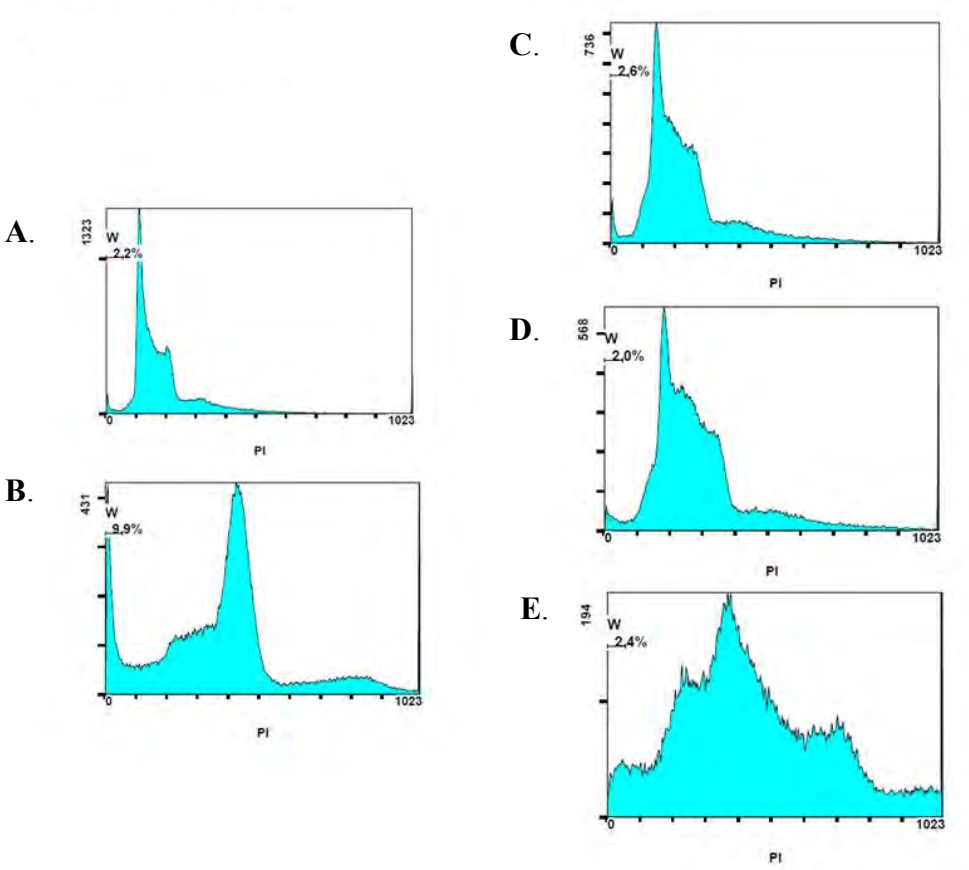


Figure 23: Representative cell cycle patterns of Ramos cells treated with ethyl acetate extract from leaves of *G. parva*. The cells were treated with 7.5, 15 and 30 µg/ml of GPE for 1 h exposure, then washed and incubated in fresh medium for 48 h. the treated cells were fixed, RNase treated, and stained with PI. The cell cycle patterns were determined by flow fluorescence cytometer . (A) 0.2% DMSO; (B) 1 µg/ml etoposide; (C, D, E) 7.5, 15 and 30 µg/ml of the ethyl acetate extract.

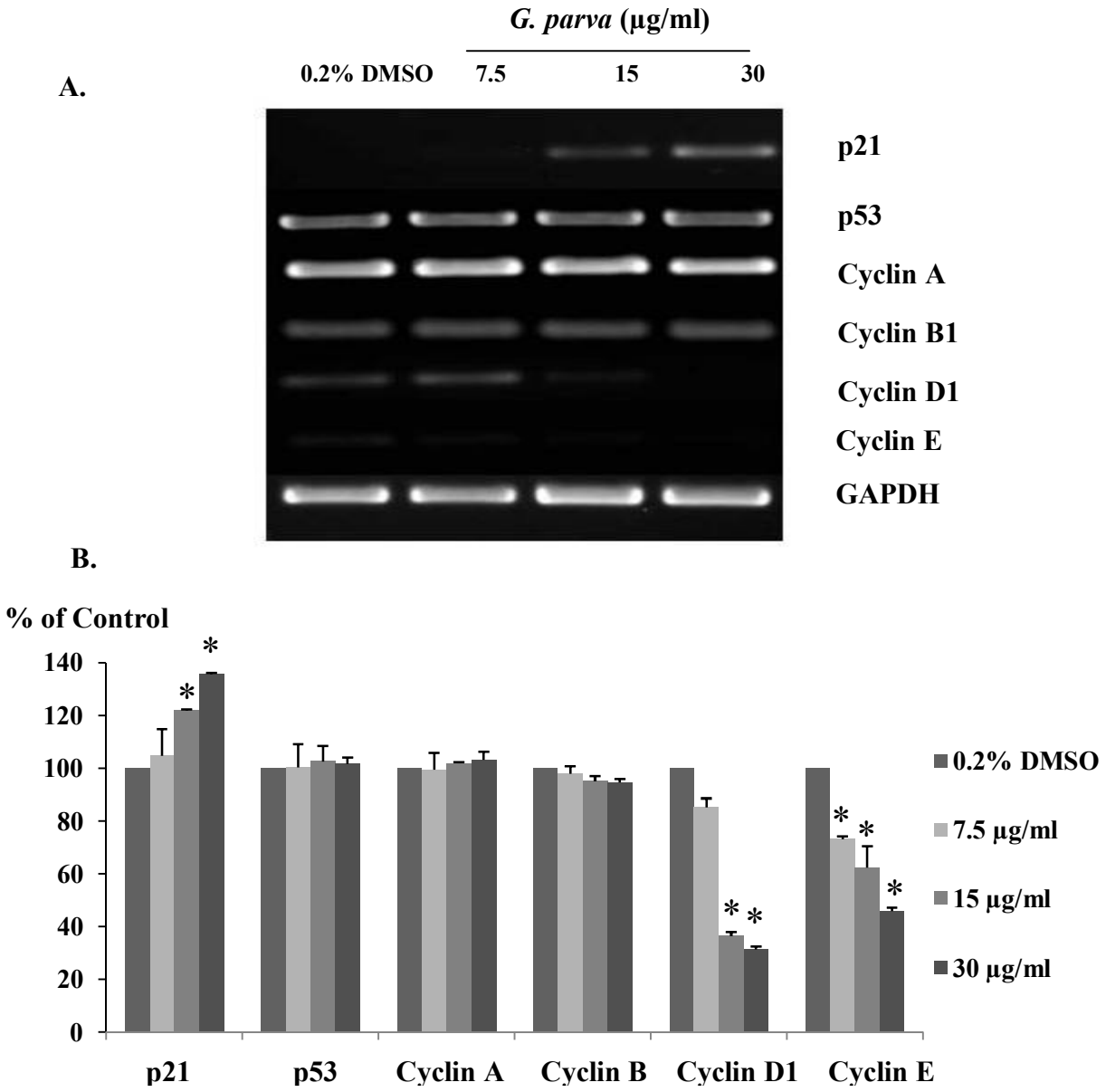


Figure 24: The effects of the ethyl acetate extract from the leaves of *G. parva* on the mRNA expression of p21, p53 and cyclins in Ramos cells. The cells were treated with 7.5, 15 and 30 µg/ml for 1 h exposure. The total RNA from the treated cells were reverse transcribed and amplified with the specific primers by RT-PCR. The PCR products were run on 1.5% agarose gell and analyzed by gel documentation. (A) A representative of PCR products of p21, p53 and cyclins from the treated cells. (B) Densitometric analysis the PCR products relative to GAPDH represented as % of control. The data expressed as mean $\pm$ S.E. of three independent experiments (n=3)

*$p < 0.05$ denotes statistically significant difference from 0.2% DMSO.

CHAPTER V
DISCUSSION AND CONCLUSION

Evaluation of new pharmacological agents from natural sources is still one of the strategies of drug discovery. In oncology, several anticancer drugs are derived from medicinal plants such as vinca alkaloids (vincristine and vinbrastine), taxane (paclitaxel). Several herbal medicines are prescribed as anticancer remedies worldwide without purifying to single compounds. Anticancer drug discovery from plants has been reported such as *Scutellaria baicalensis* which exhibited effective anticancer activity on head and neck squamous cell carcinoma both in vitro and in vivo (Xiaolu. *et al*. 2004)

It has been known that plants in the genus *Glycosmis* haves acridone alkaloids and sulfur-containing propanamides as their major active compounds in their branches and leaves (Vajrodaya. *et al*. 1998). Acridone alkaloids are rich in the branches of these plants. Several pharmacological activities of these alkaloids have been reported including antiviral activity (Yamamoto. *et al*. 1989 and Fujiwara. *et al*. 1999), antimalarial activity, anticancer and chemopreventive activities (Kawaii. *et al*. 1999, Kawaii. *et al*. 1999 and Itoigawa. *et al*. 2003). There are very few reports about pharmacological properties of sulfur-containing propanamides which are rich in the leaves of plants in this genus. Their mainly reported properties are antifungal activities (Greger. *et al*. 1993). This study evaluated the potential anticancer activities of active compounds from the leaves of *Glycosmis parva*. Activities of the solvent extracts, including the hexane, ethyl acetate, butanol and water extracts, from the leaves of this plant were investigated on human B lymphoma or Ramos cells. These extracts were screened for their cytotoxicities on Ramos cells by rezasurin assay. Only the ethyl acetate demonstrated profound cytotoxicity against these cancer cells in a concentration dependent manner with it IC50 at 15.68 μg/ml. This extract has been identified to contain 4 sulfur-containing propanamides (*S*-deoxydihydroglyparvin, *S*-deoxytetrahydroglyparvin, glyparvin-A and dihydroglyparvin) and one acridone alkaloid (arborinine). It inhibited lipopolysaccharide-induced macrophage J774A.1 cell activation by decreasing nitric oxide production and reducing the expression of pro-inflammatory cytokines (TNF-α, IL-1, and IL-6) as well as cyclo-oxygenase 2 (COX 2) (Chumseng S. 2009). This is the first study that the ethyl acetate of the leaves from *G. parva* may contain active compounds with cytotoxic activities against cancer cells. This study also

demonstrated that the ethyl acetate extract had cytotoxic effect on Ramos cells higher than on normal human PBMCs.

Many anticancer drugs induce cell death by apoptosis. Apoptosis is the program cell death which normally occurs in the human body under physiological condition for eliminating aging cells, unwanted cells or harmful cells. This type of cell death is an active process which occurs in order for changing cells to apoptotic cells and apoptotic bodies. These bodies then are recognized and eliminated by phagocytes in the body without harm to neighbor cells. Searching compounds able to induce apoptosis in cancer cells has attracted major attention. The ethyl acetate extract from the leaves of *G. parva* induced cell death mainly by apoptosis. It induced apoptosis at the higher percentage of total cell death than a well known anticancer drug etoposide which was used as the positive control in this study.

Apoptosis is in general mediated by caspase activation. Caspases are protease enzymes that play key roles in the cascade way to induce program cell death by cleaving many cellular proteins leading to DNA fragmentation (Cho. *et al*. 2002). Apoptotic effect of the ethyl acetate extract was confirmed by pretreated Ramos cells with a pan-caspase inhibitor Z-VAD-FMK which inhibits several caspases involve in apoptosis. This inhibitor markedly inhibited apoptotic effect of the extract. This result also implies that the apoptotic effect of the extract is mainly dependent on caspase activation. It is known that several chemotherapeutic agents activate caspase and induce apoptosis by the intrinsic or mitochondria pathway (Codelia. *et al*. 2008).

It is accepted that apoptosis is an important mechanism in cancer chemoprevention and chemotherapy by required anticancer agents. Mitochondria play a key role in the intrinsic pathway of apoptosis. The majority of anticancer agents induce cancer cell apoptosis by disrupting mitochondrial membrane potential leading to release of apoptogenic factors (cytochrome c, Smac/DIABLO, Omi/HtrA2, AIF and endonuclease G. These factors trigger cell death by initiating caspase activation or by acting as caspase-independent death effectors (Kiechle. *et al*. 2002). Proteins in the BCL-2 family which are divided into anti-apoptotic (e.g., BCL-2, BCL-XL) and pro-apoptotic (e.g., BAK and BAX) are main regulators of mitochondrial membrane permeability and subsequent release of the apoptotic factors. In healthy cells, BAK and BAX are suppressed their activity by binding to anti-apoptotic proteins. It has been reported that many anti-cancer agents induce

cell death by apoptosis correlating to the decrease or increase in the expression of anti-apoptotic or pro-apoptotic proteins in the BCL-2 family (Rudner. *et al*. 2010 and Adams. *et al*. 2010). The ethyl acetate extract increased the mRNA expression of pro-apoptotic BAK and decreased the expression of anti-apoptotic BCL-XL. It did not change the expression of anti-apoptotic BCL-2, pro-apoptotic BAX, as well as a transcription factor p53. The transcription factor p53 responses to diverse forms of cellular stress and mediates a variety of anti proliferative processes. It is activated by DNA damage, hypoxia, or aberrant oncogene expression leading to promotion of cell-cycle arrest, DNA repair, cellular senescence, and apoptosis. This transcription factor is encoded from a tumor suppressor gene which promotes cell death via the intrinsic pathway of apoptosis by regulating the expression of BCL-2, BAK and BAX proteins. (Fridman. *et al*. 2003). However, it has been reported that BAX and BAK can also p53-independently suppress of tumorigenesis (Degenhardt. *et al*. 2002 and Speidel D. 2009). It is suggested that the apoptotic effect of the ethyl acetate extract is p53-independence.

Anticancer drugs can act as cell cycle specific or cell cycle non-specific drugs. The ethyl acetate extract caused Ramos cells accumulated at G1 and S phase of the cycle. It is possible that this extract may act as a cell cycle specific cytotoxic agent to induce cell cycle arrest at G1 phase. This postulation was proven by determining the expression of cyclins and cyclin-dependent kinase inhibitors (CKIs). The extract decreased the mRNA expression of cyclin D and cyclin E and increased the expression of p21 which is a CKI. Cyclins are positive regulator of Cdks which are key kinases for driving the cell cycle progression. Usually all cyclins which activate Cdks are cyclically expressed and degraded during the cell cycle. They are specifically synthesized to activate their specific Cdks in different phases of the cell cycle. Cyclin D1 is known to specifically bind and activate cyclin-dependent kinase 4 (Cdk4) and Cdk6 which regulate the cell cycle at G1 phase. Cyclin E binds to and activates Cdk2 at G2 and early S phase. Cell accumulation at G1 and S phases of the cell cycle by the ethyl acetate extract correlated to its inhibitory effect on the expression of these cyclins (Garrett DM. 2001). Cdk activities are negatively regulated by CKIs which directly bind to cyclin/Cdk complexes or inhibit Cdk phosphorylation (Cunningham. *et al*. 2001). P21 is a CKI which negatively regulates Cdks at G1 and G2 phases of the cell cycle. It is essential for the onset of both G1 and G2 cell cycle arrest in damage response and cell senescence. At G1 phase, it inhibits Cdk4 activity by binding to

bind to cyclin D/Cdk4 complex during the early G1 phase (Burgess. *et al*. 2001). It expression is mediated by either p53-dependent or p53-independent mechanisms (Kerkho. *et al*. 1998 and Shinwari. *et al*. 2008). The ethyl acetate extract arrested the cell cycle and correlated to its effect on the increase in the expression of p21 expression. This effect has to be evaluated further whether it depends on p53 activation or not. This study only demonstrated that the extract did not have effect on the mRNA expression of p53.

It has been identified that the ethyl acetate extract from the leaves of *G. parva* contains an acridone alkaloid (arborinine) and 4 sulfur-containing propanamides [*S*-deoxydihydroglyparvin, *S*-deoxytetrahydroglyparvin and *N*-[(4-monoterpenyloxy) phenylethyl]-substituted sulfur-containing propanamide derivatives (glyparvin-A and dihydroglyparvin] (Chansriniyom. *et al*. 2009). Only arborinine was investigated for cytotoxic activity against some cancer cell lines. Arborinine from *Erthela bahiensis* had no cytotoxic effect on murine B cell hybridoma (Roseghini. *et al*. 2009). It inhibited the proliferation of human cell lines HeLa, MCF7 and A431 (Rethy. *et al*. 2007). It is possible that not only arborinine but also some sulfur-containing propanamides may have cytotoxic effects on Ramos cell in this study.

In conclusion, this study demonstrated that the ethyl acetate extract from the leaves of *G. parva* had cytotoxic effect on Ramos cells which are human B lymphoma cells. It induced these cell deaths mainly by apoptosis. Its apoptotic effect was mainly caspase dependent and corresponding to the increase in pro-apoptotic BAK expression and the decrease in anti-apoptotic BCL-XL expression. The extract also arrested the cell cycle of Ramos cells at G1 and S phase. This cell cycle arrest was correlated to the decrease in the expression of cyclin D1 and cyclin E and the increased in the expression of p21. These results suggest that the ethyl acetate extract from the leaves of *G. parva* induces apoptosis and causes cell cycle arrest in Ramos cells. Further study is needed to identified whether which active compound in this extract has these effects

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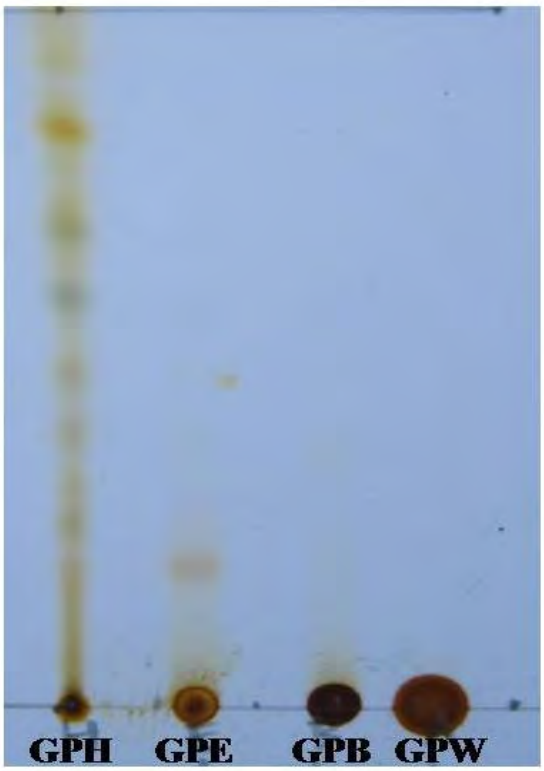
APPENDIX

APPENDIX A



Appendix A-1: Chromatograms from thin layer chromatography of the solvent extracts (GPH: hexane, GPE: ethyl acetate, GPB: butanol, and GPW: water) from the leaves of *G. parva* using 30% ethyl acetate in hexane solvent system under iodine spraying

APPENDIX B

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 0.2 sterile 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 sterilize by autoclaving

4. 10x Assay Buffer for Flow Cytometer 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 sterilize 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 sterilize by autoclaving

7. 1x TE Buffer 100 ml

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

Sterilize by autoclaving

8. 5x TBE Buffer 1 liter

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

Sterilize by autoclaving

APPENDIX C

Results

Appendix C-1: Cytotoxic activities of the solvent extracts (hexane, ethyl acetate, butanol and water extracts) from the leaves of *G. parva* on Ramos cells after 24 h exposure by using resazurin assay.

Treatment	% Cytoxicity			Mean ± S.E.
	n1	n2	n2	
20 µg/ml etoposide	73.7	80.62	74.78	76.36 ± 2.28
0.2% DMSO	0.00	0.00	0.00	0.00 ± 0.00
GPH 25 µg/ml	17.16	22.74	14.33	18.07 ± 2.47
GPH 50 µg/ml	35.11	39.81	34.16	36.36 ± 1.74
GPH 100 µg/ml	46.67	49.68	49.68	48.67 ± 1.00
GPE 25 µg/ml	99.42	99.46	62.33	87.07 ± 12.37
GPE 50 µg/ml	96.89	100.00	100.00	98.96 ± 1.03
GPE 100 µg/ml	100.00	100.00	100.00	100 ± 0.00
GPB 25 µg/ml	2.09	-10.96	3.11	-2.84 ± 0.92
GPB 50 µg/ml	-4.71	-4.85	6.66	-0.96 ± 2.22
GPB 100 µg/ml	-5.33	-3.31	9.86	0.40 ± 3.32
GPW 25 µg/ml	-5.07	-3.17	-2.47	-3.57 ± 0.00
GPW 50 µg/ml	4.27	1.22	0.32	1.94 ± 1.14
GPW 100 µg/ml	3.16	4.08	1.44	2.89 ± 0.77

hexane (GPH), ethyl acetate (GPE), butanol (GPB) and water (GPW) extracts

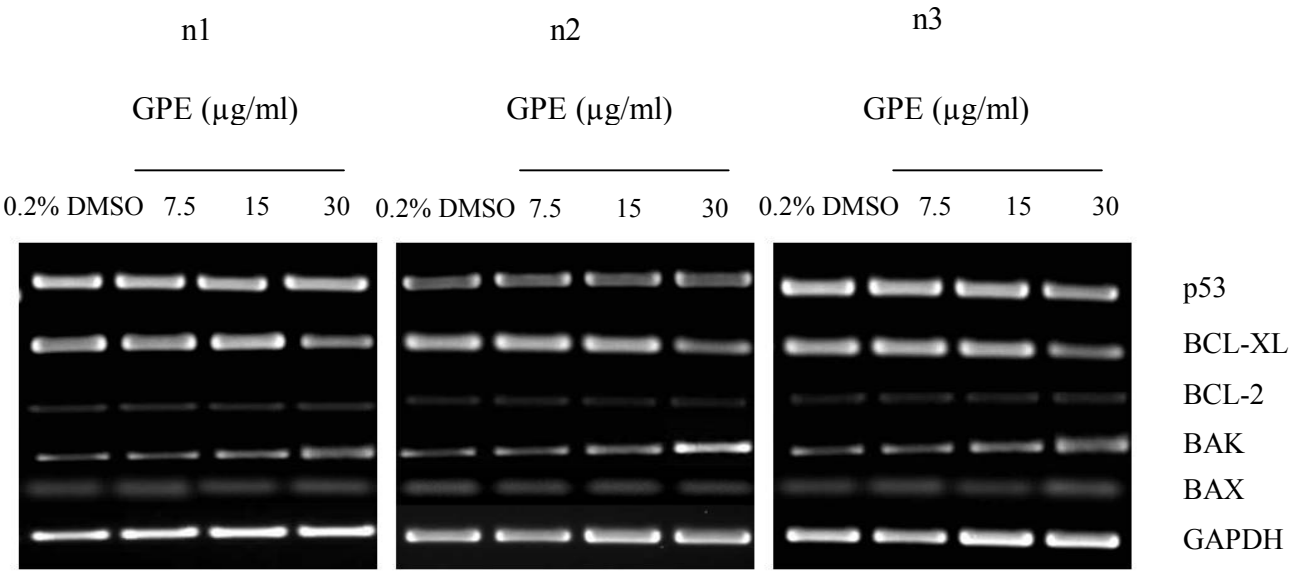
Appendix C-2: Cytotoxic effect of the ethyl acetate extract from the leaves of *G. parva* on Ramos cells and PBMCs after 24 h exposure.

Cells	GPE concentration (µg/ml)	% Cytotoxicity			Mean ± S.E
		n1	n2	n3	
Ramos cells	3.125	0.29	2.03	-0.12	0.77 ± 0.81
	6.25	6.66	7.5	7.87	7.34 ± 1.19
	12.5	36.23	29.77	27.65	31.32 ± 0.93*
	25	98.64	96.77	82.30	95.57 ± 4.14*
	50	99.94	99.45	100.00	99.80 ± 3.60*
PBMCs	3.125	4.48	6.04	3.23	4.58 ± 0.63
	6.25	8.49	10.55	6.41	8.48 ± 0.36
	12.5	17.59	15.82	14.35	15.92 ± 2.58
	25	22.17	33.98	35.17	30.44 ± 5.16
	50	38.48	50.39	47.66	45.51 ± 0.17

GPE: the ethyl acetate extract

$P<0.05$ denotes statistically significance of difference when compared to PMBCs at the same concentration of extract.

Appendix C-3: Effect of ethyl acetate extract from the leaves of *G.parva* (GPE) on the mRNA expression of p53 and BCL-2 family proteins by RT-PCR and agarose gel electrophoresis (n=3).



Appendix C-4: Effect of ethyl acetate extract from the leaves of *G. parva* (GPE) on the mRNA expressions of p53 and BCL-2 family proteins by RT-PCR, agarose gel electrophoresis and analysis by gel documentation (n=3).

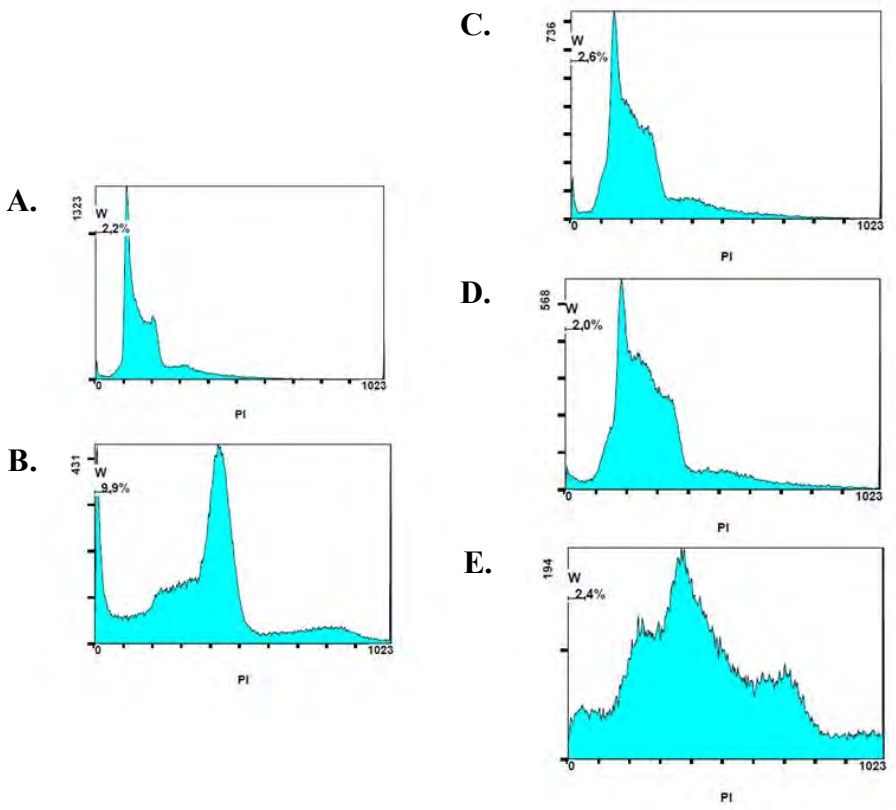
Gene	Treatment	% of control			Mean + S.E
		n1	n2	n3	
P53	0.2% DMSO	100.00	100.00	100.00	100.00 ± 0.00
	7.5	101.00	100.56	99.59	100.38 ± 0.42
	15	105.49	102.42	99.53	102.48 ± 3.90
	30	103.59	100.19	101.28	101.69 ± 6.69
BCL-XL	0.2% DMSO	100.00	100.00	100.00	100.00 ± 0.00
	7.5	103.13	101.38	87.53	97.35 ± 4.93
	15	94.66	100.32	86.87	93.95 ± 3.90
	30	81.48	78.50	60.09	73.36 ± 6.69*
BCL- 2	0.2% DMSO	100.00	100.00	100.00	100.00 ± 0.00
	7.5	100.08	100.57	99.28	99.98 ± 0.38
	15	104.52	101.89	99.97	102.13 ± 1.32
	30	102.81	102.13	100.04	101.66 ± 0.83
BAK	0.2% DMSO	100.00	100.00	100.00	100.00 ± 0.00
	7.5	110.82	111.60	107.51	109.98 ± 1.25
	15	124.87	118.81	116.04	119.91 ± 2.54*
	30	157.89	140.13	141.98	146.67 ± 5.57*
BAX	0.2% DMSO	100.00	100.00	100.00	100.00 ± 0.00
	7.5	101.57	99.45	99.13	100.05 ± 2.53
	15	101.88	99.31	101.99	101.06 ± 0.87
	30	105.50	100.40	105.83	103.91 ± 1.76

*P < 0.05 denotes statistically significant difference from the solvent control (0.2 % DMSO).

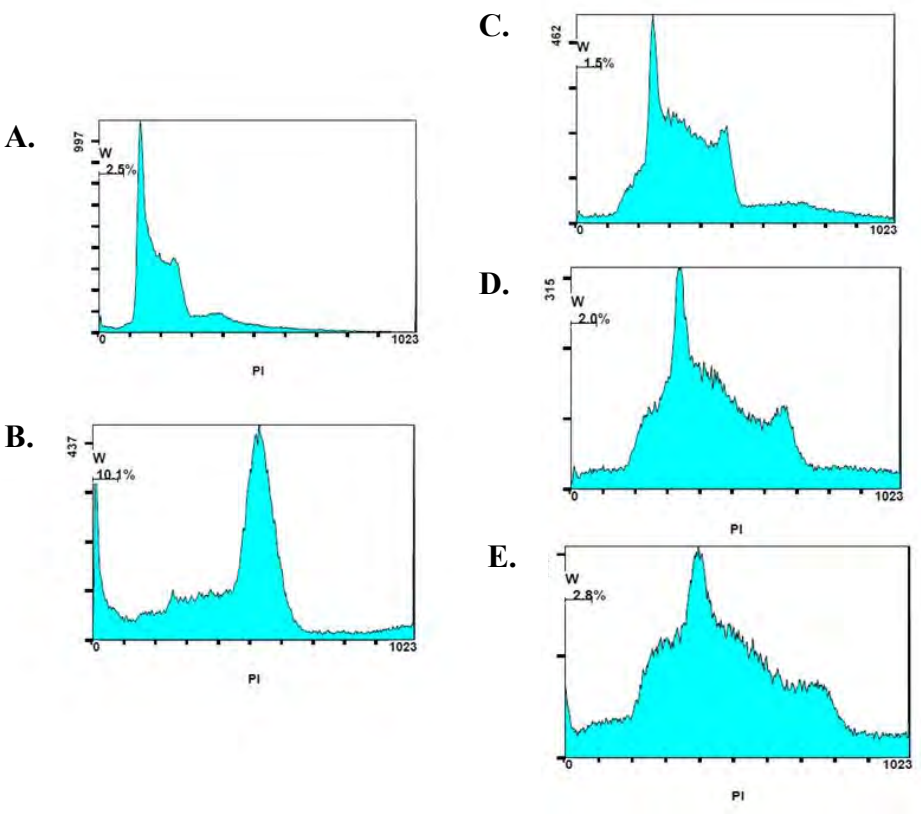
Appendix C-5: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on caspase dependent apoptosis in Ramos cells by using a pan caspase inhibitor Z-VAD-FMK . Data express as mean ± S.E. of two independent experiments (n=2)

Treatment	% Viable	% Death cells		
		Apoptosis (Annexin Positive)	Necrosis (PI positive)	Late apoptosis & necrosis (Double positive)
Without Z-VAD-FMK				
0.2% DMSO	95.30 ± 0.40	1.65 ± 0.15	0.75 ± 0.15	2.30 ± 0.10
GPE 15 µg/ml	88.45 ± 0.05	7.35 ± 0.85	1.90 ± 1.60	2.25 ± 0.65
GPE 30 µg/ml	43.15 ± 0.15	41.3 ± 0.30	8.05 ± 6.35	7.55 ± 6.25
With Z-VAD-FMK				
0.2% DMSO	95.30 ± 0.40	1.65 ± 0.15	0.75 ± 0.15	2.30 ± 0.10
GPE 15 µg/ml	94.95 ± 0.05	2.85 ± 0.05*	0.15 ± 0.05	2.00 ± 0.10
GPE 30 µg/ml	92.95 ± 0.35	4.05 ± 0.15*	0.30 ± 0.00	2.90 ± 0.00

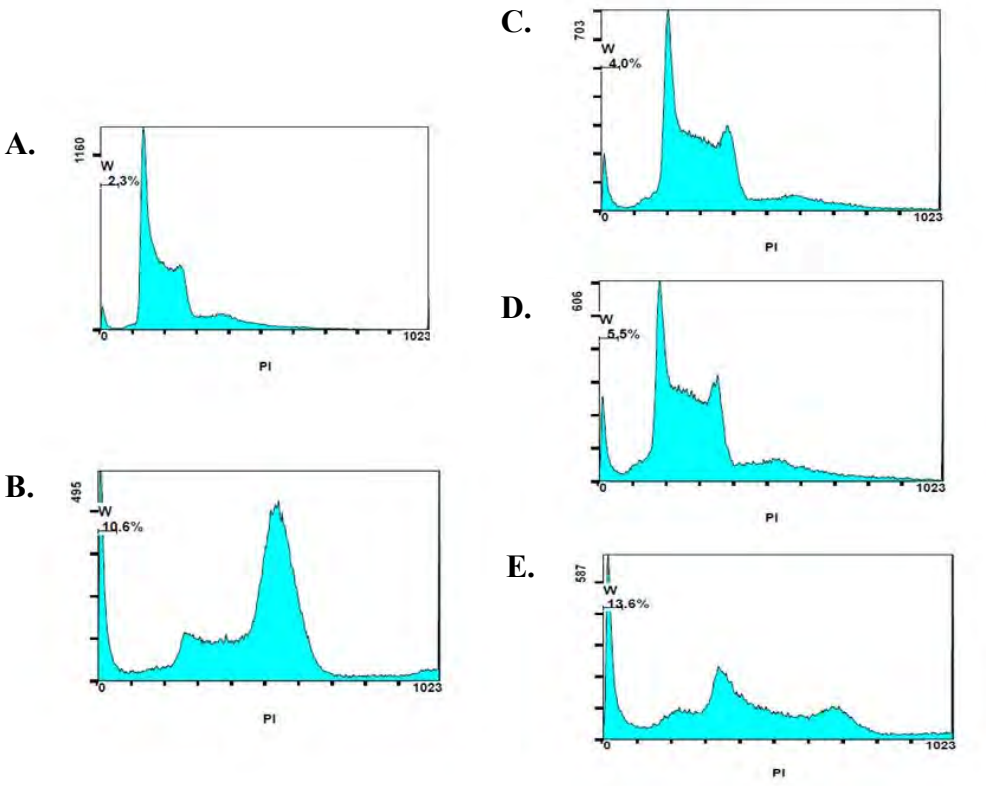
*$P < 0.05$ denotes statistically significant difference from without Z-VAD-FMK conditions.



Appendix C-6: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on the cell cycle of Ramos cells (n1). (A) 0.2% DMSO; (B) 1µg/ml etoposide; (C, D, E) 7.5, 15 and 30 µg/ml of GPE.

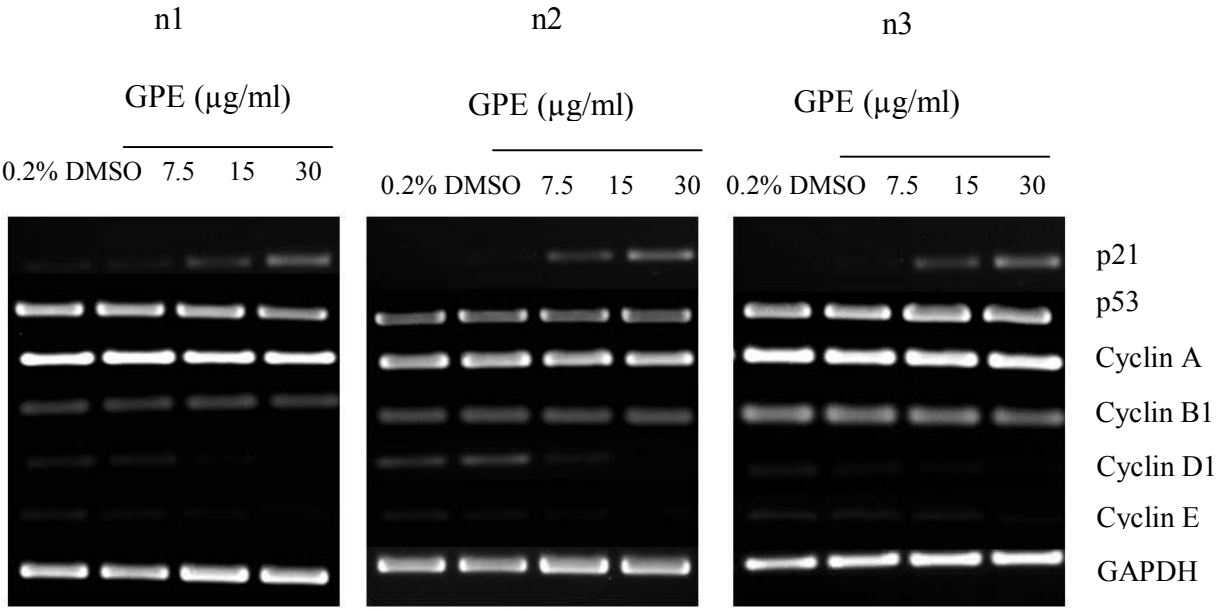


Appendix C-7: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on the cell cycle of Ramos cells (n2). (A) 0.2% DMSO; (B) 1µg/ml etoposide; (C, D, E) 7.5, 15 and 30 µg/ml of GPE.



Appendix C-8: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on the cell cycle of Ramos cells (n3). (A) 0.2% DMSO; (B) 1µg/ml etoposide; (C, D, E) 7.5, 15 and 30 µg/ml of GPE.

Appendix C-9: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on the mRNA expression of p21, p53, and cyclins by RT-PCR and agarose gel electrophoresis (n=3).



Appendix C-10: Effect of the ethyl acetate extract from the leaves of *G. parva* (GPE) on the mRNA expression of p21, p53 and cyclins by RT-PCR, agarose gel electrophoresis and analysis by gel documentation (n=3).

Gene	Treatment	% of control			mean ± S.E		
		n1	n2	n3			
P21	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	103.30	101.23	109.89	104.81	±	10.00
	15 µg/ml GPE	113.85	125.85	125.96	121.89	±	0.40
	30 µg/ml GPE	127.31	132.24	147.23	135.59	±	0.46
P53	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	101.00	100.56	99.59	100.38	±	8.76
	15 µg/ml GPE	105.49	102.42	99.93	102.61	±	5.79
	30 µg/ml GPE	103.59	100.19	101.28	101.69	±	2.41
Cyclin A	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	100.69	98.08	99.53	99.43	±	6.40
	15 µg/ml GPE	104.28	100.10	101.08	101.82	±	0.39
	30 µg/ml GPE	108.47	98.33	102.73	103.18	±	3.02
Cyclin B1	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	99.58	96.99	97.44	98.00	±	2.71
	15 µg/ml GPE	96.26	94.83	94.29	95.13	±	1.84
	30 µg/ml GPE	97.40	93.24	93.20	94.61	±	1.23
Cyclin D1	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	89.53	81.92	83.85	85.10	±	3.47
	15 µg/ml GPE	38.25	34.92	36.03	36.40	±	1.51*
	30 µg/ml GPE	36.80	20.08	31.22	29.37	±	1.19*
Cyclin E	0.2% DMSO	100.00	100.00	100.00	100.00	±	0.00
	7.5 µg/ml GPE	71.47	72.92	74.72	73.04	±	1.09*
	15 µg/ml GPE	61.96	56.31	68.72	62.33	±	8.14*
	30 µg/ml GPE	57.06	46.59	34.07	45.91	±	1.21*

*$P < 0.05$ denotes statistically significant difference from 0.2% DMSO

BIOGRAPHY

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